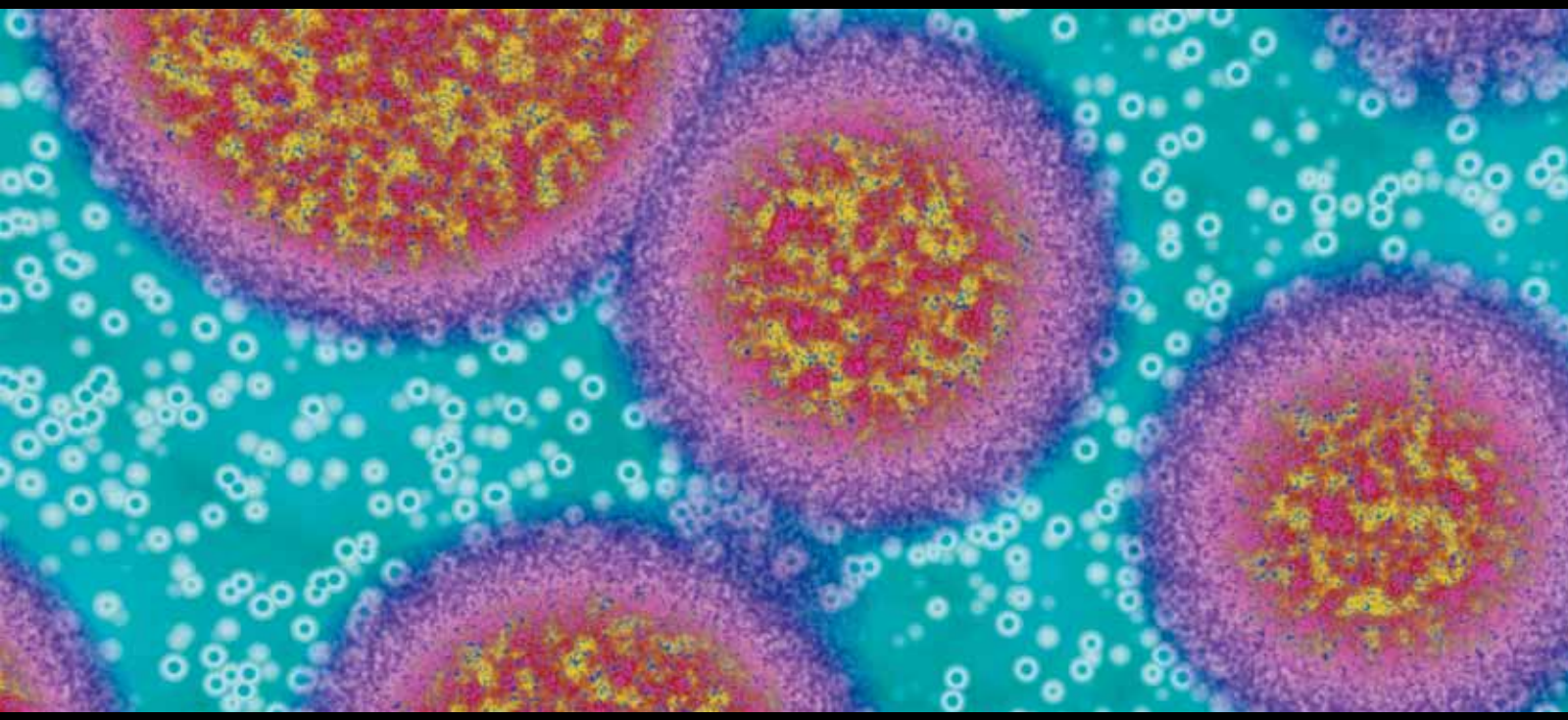
International Journal of Cell Biology

Cell Biology of Cysteine-Based Molecular Switches

Guest Editors: Christian Appenzeller-Herzog, Kenji Inaba, and Agnès Delaunay-Moisan



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Editorial Board

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Editorial

Cell Biology of Cysteine-Based Molecular Switches

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Reversible posttranslational protein modifications form the mechanistic basis for the reception and propagation of biological signals in cells. Besides other modifications such as phosphorylation, acetylation, ADP-ribosylation, and ubiquitylation, reduction-oxidation (redox) processes allow reversible structure-function modulation of proteins, which serve as molecular on-off switches in cell biology. Although many protein-bound amino acids and even the peptide backbone can react with oxidizing metabolites during oxidative stress, only three amino acids adopt reversible redox modifications: cysteine, selenocysteine, and methionine. Among these, cysteine-based molecular switches are by far the most prevalent and best studied. Cysteine switches (or "sulfur switches") respond in heterogeneous, context-dependent manner to a variety of stimuli (endogenous metabolites, chemicals from the diet, xenobiotics, or air oxidants) by direct modification. Common covalent modifications of cysteines include intra- or intermolecular protein-protein disulfide-bond formation, S-glutathionylation, S-cysteinylation, S-nitrosylation, sulfoxidation, and sulfhydration.

Catalyzed, redox-dependent on-off cycles of cysteine centers in proteins regulate processes as diverse as protein folding, aggregation, and trafficking, enzymatic activity, metal chelation, DNA, RNA, protein, or membrane binding, and channel opening. In this special issue, we have attempted to illustrate the versatility of cysteine-based protein regulation and its impact on the physiology of cells and organisms.

In both the secretory pathway and the mitochondrial intermembrane space (IMS), protein maturation often requires the introduction of disulfide crosslinks to promote or maintain protein structure. During this process known as oxidative protein folding, introduced disulfide bridges can be reshuffled, until the native conformation is achieved. Dedicated oxidative folding catalysts, as reviewed by Y. Onda, exist in the endoplasmic reticulum (ER), IMS, and chloroplasts in plant cells as well as in the extracellular space. The disulfide-generating machineries in ER and IMS are conserved in plants, fungi, and animals. Evolutionary and mechanistic aspects of disulfide-bond formation in IMS are discussed by M. Fischer and J. Riemer. Interestingly, the core components of this machinery, Erv1/ALR and Mia40, have additional, poorly understood functions in liver regeneration and hypoxia response, which are likely fulfilled through mechanisms other than oxidative folding in IMS.

Two contributions are concerned with the involvement of cysteines in the regulation of antibody secretion and differentiation of B lymphocytes. The review article by T. Anelli and E. van Anken enlightens how cysteine redox status acts as a quality control checkpoint to ensure that only mature IgM antibodies leave the compartments of the early secretory pathway en route to the blood stream. Immature antibodies are tagged with a free cysteine, which is covalently trapped by interchain disulfide formation with the retrieval factor ERp44 at the lowered pH of post-ER compartments. In this process, the active site cysteine in ERp44 apparently acts as a pH sensor. Antibody production in activated B lymphocytes is a bulk process, which—besides rigorous quality control—relies on massive ER expansion and disulfide-bond formation. How far the differentiation of resting B cells into antibody-secreting plasma cells affects the overall levels of protein disulfides

and of S-glutathionylation relative to the levels of reduced cysteines was addressed by J. R. Winther and I. Braakman and colleagues. They report that ER expansion per se does not lead to a global increase in oxidation of protein-bound cysteines, which is only observed later in differentiation upon induction of antibody synthesis. Furthermore, the fraction of disulfides present as S-glutathione adducts remains constant.

Hydrogen peroxide (H_2O_2) is a signaling molecule controlling essential aspects of cellular life and death. Peroxiredoxins are critical H_2O_2-reducing enzymes, which shape the amplitude and duration of local accumulation of this second messenger. Importantly, their active site cysteine is inherently prone to H_2O_2-driven inactivation by sulfoxidation. As a consequence, H_2O_2 signals can transiently override the peroxiredoxin defense and mediate downstream signaling events. The review of S. W. Kang and colleagues focuses on typical 2-cysteine peroxiredoxins and provides a comprehensive overview of their cell biological functions. Interestingly, some of these are also linked to cellular signaling events without being directly related to H_2O_2 reduction. On a different note, M. Molin and A. B. Demir address the function of a cytosolic peroxiredoxin in calorie restriction-mediated life span extension. Under these conditions, ATP-driven reactivation of the hyperoxidized form of this peroxiredoxin is stimulated. As the authors hypothesize, this nutrient-sensing mechanism is likely intertwined with other nutrient-responsive processes such as vacuolar proton pumping, mitochondrial function, and iron metabolism. A third contribution to peroxidases by T. Ramming and C. Appenzeller-Herzog summarizes the nonoverlapping functions of the three known ER-resident H_2O_2-reducing enzymes, peroxiredoxin IV and glutathione peroxidases 7 and 8.

Finally, two articles—a review by J. D. Atkin and colleagues and a research paper by A. Odermatt and colleagues—represent links of cysteine-dependent regulation with disease. M. Halloran et al. explain how S-nitrosylation and S-glutathionylation of the active site cysteines in protein disulfide isomerase are implicated in various neurodegenerative diseases. L. G. Nashev et al. examine the role of a conserved cysteine in the NADPH-binding region of 17-hydroxysteroid dehydrogenase 1, a prognostic marker for tumor progression and survival of patients with breast cancer and other estrogen-dependent cancers. While mutation of this cysteine does not change the kinetic parameters of the enzyme, its alkylation by sulfhydryl-modifying agents irreversibly inhibits dehydrogenase activity.

Acknowledgment

We would like to conclude by thanking all the authors and, in particular, the anonymous reviewers who contributed to the realization of this special issue and the promotion of its scientific quality.

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Review Article

2-Cys Peroxiredoxins: Emerging Hubs Determining Redox Dependency of Mammalian Signaling Networks

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Mammalian cells have a well-defined set of antioxidant enzymes, which includes superoxide dismutases, catalase, glutathione peroxidases, and peroxiredoxins. Peroxiredoxins are the most recently identified family of antioxidant enzymes that catalyze the reduction reaction of peroxides, such as H_2O_2. In particular, typical 2-Cys peroxiredoxins are the featured peroxidase enzymes that receive the electrons from NADPH by coupling with thioredoxin and thioredoxin reductase. These enzymes distribute throughout the cellular compartments and, therefore, are thought to be broad-range antioxidant defenders. However, recent evidence demonstrates that typical 2-Cys peroxiredoxins play key signal regulatory roles in the various signaling networks by interacting with or residing near a specific redox-sensitive molecule. These discoveries help reveal the redox signaling landscape in mammalian cells and may further provide a new paradigm of therapeutic approaches based on redox signaling.

1. Introduction

It is generally accepted that the cellular antioxidant enzymes belong to a group of the oxidoreductase enzymes maintaining the cellular redox homeostasis. However, the importance of antioxidant enzymes is given a spotlight after a paradigm shift of the cellular function of reactive oxygen species (ROS) from toxic respiratory by-products to a signaling second messenger. Peroxiredoxin (Prx) is a family of antioxidant enzymes exhibiting peroxidase activity which reduces the hydroperoxides to water in the presence of proper electron donors. Prxs are classified by the number of cysteine residues involved in the peroxidase activity: 2-Cys Prxs and 1-Cys Prx. The 2-Cys Prxs form a disulfide bond by reacting with the peroxides and the disulfide is reduced by thioredoxin which is coupled with thioredoxin reductase and NADPH. Therefore, 2-Cys Prxs are the first thioredoxin-dependent peroxidase enzymes [1, 2]. The 2-Cys Prxs are purely cysteine-based peroxidase enzymes with no cofactor or selenocysteine requirement. They are divided into typical and atypical groups based on the catalytic mechanism. Typical 2-Cys Prxs (Prx1–Prx4) are active as dimers organized in antiparallel fashion: that is, the peroxidatic cysteine residue (C_P) in the amino terminus of one subunit reacts with the hydroperoxides and the resulting C_P sulfenic acid forms a disulfide linkage with the sulfhydryl group of resolving cysteine residue (C_R) in carboxyl terminus of another subunit [3]. In contrast, an atypical 2-Cys Prx (i.e., Prx5) catalyzes the H_2O_2 reduction reaction through the formation of intramolecular disulfide linkage [4]. The 2-Cys Prx enzymes have distinct roles in diverse cellular processes, such as proliferation, migration, apoptosis, and metabolism, and are fundamentally supported by a broad distribution of the isoforms throughout the subcellular compartments. For example, Prx1 and Prx2 are the most abundant antioxidant enzymes in cytosol. Prx3 is a major mitochondrial peroxidase responsible for efficient elimination of H_2O_2, which is continuously produced by the dismutation of superoxide anions formed as a result of a partial reduction of the dissolved

oxygen molecules during mitochondrial respiration. Prx4 is in both endoplasmic reticulum (ER) and extracellular fluid. Recent studies indicate that Prx4 is involved in the oxidative protein-folding pathway by the reoxidation of protein disulfide isomerase [5, 6]. The distribution of Prx5 is somewhat complex: high in mitochondria, some in peroxisome, and low in cytosol [4]. Hence, the cellular abundance and broad distribution of 2-Cys Prxs mark them as a major antioxidant system in mammalian cells.

Beside their primary function as antioxidant enzymes, the observation that 2-Cys Prxs peroxidase activity can be readily inhibited by overoxidation of the active site cysteine residue (C_P) and reactivated by sulfiredoxin-dependent reduction [7] highlights novel and unforeseeable functions of these enzymes. In both *in vitro* enzyme reaction with high concentration of H_2O_2 and oxidatively-stressed cells, the C_P-sulfenic acid at the active site of typical 2-Cys Prxs is overoxidized to sulfinic/sulfonic acids [8]. Unlike bacterial homologs, the typical 2-Cys Prxs in eukaryotes have been characterized to show a structural feature that the resolving cysteine (C_R) buries away in latent enzyme and then reacts with C_P-sulfenic acid by local unfolding of the C-terminus [9]. It is therefore interpreted that such conformational change of the C-terminus in eukaryotic 2-Cys Prxs necessary for forming a disulfide linkage with the C_P-sulfenic acid tolerates an additional reaction of the C_P-sulfenic acid with the second molecule of H_2O_2. Consequently, the 2-Cys Prxs can be inactivated by overoxidation during the reaction cycle and, if the inactive enzymes are accumulated, the local H_2O_2 concentration may be raised ("Floodgate" hypothesis).

Subsequent studies state that the overoxidation of C_P most probably corresponds to a gain of function of 2-Cys Prxs in eukaryotes. The first surprising result is that the overoxidized 2-Cys Prxs are multimerized and function as a molecular chaperone to prevent unfolded proteins from irreversible aggregation [10]. Hence, the evolution of the eukaryotic 2-Cys Prxs sensitive to overoxidation implies a highly efficient survival tactic in eukaryotes adapting oxidative stress. Recently, Veal and her colleagues reported that the overoxidation of 2-Cys Prxs plays a role in cell survival other than as a molecular chaperone [11]. In this study, the inactivation of 2-Cys Prx by overoxidation discharged a key coupling redox protein, thioredoxin, which in turn rescued other oxidized client proteins by reduction. Another compelling biological role of the 2-Cys Prx overoxidation is a correlation with circadian rhythm in normal physiology. O'Neill and Reddy have shown that the overoxidation of 2-Cys Prxs exhibits a circadian oscillation with a period of about 24 hours in human red blood cells [12]. Later, it turned out to be a transcription-independent circadian marker universally conserved from bacteria to eukaryotes [13]. Consequently, the intrinsic susceptibility of 2-Cys Prxs to inactivation by overoxidation is seemingly to be a part of the important redox mechanism in both normal and abnormal physiology. In addition, a study using yeast mutant strains lacking multiple thiol peroxidases including all five Prxs and three glutathione peroxidase genes suggests that the thiol peroxidases may transfer the ROS signals to gene expression by transcriptional regulation [14]. Therefore, in this review, we collect the evidence for specific signaling functions of typical 2-Cys Prxs with low K_m for H_2O_2 and discuss its implication as a conceptually new hub in signaling networks.

2. 2-Cys Prxs in Protein Phosphorylation Signaling Networks

Protein phosphorylation is one of the most important posttranslational modifications in the membrane receptor-mediated growth factor and cytokine signaling and as such modulates protein-protein interaction, enzyme activity, and protein stability and structure. Human genome encodes over 500 putative kinase genes and more than 150 protein phosphatases including dual-specificity phosphatases and protein tyrosine phosphatases (PTP). With the exception of the *EYA* subfamily, most protein phosphatases contain a low-pKa cysteine residue at the active site. The sulfhydryl group is thus deprotonated to the thiolate anion at the physiological pH [15, 16], which renders it susceptible to oxidation by H_2O_2 *in vitro* and *in vivo* [17, 18]. Since H_2O_2 was proposed as a novel intracellular second messenger in the platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) signaling pathways [19, 20], the H_2O_2-mediated reversible oxidation of PTPs has become an important regulatory mechanism controlling protein tyrosine phosphorylation [21, 22]. Recently, plausible evidence also indicates that the protein kinases are redox-regulated by a reversible oxidation of the cysteine residues in the regulatory region, rather than on their active sites. For example, IκB kinases α/β (IKKα/β) were shown to harbor a reactive cysteine between two serine residues, which are the dual phosphorylation sites critical for activation, in T-loop [23–25]. The ataxia-telangiectasia mutated (ATM) kinase and a Src kinase Lyn were shown to be activated by a H_2O_2-mediated cysteine oxidation [26, 27]. Such evidence concertedly indicates that the phosphorylation signaling network involves redox-regulated kinases/phosphatases and therefore it is associated with the dynamics of intracellular H_2O_2 level. In a live cell, the intracellular H_2O_2 level is determined by balancing the H_2O_2 generators (e.g., mitochondria, oxidases, and heavy metals) and antioxidants (e.g., catalase, glutathione peroxidases, and peroxiredoxins). Among cellular peroxidases, 2-Cys Prxs are the most abundant enzymes and versatile in the subcellular distribution. In particular, the evidence indicates that the two cytosolic forms, Prx1 and Prx2, are likely the key enzymes in the phosphorylation signaling pathway (Figure 1). The first indication of the signaling function of Prx was made in 1998 and showed that the overexpression of Prx1 and Prx2 eliminated intracellular H_2O_2 increased by growth factors, such as PDGF-B and EGF, and cytokine tumor necrosis factor (TNF)-α [1]. Since then, many investigations indicate the important regulatory role of Prx1/2 in phosphorylation signaling. The Prx1 ablation was shown to result in the Akt hyperactivation in H_2O_2-treated cells, but not in PDGF-treated cells [28]. Prx1 interacted with phosphatase and tensin homolog (PTEN) in H_2O_2-treated cells and thus promoted the Ras- or ErbB2-drived cell transformation. Hence, it was proposed that Prx1 might contribute someway to the tumorigenesis.

However, the function of Prx2 as a signal regulator was initially proposed by the differential regulation of TNF-α-induced MAP kinase activation [29]. Also, Prx2 negatively regulates the PDGF-induced tyrosine phosphorylation in fibroblast and vascular smooth muscle cells [30]. In this case, the deletion of Prx2, not Prx1, selectively increased the autophosphorylation of PDGFRβ only at two tyrosine sites (Y579 and Y857), which was not mimicked by addition of exogenous H_2O_2. Such selective regulation was achieved by the stimulation-dependent interaction of Prx2 and PDGFRβ proteins, which allowed the reactivation of a membrane-associated PTP. This is the first report showing the selective action of endogenous H_2O_2 distinguished from the exogenous source of H_2O_2. Recently, Prx2 was also shown to preserve the VEGFR2-dependent tyrosine phosphorylation in vascular endothelial cells by protecting the receptor from oxidative inactivation by both the endogenous and exogenous H_2O_2 [31]. This function appeared to be due to the colocalization of Prx2 and vascular endothelial growth factor receptor-2 (VEGFR2) in endothelial caveolae. Although the source of endogenous H_2O_2 was not identified, it is an important finding that Prx2 functions upstream of the receptor tyrosine kinase whose activity is regulated by an oxidation-sensitive cysteine residue.

The cytosolic 2-Cys Prxs are themselves linked to the phosphorylation networks as their activities are regulated by phosphorylation. Chang et al. reported that the 2-Cys Prxs contain the conserved CDK phosphorylation sequence (Thr^{90}-Pro-Arg-Lys), and among them the Prx1 and Prx2 were indeed phosphorylated by Cdk1/Cdc2 [32]. Although such threonine phosphorylation caused the loss of peroxidase activity of both 2-Cys Prxs *in vitro*, it was observed only in Prx1 *in vivo* using the mitotic arrested HeLa cells. However, its biological significance remains unsolved. Other studies also showed that the Prx1 threonine phosphorylation is mediated by serine/threonine kinase Mst1/2 [33, 34]. Similarly, this phosphorylation inactivated the peroxidase activity and therefore resulted in an increase in the intracellular H_2O_2 level. In contrast, the serine phosphorylation of Prx1 by a T-cell-originated protein kinase (TOPK) increased the peroxidase activity [35]. TOPK binds to and phosphorylates Prx1 at Ser^{32} *in vitro* and in human melanoma cells. It is noteworthy that the activated TOPK colocalized with Prx1 in nucleus, which is the first indication of nuclear Prx1. Later, both Prx1 and Prx2 were found in the nucleus and, particularly, Prx2 protects the cancer cell death against DNA damaging agents [36]. The threonine phosphorylation of Prx2 correlates with an increased loss of dopaminergic neurons by mitochondrial damage [37]. Interestingly, in this case, the phosphorylation was mediated by Cdk5/p35 and increased in nigral neurons from postmortem tissue of Parkinson's disease patients. Related to Parkinson's disease, there was another interesting report that a mutation of leucine rich repeat kinase 2 (LRRK2), where glycine-2019 is mutated to serine, increased the phosphorylation of a mitochondrial Prx3 [38]. The phosphorylation of Prx3 was associated with the increased cell death in neuronal cells by a mitochondrial stress and significantly detected in Parkinson's disease patients with the LRRK2 mutation. Consequently, the phosphorylation-dependent inactivation of mitochondrial Prx3 and cytosolic Prx2 seems to be coordinately involved in the loss of dopaminergic neuronal cells by mitochondrial damage.

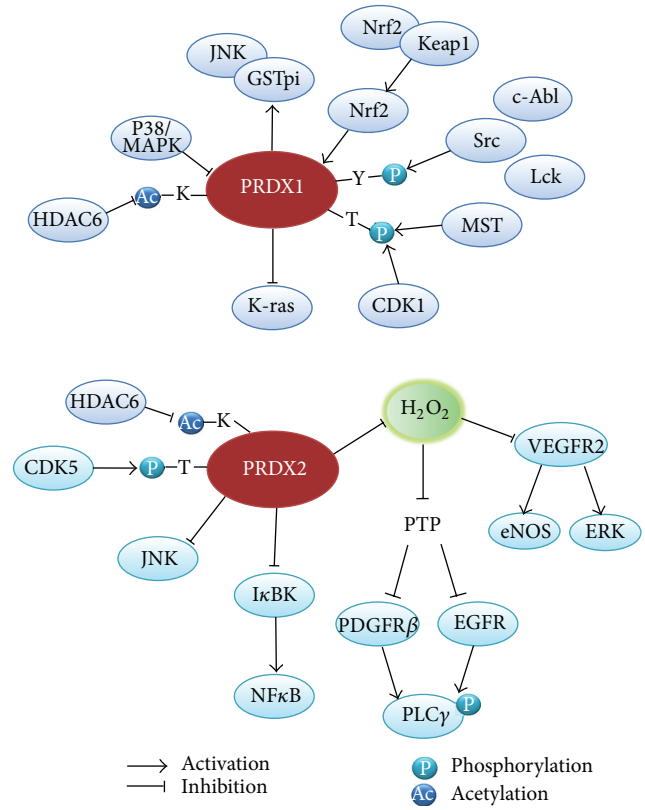

Figure 1: Interaction of typical 2-Cys Prxs with signaling molecules in the phosphorylation and acetylation networks. Prx1 and Prx2 interact directly or indirectly via ROS with the kinases/phosphatases and regulate their activation. In addition, the activities of these two Prxs are also controlled by phosphorylation and acetylation. Note that the Prx1 expression is known to be controlled by a transcription factor Nrf2 (nuclear factor E2-related factor 2) under oxidative stress condition.

Recently, Prx1 was shown to be phosphorylated at Tyr^{194} by protein tyrosine kinases, such as Lck and Abl, *in vitro* and in various mammalian cells treated with growth factors [39]. This evidence is significant in terms of that the inactivation of Prx1 by phosphorylation in caveolae membrane microdomain could alter the local redox status. Although the authors showed the phosphorylated Prx1 in the margin of healing wounds in C57BL/6 mice, the physiological relevance of the selective Prx1 phosphorylation to wound healing process remained uncertain. It is however clear that the phosphorylation-dependent inactivation takes a physiological advantage of the dynamic regulation linked to the intracellular kinase/phosphatase signaling network compared to Prx inactivation by overoxidation, as the reversal by sulfiredoxin of the latter is a slow reaction requiring an ATP energy demand [40, 41].

How does such phosphorylation regulate Prx peroxidase activity? Phosphorylation at Thr^{90} and phosphorylation Tyr^{194} have both been shown to regulate Prx activity by a similar mechanism, potentially involving a perturbation of the active site conformation after the introduction of a negatively charged phosphate moiety at the vicinity of the active site C_P residue [32, 39]. The crystal structure of Prx1 also suggests that the introduction of negative charges may destabilize Prx1 homodimer further causing the reduction of Prx activity toward H_2O_2.

Considering that the 2-Cys Prx isoforms are widely distributed in subcellular compartments, such modification-dependent inactivation of the 2-Cys Prxs may be an important mechanism in determining a localized elevation of H_2O_2 levels.

3. 2-Cys Prxs in Acetylation Signaling Networks

The reversible acetylation of protein lysine residues is an important posttranslational modification that regulates enzyme activity, protein-protein interaction, and protein conformation [42]. The majority of the initial studies focused on the histone acetylation, which directly regulates gene transcription and chromatin remodeling [43]. Since the microtubule-associated HDAC6 and mitochondrial Sirt3 were discovered [44–46], the reversible acetylation has been considered to be a general modification involved in the cellular signaling.

There are several studies implicating the redox regulation of lysine acetylation network. One study showed that H_2O_2 inhibits IL-1β-induced HDAC2 activity in airway epithelial cells, which is associated with the tyrosine nitration of HDAC2 [47]. Another study showed that H_2O_2 and hypertrophic stimuli induce a cysteine oxidation on HDAC4 in myocytes [48]. Upon oxidation, HDAC4 forms an intramolecular disulfide linkage and then the oxidized HDAC4 is exported to the cytoplasm. When the disulfide was reduced by Trx1, the reduced HDAC4 reenters into the nucleus. Consequently, the nucleocytoplasmic shuttling of HDAC4 is determined by its cysteine oxidation status. A member of class II HDACs, Sirt1, was shown to be sensitive to oxidation, especially S-glutathionylation on the Cys^{67} residue by S-nitrosoglutathione (GSNO) [49]. Interestingly, the GSNO inhibited the resveratrol-stimulated, not the basal, Sirt1 activity, which suggests that the redox-sensitive Cys residue could be exposed to the modification upon activation. The Sirt3 knockout mice showed oxidative stress phenotype in skeletal muscle and its knockdown in cultured myoblasts increased the ROS level [50].

Many studies show that the 2-Cys Prx activity is regulated by acetylation (Figure 1). A recent high-resolution mass spectrometric analysis combined with the stable isotope labeling by amino acids in cell culture (SILAC) revealed the lysine acetylation of Prx enzymes in various cell types [51]. A previous study showed that the Prx1 and Prx2 were among the substrates of cytoplasmic HDAC6 and their acetylation increased peroxidase activity and resistance to overoxidation [52]. It was shown that a lysine residue in the C-terminus of Prx1 and Prx2 enzymes (Lys197 in Prx1 and Lys196 in Prx2) is a site of acetylation. Thus, although the molecular mechanism underlying the acetylation-dependent activity increase is currently unknown, it is possible that the C-terminal acetylation may influence the resolving step accompanied with a conformation change of the C_R residue [9]. In the case of Prx2, the lysine-independent acetylation at its demethionylated N-terminus conferred a resistance to overoxidation in HeLa cells treated with high concentrations of H_2O_2 [53]. It is noteworthy that the acetylation of 2-Cys Prxs increases the enzyme activity and protects against overoxidation in contrast to enzyme inactivation by phosphorylation.

Although there is no evidence showing a direct regulatory role of 2-Cys Prxs in the lysine acetylation network, it will be interesting to investigate the mechanism of how the acetylation and deacetylation network is associated with 2-Cys Prxs in various subcellular compartments.

4. 2-Cys Prxs in Cell Death Signaling Networks

The role of ROS in cell death has been a long-standing issue because mitochondria are the key players in both apoptotic and necrotic cell death pathways. Indeed, mitochondria are the site where the electron transport takes place and leakage of the high energy electrons from the electron carrier complexes can combine with molecular oxygen to produce ROS [54]. Higher organisms with an aerobic respiratory system have evolved apoptotic cell death programs utilizing mitochondrial proteins, which include cytochrome c [55, 56]. In principle, the mitochondrial release of cytochrome c results in a disruption of the electron transport in the respiratory chain and causes an increase of mitochondrial ROS via the leakage of high energy free electrons. The resulting ROS burst may oxidatively damage the cellular macromolecules, such as proteins, membrane lipids, and DNA. However, the evidence indicates that the mitochondrial ROS is not a causative factor in apoptotic cell death, but rather it is the consequence of the disruption of mitochondrial transmembrane potential ($\Delta\psi_m$) [57]. The involvement of ROS in the apoptotic death pathway could be challenged by the fact that the active site of caspase is a reactive cysteine residue, which can be inactivated by oxidation [58–60]. Contrary to apoptosis, there may be a function of ROS in necroptosis. Necroptosis, also called programmed necrosis, is a type of necrotic cell death involving the activation of death receptor but occurring independently of caspase activation [61]. It has been shown that activation of death receptors, such as the TNF-α receptor (TNFR)-1 and Fas (CD95), induces necroptosis in some cell types [62, 63]. For example, mouse fibrosarcoma cells L929 underwent caspase-independent necrosis when stimulated with TNF-α [64]. Human Jurkat T lymphoma cells deficient in Fas-associated death domain (FADD) adaptor protein died via necrosis when death receptors, such as TNFR and Fas, were activated in an RIP1-dependent manner [65]. Therefore, the necroptosis was found to require the RIP1 kinase activity [66]. Further evidence indicates that ROS accumulates in

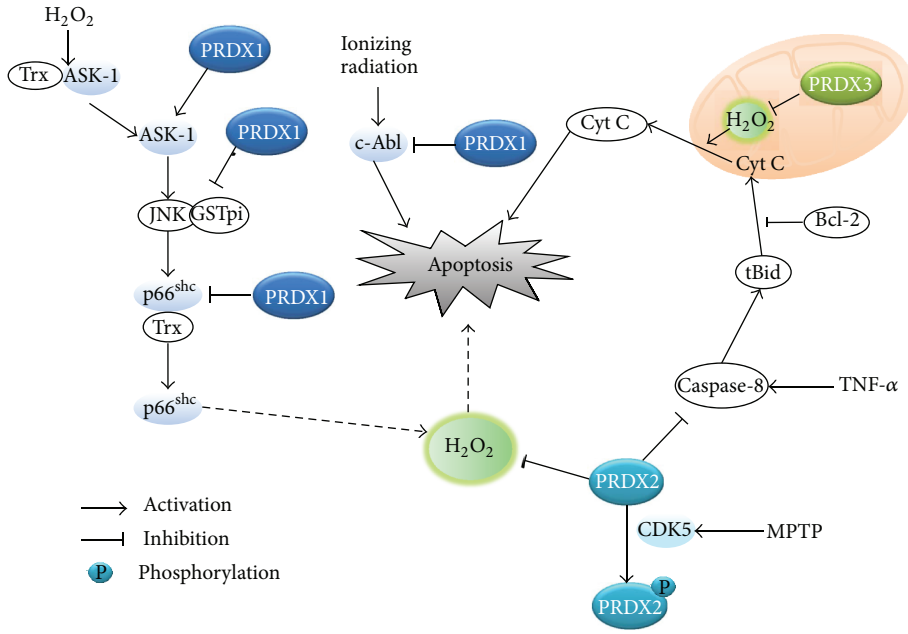

Figure 2: Role of typical 2-Cys Prxs in the apoptotic death pathways. The schematic drawing illustrates that each Prx interacts with proapoptotic molecules and regulates various apoptotic pathways. MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

a RIP1 and FADD-dependent manner and is required for the necroptosis [67]. The activation of NADPH oxidase-1 via RIP1 is involved in the TNF-α-induced necrosis in L929 cells [68]. In addition, RIP3, which was shown to be the most essential factor for necroptosis [69, 70], was involved in the production of mitochondrial ROS via energy metabolism [71].

The cell death studies by modulation of cellular antioxidant enzymes reveal a clear role of intracellular ROS in apoptosis. Particularly, the 2-Cys Prxs play a regulatory role in apoptotic, not necrotic, cell death (Figure 2). Prx1 was shown to protect lung cancer cells from radiation-induced apoptotic cell death by reducing JNK activation [72]. Interestingly, Prx1 prevented the JNK activation by retaining the JNK associated with glutathione S-transferase (GST)-pi, but not through the peroxidase activity. It was also shown that the expression of Prx1 in dopaminergic neuronal cells inhibited 6-hydroxydopamine-induced apoptotic death by reducing the p38/caspase-3 activation [73]. The level of Prx1 was obviously upregulated in human lung cancer patients and the Prx1 knockdown in hepatocarcinoma cells accelerated the TNF-related apoptosis-inducing ligand (TRAIL)-induced cell death via caspase-8/-3 activation [74]. Prx1 also mediated the disulfide-linked activation of the apoptosis signaling kinase ASK1 by forming a mixed disulfide intermediate with ASK1 in the peroxide-treated cells [75]. It has been shown that Prx2 and Prx3 reduce apoptotic cell death via mitochondrial-dependent intrinsic pathway [76, 77]. Interestingly, the redox cycle of the Prx3 activity shifted to the disulfide-containing oxidized state during Fas-mediated apoptosis of Jurkat and U937 monocytic cells [78]. Collectively, the evidence related to the 2-Cys Prxs strongly indicates that ROS is connected to the apoptotic cell death. Further exploration is needed to determine the molecular mechanism underlying antiapoptotic role of 2-Cys Prxs.

5. Signaling Role of 2-Cys Prxs Beyond Peroxidase Enzyme

Despite the 2-Cys Prx being a sophisticated peroxidase enzyme with a high affinity to H_2O_2 [79], recent studies also suggest that 2-Cys Prx can function as redox protein that regulates the activity of various client proteins by direct protein-protein interaction or interprotein disulfide linkage. In 1997, it was reported that Prx1 interacts with the SH3 domain of c-Abl and inhibits its tyrosine kinase activity [80]. It was the first report showing that the 2-Cys Prx is one of the redox proteins capable of regulating a key signaling kinase. Subsequently, Prx1 has been found to interact with the Myc Box II (MBII) domain of c-Myc by a yeast two-hybrid screen [81]. By this interaction, Prx1 contributed to an antioxidative stress function and it did also inhibit the c-Myc-dependent target gene expression and tumorigenesis. Park and her colleagues showed that Prx1 interacts with androgen receptor in various prostate cancer cell lines and GST-pi in lung cancer cell lines [72, 82]. The Prx1 interaction with androgen receptor promotes the receptor's transactivation activity. Later, it turned out that Prx1 increases the receptor affinity to dihydrotestosterone [83]. The findings seem to be important in relation to the high Prx1 expression in the prostate cancer patients [84]. Another interesting result was that Prx1 interacts and forms a mixed disulfide linkage with the GDE2 activation in spinal motor neurons [85]. In motor neuron progenitors, Prx1 promotes the GDE2 activity to drive a neuronal differentiation by reducing an

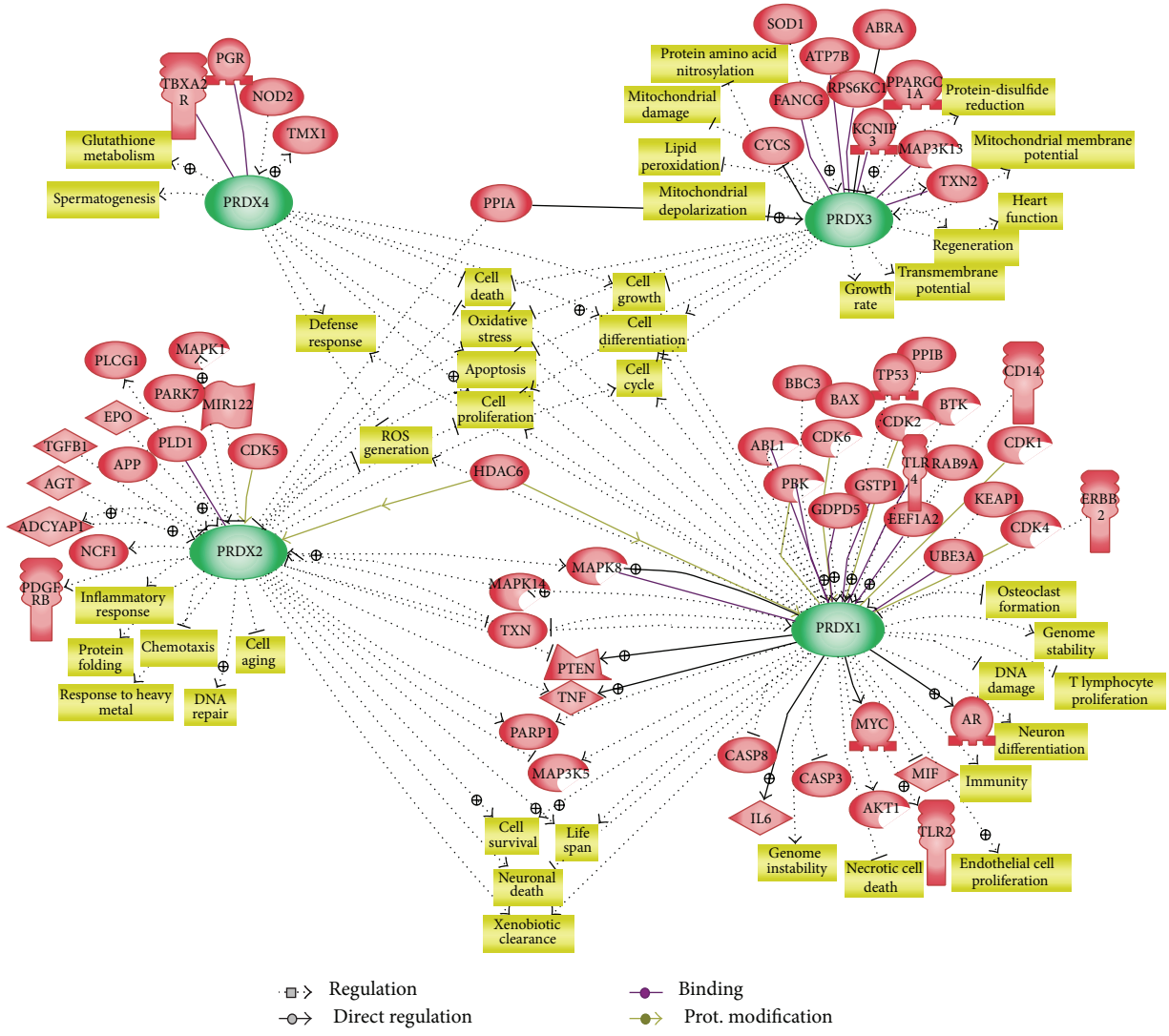

FIGURE 3: Summary of regulatory networks centered by typical 2-Cys Prxs. Functional correlation and interaction among the signaling proteins and typical 2-Cys Prxs are reconstituted into a network model using the *Pathway Studio* software (Ariadne Genomics Inc., USA). All molecules are shown as gene symbol. Direct and indirect regulations were indicated in gray lines and dotted gray lines, respectively. Green arrows indicate regulation by protein modifications. Purple lines indicate direct protein-protein binding.

intramolecular disulfide linkage in the cytoplasmic tail of the transmembrane protein. This evidence indicates that the Prx1 can function as a protein disulfide reductase (PDI). Other example of PDI activity among 2-Cys Prxs is Prx4 in endoplasmic reticulum. It was shown that the oxidized Prx4 transfers the disulfides to PDI [5, 6]. The Prx4 reoxidation is achieved by metabolizing H_2O_2 produced by Ero1, which is known as the main ER enzyme responsible for reoxidation of protein disulfide reductase [86]. This evidence defines a new role of Prx4 in oxidative protein folding along with Ero1.

In contrast to the case of Prx1, the closest isoform Prx2 has barely been shown to directly interact with any protein. Actually, few reports are stating that Prx2 colocalizes and interacts with phospholipase D1 in phorbol ester-stimulated cells [87] as well as interacts with the PDI family member, ERp46, when under its overoxidized form [88]. The *in vitro* activity assays showed that Prx2 is less active as a peroxidase enzyme than Prx1 [1, 39]. Given the *in vitro* evidence that the 2-Cys Prxs is inactivated by overoxidation during the reaction cycle proportional to the enzyme activity [8], it is conceivable that Prx1 is the peroxidase enzyme acting as the first line of antioxidant defense under H_2O_2 stress. Nonetheless, it turned out that Prx2 is more susceptible for overoxidation in the animal cells under H_2O_2 stress than Prx1 [39]. The same study shows that Prx1 rather prefers to be tyrosine phosphorylated under H_2O_2 stress *in vivo*. This discrepancy between *in vitro* and *in vivo* properties of Prx1 and Prx2 could be explained as a paradox: unlike the potential function assumed from the *in vitro* characterization, the Prx2 can be the real peroxidase enzyme in the cells while the Prx1 primarily functions as a redox regulator of diverse client proteins by

interaction. This idea is supported to some extent because it was observed that Prx1 was more abundant in protein amount than was Prx2 in certain cell types like fibroblasts and HeLa cells.

In an effort to obtain a global picture of regulations by 2-Cys Prxs, we finally produced a network model among typical 2-Cys Prxs using the *Pathway Studio* software. We obtained the network relations for all four Prxs in the knowledge base of Pathway Studio, which were built by text-mining of literature texts. False positives or indirect relations were removed by inspecting the relevant sentences manually. Figure 3 shows the resulting network model where relations specific to each Prx were located near the corresponding Prx and entities involved in more than one Prxs were positioned in the intervening space. This network includes the direct interaction of 2-Cys Prxs and their client proteins as mentioned above. As expected, it is evident that all four Prxs are closely related to apoptosis and cell death, ROS generation and oxidative stress, cell proliferation, growth, and differentiation. This diagram also illustrates biological processes and functions specific to each Prx or common between two Prxs. For example, Prx3 is specifically related to mitochondrial damage and lipid peroxidation. It can be readily seen that cytosolic enzymes Prx1 and Prx2 are related to cell survival via PTEN, TNF, MAP kinases, and PARP1. Overall, the network model emphasizes the importance of typical 2-Cys Prxs as hub molecules connecting cellular signaling pathways and biological processes.

6. Conclusion Remarks

Four members of typical 2-Cys Prx subfamily are present in various cellular compartments, including cytosol, plasma membrane (especially caveolae), nuclei, mitochondria, and endoplasmic reticulum. The majority of the abundant 2-Cys Prx enzymes primarily function as general antioxidant systems that maintain the intracellular ROS level within a safety zone in both normal and stressed cells. However, some part of the enzymes functions as the signal regulator at specific locations by modulating the local ROS change or by regulating the activity of the interacting/neighboring proteins in a redox-dependent manner. Since H_2O_2 is an important second messenger in a signaling network, the discovery of the 2-Cys Prx function related to signal transduction should provide clues necessary to understand redox signaling architecture and further solve medical problems in ROS-mediated chronic diseases.

Conflict of Interests

No potential conflict of interests was disclosed.

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Review Article

Linking Peroxiredoxin and Vacuolar-ATPase Functions in Calorie Restriction-Mediated Life Span Extension

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Calorie restriction (CR) is an intervention extending the life spans of many organisms. The mechanisms underlying CR-dependent retardation of aging are still poorly understood. Despite mechanisms involving conserved nutrient signaling pathways proposed, few target processes that can account for CR-mediated longevity have so far been identified. Recently, both peroxiredoxins and vacuolar-ATPases were reported to control CR-mediated retardation of aging downstream of conserved nutrient signaling pathways. In this review, we focus on peroxiredoxin-mediated stress-defence and vacuolar-ATPase regulated acidification and pinpoint common denominators between the two mechanisms proposed for how CR extends life span. Both the activities of peroxiredoxins and vacuolar-ATPases are stimulated upon CR through reduced activities in conserved nutrient signaling pathways and both seem to stimulate cellular resistance to peroxide-stress. However, whereas vacuolar-ATPases have recently been suggested to control both Ras-cAMP-PKA- and TORC1-mediated nutrient signaling, neither the physiological benefits of a proposed role for peroxiredoxins in H_2O_2-signaling nor downstream targets regulated are known. Both peroxiredoxins and vacuolar-ATPases do, however, impinge on mitochondrial iron-metabolism and further characterization of their impact on iron homeostasis and peroxide-resistance might therefore increase our understanding of the beneficial effects of CR on aging and age-related diseases.

1. Introduction

Caloric restriction (CR; or dietary restriction [DR]) is the only known intervention that extends the life span of organisms as divergent as yeast, worms, flies, fish, and primates [1, 2], an observation which might indicate the existence of a universal, conserved mechanism of aging. Despite almost 80 years of research since McCay's initial discovery that caloric restriction without malnutrition extended the life span of rats [3], the mechanisms underlying its retardation of the rate of aging are still incompletely understood [4–7]. Since decreased nutrient intake also lowers the incidence of many age-related maladies such as diabetes, cancer, and cardiovascular diseases in several organisms [8], intense efforts at identifying the molecular processes underlying these beneficial effects are underway in the aging researcher community.

Decreased signaling through nutrient-sensing pathways, for example, protein kinase A (PKA), target-of-rapamycin (TOR), or insulin-like growth factor (IGF) pathways [2, 9], is in several model organisms required for life span extension upon CR. These pathways regulate many downstream target processes important for cell growth and stress resistance [10]. However, the many targets and their highly interconnected nature have prevented the identification of targets important for aging. The views of some researchers in the field were, at least until rather recently, that life span extension by CR depends on the combined activity of many gene products acting through multiple pathways [2].

In contrast to this hypothesis, two recent reports have pointed to two unique target mechanisms for how CR postpones replicative aging in yeast by counteracting detrimental processes acting at the genesis of aging [11, 12]. We demonstrated that CR, through reduced PKA signaling, activates

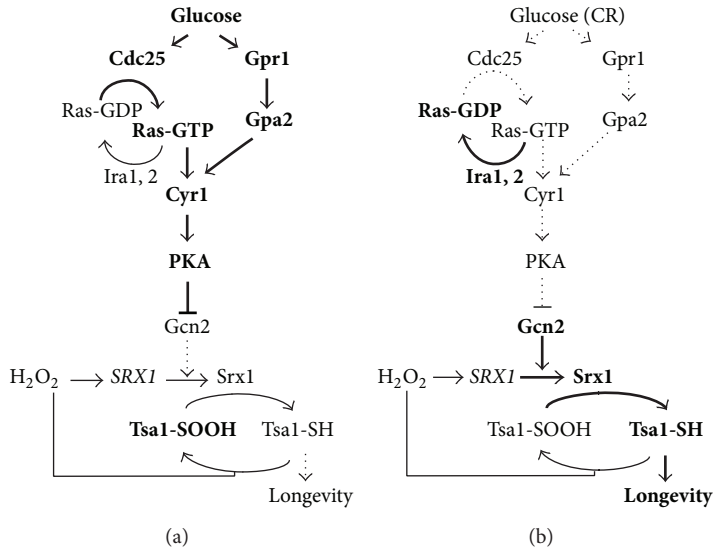

Figure 1: Model for how CR elicits Tsa1 and Srx1-dependent H_2O_2 resistance and life span extension. (a) At a high concentration of glucose, when increased signaling through both the Ras-Cyr1 and the Gpr1-Gpa2-Cyr1 signaling branches stimulate PKA activity (Box 1), H_2O_2 stress activates Yap1/Skn7-dependent transcription of the *SRX1* mRNA but its translation is attenuated by PKA. As a consequence, Srx1 production is diminished and Tsa1 hyper-oxidized and inactivated. (b) During CR, PKA activity is reduced relieving the translational inhibition of the *SRX1* mRNA in a Gcn2-dependent manner to provide more Srx1 protein and, as a consequence, more reduced, peroxidase-active Tsa1.

the peroxiredoxin (Prx) Tsa1, an antioxidant protein reducing H_2O_2, to prolong yeast life span [12]. Strikingly, whereas wild-type cells responded to CR (and reduced PKA activity) by an increased life span, *TSA1* mutants did not, identifying Tsa1 as a key enzyme extending life span during CR. In line with this, CR stimulated Tsa1 activity through increasing the levels of the Prx reducing enzyme Srx1, which reduces hyperoxidized (sulfinylated) Tsa1, and ectopically increasing Srx1 levels was sufficient to retard aging in calorie-replete medium (Figure 1). Interestingly, the CR-induced increase in Srx1 levels did not involve increased transcript levels, but rather appeared to result from Gcn2-dependent increased translation of the *SRX1* mRNA (Figure 1(b)). Increased Srx1 levels are expected to increase both the recycling of hyperoxidized Tsa1 and as a consequence also the ability of Tsa1 to reduce peroxide (peroxidase activity). However, Prx are not only H_2O_2-reducing enzymes but also function in H_2O_2-signaling and in proteostasis (see below) and it is currently not clear which facet(s) of peroxiredoxin function that is/are required for CR-induced longevity.

Interestingly, a recent report from the Gottschling lab identified increased vacuolar pH as an early-age promoter of age-induced mitochondrial depolarization and fragmentation leading to replicative aging in yeast (Figure 2(a), [11]). Caloric restriction, as well as reducing the activities of conserved nutrient signaling pathways (e.g., Ras-cAMP-PKA and TORC1), delayed an age-induced loss of vacuolar acidity suggesting that the control of vacuolar pH also constitutes a target process regulated by CR (Figure 2, arrows i and ii, [11]). Mutating subunits of the vacuolar proton-translocating ATPase (v-ATPase) leads to increased vacuolar pH and accelerated aging (Figure 2(a)). Conversely, restoring vacuolar acidity in aging cells retarded aging (Figure 2(b)), suggesting that the control of vacuolar pH critically regulates aging. The authors furthermore observed that increasing vacuolar pH decreased the import of cytosolic amino acids into the vacuole through the H^+-neutral amino acid antiporter Avt1, and both mitochondrial dysfunction and replicative aging were in part coupled to this reduced transport (Figure 2(a)). How increased levels of cytosolic amino acids induce mitochondrial dysfunction and replicative aging was not addressed. As we will discuss later, however, both v-ATPase and a cytosolic neutral amino acid (leucine) have been implicated in the regulation of nutrient signaling [13–15], raising the question whether v-ATPase and cytosolic amino acids affect aging via feedback regulation of nutrient signaling pathways.

The purpose of this paper is to review the physiological impact of both Prx-mediated stress defense and vacuolar pH control and to pinpoint possible common denominators between these two apparently distinct target mechanisms proposed for CR-mediated life span extension. We will then point to outstanding questions that need to be resolved to further understand the beneficial impact of caloric restriction on aging and age-related diseases. To achieve this, we will first review the roles of ROS and peroxiredoxins in aging and in CR-mediated longevity. Next, we will shortly consider nutrient and glucose signaling in general and through the lenses of the free radical theory of aging in particular, since these signaling pathways are conserved mediators of CR life span extension in most organisms. Following this, we will discuss the cellular roles of v-ATPases including recent observations that these enzymes, via regulating intracellular pH homeostasis, are implicated in the regulation of nutrient signaling. On a final note, we will discuss points of intersection between v-ATPases and Prxs identified here, which

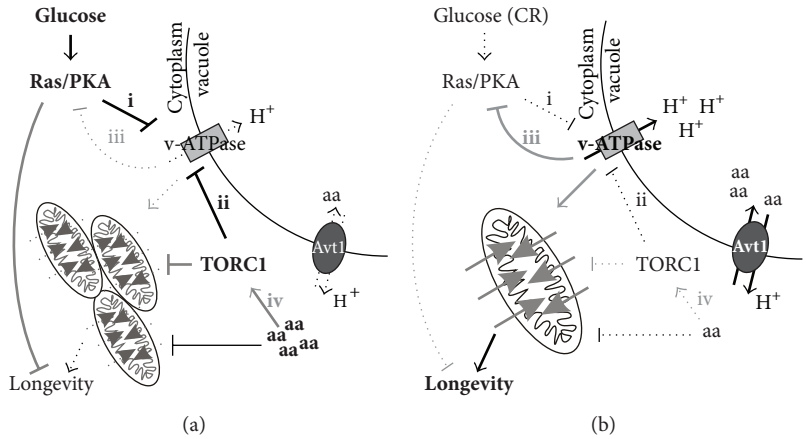

Figure 2: Model for how CR postpones mitochondrial deficiency and aging via stimulating the function of vacuolar ATPase (v-ATPase) and the import of neutral amino acids into the vacuolar lumen. (a) Upon high glucose levels increased Ras-cAMP-PKA (Ras/PKA) activity inhibits v-ATPase function (arrow i) and thereby also the neutral aminoacid transporter Avt1 leading to the accumulation of neutral amino acids in the cytoplasm. Among these, leucine has been proposed to activate TORC1 through the leucyl-tRNA synthetase LeuRS and the Gtr1 GTPase (arrow iv). Cytosolic amino acid accumulation is thought to decrease mitochondrial function and to stimulate aging, possibly via increased TORC1 signaling. Increased TORC1 activity would also be expected to further inhibit v-ATPase function (arrow ii). Reduced v-ATPase function might also be expected to releive v-ATPase inhibition of Ras-PKA via cytosolic alkalinization (arrow iii). (b) CR and reduced glucose inhibition of v-ATPase function by Ras-PKA (arrow i) stimulates Avt1-mediated uptake of neutral amino acids. Lower cytoplasmic leucine levels would be expected to reduce TORC1 activity (arrow iv) and TORC1-mediated repression of v-ATPase activity (arrow ii). Similarly, increased v-ATPase function might be hypothesized to increase the inhibition of Ras-PKA activity, possibly via cytosolic acidification. Mitochondrial functions are maintained under conditions of reduced Ras/PKA and TORC1 activity as well as reduced levels cytosolic amino acids, which stimulates longevity. Arrows in black represent mechanisms at least in part experimentally verified to be in operation in aging cells [11] whereas arrows in grey indicate mechanisms inferred based on recent data implicating cytosolic pH and v-ATPase in the regulation of Ras-PKA [14, 16] as well as cytoplasmic leucine levels in the regulation of TORC1 signaling [13].

include recent papers implicating both pathways in iron metabolism.

2. Reactive Oxygen Species (ROS) in Aging and in Caloric Restriction-Mediated Life Span Extension: Damaging Agents or Signal Transducers?

In aerobic organisms, incomplete reduction of oxygen in the mitochondrial respiratory chain leads to the production of reactive molecules called reactive oxygen species (ROS) [17]. These reactive molecules mainly include hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and hydroxyl radical ($OH^\cdot$) and they may damage macromolecules, including proteins, lipids, and nucleic acids [18].

The most widely accepted aging theory, the free radical theory, proposed by Denham Harman in 1956, states that damage caused on biomolecules by ROS/free radicals might be an underlying cause of the aging process [19]. Consistent with this hypothesis, H_2O_2 levels have been shown to increase with the replicative age of yeast mother cells [20, 21] and with the chronological age of both rats and mice [22, 23]. Whereas H_2O_2 in itself mainly oxidizes cysteine or methionine residues, its conversion into the highly reactive $OH^\cdot$ via metal-catalyzed oxidation reactions, may inflict widespread damage [18]. Macromolecular damage has been observed to increase with age in many organisms [18, 24–26]. Furthermore, a correlation between the amount of oxidized proteins and the rate of aging was reported in flies and in cultured human fibroblasts [18, 27]. The levels of oxidized proteins have also been shown to increase with age in rat liver and brain extracts. In many organisms studied, CR postpones the increased production of ROS seen with age [28, 29], increases the resistance to oxidative insults [12, 30, 31], and reduces oxidative damage [32, 33].

On the other hand, observations that too low levels of ROS lead to deficiencies in proliferation and immune responses [34], suggest that a balanced level of oxidants is necessary for the normal functions of cells and organisms. Illustrating this, proliferation and growth factor signaling in multicellular organisms involve controlled production of O_2^- or H_2O_2 by, for example, NADPH oxidase enzymes [35]. Superoxide anion is instable due to its reactivity and it cannot diffuse through membranes, which makes it a poor signaling molecule [17, 36]. In contrast, the half-life of H_2O_2 is longer and since it is an uncharged molecule it can diffuse through membranes [37]. H_2O_2 can also be transported across biological membranes through aquaporin channels. Therefore, it is not surprising that most ROS signaling involves signal transmission via H_2O_2 [17].

ROS signaling has been implicated in a variety of cellular events, for example, development and growth, oxidative stress resistance and even in the retardation of aging [38]. In particular, adaptive responses to moderately increased ROS levels have been proposed to induce antioxidant defences and hence resistance to ROS and longevity upon CR [38, 39]. In a recent study, thiol-peroxidases were shown to be the main

receptors for H_2O_2 signaling in yeast cells, since the absence of all the eight thioredoxin-dependent peroxidases (five Prxs and 3 glutathione-peroxidase homologs) resulted in an inability to sense and respond to externally added H_2O_2 [40]. However, for most of the thiol peroxidases/Prxs, the signal transduction pathways regulated are not known. A dual role in peroxide scavenging and signaling has been proposed for thiol peroxidases in several organisms [41–43].

Stimulation of longevity by ROS signaling is suggested by the observation that treatment of *C. elegans* worms with low levels of the superoxide anion generator paraquat increased lifespan by more than 50% [44]. Such a prolongevity role of ROS is, however, only seen at moderately increased ROS levels, since higher doses of paraquat reduced life span and low levels of the drug were toxic in worms lacking detectable SOD activity. Growing yeast cells under CR (0.5% glucose or lower) appears to lead to both higher respiration [45] and higher mitochondrial ROS production, as suggests the life span of cells lacking the mitochondrial SOD, *SOD2*, that is normal at high glucose levels but drops precipitously at glucose levels of 0.5% or lower [46]. This deficiency could be suppressed by the addition of the antioxidant ascorbic acid supporting the idea that *sod2* mutants suffer excessive ROS levels at reduced glucose levels. Similarly, CR has been observed to increase respiration and mitochondrial ROS production in mice and worms and these increases were reported to be required for the induction of both antioxidant defences and longevity [47, 48].

In summary, ROS appear to extend life-span, presumably by inducing adaptive ROS signaling, at low levels, but are toxic at higher levels. CR may mitigate oxidative damage by stimulating the cellular ROS defence capability through an increase in mitochondrial ROS production.

3. Peroxiredoxins in ROS Defence and in the Retardation of Aging

Prxs are an evolutionarily highly conserved antioxidant enzyme family which members reduce intracellular peroxide levels through a cysteine-based mechanism [40, 49]. Prxs were first discovered in yeast [50], *E. coli* and *S. typhimurium* [50, 51] but are now found to be widespread across phylogeny with one or more members typically being abundantly expressed in many aerobic organisms. Prxs are divided into groups based on the number and the position(s) of catalytic cysteine residues. The major subclass is the 2-Cys Prxs that form homodimers and catalyze the reduction of peroxide via two conserved catalytic cysteine residues [52, 53]. Catalysis is initiated by the reduction of H_2O_2 by a conserved N-terminal cysteine residue (the peroxidatic cysteine) which, in turn, oxidizes to the sulfenic acid form (Cys-SOH). In 2-Cys Prxs, the peroxidatic Cys-SOH then condenses with the second catalytic cysteine residue present in the other monomer to form a disulfide that is reduced by thioredoxin, thus completing the catalytic cycle. However, the peroxidatic Cys-SOH might react again with H_2O_2, when the levels of it are high, which leads to the formation of a sulfinic acid (Cys-SOOH), and enzyme inactivation (Figure 1). Such inactive form of Prx is slowly reactivated by ATP-dependent reduction of the sulfinic acid by sulfiredoxin (Srx1, [54]).

Prx deficiency accelerates aging in yeast, worms, flies, and rodents [42, 55–57]. In addition, increasing the levels of a neuronal Prx in flies [55] or increasing sulfiredoxin levels in yeast [12], extends life span (Figure 1(b)), suggesting that the anti-aging function of Prxs is conserved [58]. In addition, mice deficient in PrxI age prematurely and develop several malignant cancers indicating that Prxs prevent a prevalent group of age-related diseases [56]. Yeast cells lacking Tsa1 suffer from increased mutation rates and genome instability [59], which at least to some extent has been linked to increased oxidative DNA damage [58, 60].

The genome of the budding yeast *S.cerevisiae* encodes for eight thioredoxin-dependent peroxidases. Five of these are Prxs proper (Tsa1, Tsa2, Ahp1, Dot5, and Prx1) and the other three Prx-like enzymes with sequence homology to glutathione peroxidases (Gpx1, Gpx2, and Gpx3) [40, 61, 62]. Although these Prxs are thought to constitute an important part of the antioxidant arsenal, *S. cerevisiae* cells lacking the eight yeast thioredoxin-dependent peroxidases are viable, do not appear to contain increased levels of ROS and can withstand a certain level of oxidative stress [40]. Interestingly, this mutant still suffers from a reduced replicative lifespan which might therefore be caused by the lack of functions other than direct ROS detoxification. However, these data somewhat contradict earlier reports that yeast cells lacking all five Prxs contain increased ROS levels and are genomically unstable [63]. To settle this issue, specifically in the context of aging, it will be important to monitor intracellular ROS levels using modern, more sensitive and specific ROS sensors [64, 65] in aging Prx-sufficient and -deficient cells [20, 21].

Tsa1 contributes 91% of all Trx dependent peroxidase activity in yeast cells [62]. Yeast Tsa1 and mammalian Prx1 share 65% amino acid identity [66]. Similar to Tsa1, the 2-Cys Prxs Prx1, and Prx2 are also very abundant in mammalian cells, which despite poor catalytic efficiency might indicate that they are important in H_2O_2 scavenging [67].

Tsa2 is a 2-Cys Prx 86% sequence identical to Tsa1 but that is expressed only at ~80-fold lower levels than Tsa1 [68]. In agreement with its low expression, cells lacking Tsa2 are not deficient in ROS defence, but in fact, paradoxically, appear to grow better than wild-type cells in the presence of H_2O_2 [12]. However, in cells lacking *TSA1,* the levels of Tsa2 are increased [69] and cells lacking both *TSA1* and *TSA2* are more sensitive to H_2O_2 than the *TSA1* single mutant [70], indicating that the two cytosolic Prxs cooperate in peroxide defence.

2-Cys Prxs are multifunctional enzymes which, in addition to their peroxidase activity required to cope with oxidative stress, can act as chaperone holdases to counteract protein damage and as H_2O_2 signaling devices [71, 72]. During peroxidase catalysis, further oxidation of the peroxidatic cysteine of a 2-Cys-Prx from sulfenic acid into sulfinic acid leads to the inactivation of its peroxidase activity. Interestingly, hyperoxidation stabilizes the formation of high-molecular-weight Prx complexes (dodecameric and higher order decameric derivative forms) [73, 74], which carry increased chaperone activity [75]. Similar alterations in quaternary structure were observed upon increased temperature

or reduced pH and were also found to stimulate the ability of Prxs to prevent the aggregation of model proteins *in vitro* [74, 76]. Reversion of the sulfinylated form of Tsa1 by ATP- and sulfiredoxin-dependent reduction indicates that sulfinylation is a redox-switch that alters the function of Prxs from a peroxidase to a chaperone. Prxs have been shown to interact with a multitude of signaling proteins and the redox-dependent oligomerization of 2-Cys-Prxs may therefore be important also in the regulation of signaling [75].

Interestingly, accumulation of the hyperoxidized forms of the yeast and rat Prxs, Tsa1, and PrxIII, have been reported upon aging [12, 77], indicating that Prx inactivation may be a common phenotype in aging organisms (Figure 1(a)). It is, however, not clear whether increased hyperoxidation with age is due to increased levels of H_2O_2 [20–23] or deficient Srx1-mediated Prx de-sulfinylation [12] and whether this in turn controls a switch to the chaperone-function [76] or modulates Prx-dependent H_2O_2 signaling [40, 78].

In summary, Prxs are multifunctional enzymes that retard aging and it is currently unknown which of their functions is/are important to retard aging. It appears that the signaling role of Tsa1 may be discarded, however, since cells lacking *TSA1* do not appear to display altered gene expression [12, 40] yet age at a faster rate and fail to extend life-span upon CR [12]. The function of yeast Tsa1 in CR-mediated longevity appears to require Srx1 since the recycling of hyperoxidized Tsa1 is necessary and sufficient for CR life span extension. However, it is currently not clear what is the beneficial effect of Tsa1 recycling during aging; the consequential increased Tsa1 peroxidase activity or a function linked to enzyme chaperone activity? Given the unique ability of Prx peroxidase activity to scavenge low levels of endogenous peroxide to protect the genome [67], the determination of mutation rates and/or DNA damage in aging CR cells would give an indication of the importance of the scavenging function during aging.

4. Calorie Restriction, Glucose Signaling, and the Link to the Free Radical Theory of Aging

The three conserved nutrient sensing kinases that mediate life span extension by CR in yeast are the protein kinase A (PKA), the target-of-rapamycin-complex 1 (TORC1) kinase, and the Akt/protein kinase B/ribosomal S6 kinase homologue Sch9 [4, 79, 80]. Partly reduced activites in any of these three pathways mimic the effect of calorie restriction on aging. Accordingly, partial inactivation of PKA catalytic subunits, cAMP synthesis, or regulatory proteins of the pathway (e.g., Cdc35/Cyr1, Cdc25, Gpr1, and Gpa2 in yeast) are frequently used as calorie restriction mimetics and mitigate aging and/or age-related diseases in mice [9, 81, 82], *Drosophila* [83], and yeast ([4, 84], Figure 1(b)). Since both the TORC1/Sch9 and Ras-cAMP-PKA pathways control mitochondrial activity and stress-defenses, nutrient signaling clearly impinges on pathways relevant in the context of the free radical theory of aging [10]. For example, in both yeast cells and mouse adipose tissue TORC1 negatively controls respiration [10, 85]. Similarly, the reprogramming of yeast metabolism upon glucose depletion and growth on respiratory carbon sources requires reduced activity of the Ras-cAMP-PKA pathway, which inhibits of mitochondrial biogenesis and mitochondrial activity [10]. Many yeast antioxidant enzymes are also under negative control of the Ras-cAMP-PKA pathway, for example, cytosolic catalase [86], all five yeast Prxs [12, 87–89] and the mitochondrial SOD [90]. TORC1 similarly represses at least some of the yeast antioxidants. Most of the studies on CR-mediated longevity in yeast have focused on the impact of reduced glucose levels on aging and a short review of yeast glucose signaling can therefore be found below (Box 1).

In conclusion, the major regulatory impact of the nutrient signaling pathways on mitochondrial activity and on antioxidant defence pathways indicate that the mechanisms underlying their effects on the rate of aging and CR-induced longevity is compatible with the free radical theory of aging. In agreement with this, decreased nutrient signaling increases the resistance to oxidative stress, presumably at least in part because increased ROS defences more than offset an increased mitochondrial activity.

Box 1 (*Yeast Glucose Signalling*). Glucose is the most abundant monosaccharide in nature and a rich carbon source that is preferred as a primary energy source by a variety of organisms, from yeast to humans. In *S. cerevisiae*, it has been shown that upon glucose addition the expression of about 40% of the genes in the genome is altered, indicating a substantial reprogramming of gene expression [91]. The response to glucose is mainly regulated by the Ras-cAMP-PKA pathway [92]. Yeast has a G protein coupled receptor (GPCR) (Gpr1) that can accommodate glucose or sucrose as ligands to activate the G-protein Gpa2 [93]. Gpa2 in turn stimulates the PKA signaling pathway via adenylate cyclase (Cyr1) and cAMP levels (Figure 1, [93]). However, the most important part of the response to glucose is thought to involve the stimulation of Ras activity (Figure 1, [94, 95]), the mechanism of which still remains elusive despite extensive research. Ras2 (and to a minor extent Gpa2) activation more or less fully recapitulates the transcriptional response to glucose addition through stimulating PKA activity [91], arguing that PKA activation is sufficient for the response to glucose. Whereas glucose still stimulates cAMP synthesis in the absence of Gpr1 or Gpa2, the cAMP increase seen upon glucose addition is lost in cells deficient in Ras [96]. Of note, glucose phosphorylation is required for Ras activation and cAMP synthesis indicating that the Ras proteins may respond to the levels of a glucose metabolite.

5. Roles for Intracellular pH Homeostasis and Vacuolar ATPases in Nutrient Signaling

Recent data on yeast nutrient sensing suggest that nutrient signaling is intimately connected to v-ATPase function and intracellular pH homeostasis. V-ATPase deficiencies in aging cells thus likely directly impinge on the activation of nutrient signaling pathways. To better understand this connection, we will next shortly review the cellular roles of v-ATPases.

H^+-ATPases are important contributors to cellular pH homeostasis. In addition to being localized at the vacuolar

membrane, where they (v-ATPases) acidify the vacuole, they are also found at the plasma membrane of several mammalian cell types [97–100], where they pump protons towards extracellular space [101]. In yeast cells, pH homeostasis is maintained by two proton pumps; Pma1, which resides at the plasma membrane, and v-ATPases, which reside within the membranes of multiple organelles (in particular the vacuole and the Golgi). These two H^+-ATPase pump systems seem to function coordinately since any defect in the v-ATPase causes altered localization of the plasma membrane H^+-ATPase, Pma1, presumably to maintain intracellular pH homeostasis [102].

All eukaryotic v-ATPases consist of two subcomplexes, V_0, and V_1, that are formed from 6 and 8 subunits, respectively [103]. Deletion of any v-ATPase subunit is lethal in all organisms except fungi [104]. In yeast cells, v-ATPase subunit deletions lead to a growth phenotype called the "*vma* phenotype," which is characterized by an inability to grow on respiratory carbon sources, a sensitivity to increased extracellular pH, to heavy metals and to oxidants [104]. Accordingly, several genes encoding vacuolar proteins, for example, those important for vacuolar acidification, were identified in a genome-wide screen for genes important for oxidant tolerance suggesting that v-ATPase function is required for proper antioxidant defences [105].

In yeast, pH homeostasis is intimately linked to glucose utilization. The addition of glucose to starved cells leads to a rapid and very transient cytosolic acidification (30 seconds, [106, 107]), which is thought to be caused by the initiation of glycolysis, followed by its alkalinization through the H^+-ATPase activities of Pma1 [108] and the v-ATPase [102] upon which growth recommences [106] and cAMP levels increase [14]. Cytosolic alkalinity correlates closely with glycolytic rates [14] and would eventually be expected to inactivate v-ATPase function because of reduced substrate availability (Figure 2(a), [102]). Mutants lacking v-ATPase activity, grown at steady state in high glucose, display acidic cytosolic pH [14, 102], in agreement with a role for v-ATPase in cytosolic alkalinization. Upon glucose withdrawal, the v-ATPase is inactivated by disassembly of its two subunits, which lowers cytosolic pH and reduces the activity of the PKA pathway [14]. Presumably, upon reduced glucose (CR), the v-ATPase is subsequently reactivated to promote vacuolar acidification and/or to maintain cytosolic pH homeostasis (Figure 2(b), [11]). In agreement with this, cytosolic pH drops continuously as yeast cells grow and consume glucose [16], but cytosolic and vacuolar pH have not been measured during replicative aging of CR cells. In addition, although the measurement of vacuolar pH was used to argue for a role for v-ATPase mediated vacuolar acidification in CR-mediated longevity [11], cytosolic pH was never measured and thus the full scope of increased v-ATPase activity in aging CR cells was not assessed. These data thus raise the question whether v-ATPase-mediated regulation of cytosolic pH contributes to CR life span extension by influencing nutrient signaling (Figure 2). Importantly, cytosolic pH appears intimately connected to the cellular nutrient status since both glucose starved cells and cells grown on respiratory carbon sources show reduced cytosolic pH [16]. In fact, cytosolic pH was proposed to control the cell division rate both in yeast cells [16] and in *Xenopus* oocytes [109]. Thus, to better understand life span extension upon CR it would be important to monitor also cytosolic pH as cells age.

The assembly/disassembly of v-ATPases is fast and constitutes an important regulatory mechanism of v-ATPase function. The role of the Ras-cAMP-PKA pathway in these events is, however, controversial. As mentioned above, v-ATPase assembly is stimulated by glucose *in vivo* [110] and this process was reported independent on cAMP-PKA and the conventional glucose signaling pathways, but dependent on glucose metabolism beyond glucose-6-phosphate [111]. V-ATPase complex integrity independent on the Ras-cAMP-PKA pathway is supported by the unaltered disassembly of the Vma5 subunit of the V_1 domain upon glucose withdrawal in *ira2* mutant cells, as observed by microscopical observation of a Vma5-RFP fusion protein [14]. In contrast, a genetic screen in yeast for mutants lacking v-ATPase disassembly upon glucose withdrawal identified loss of *IRA1*, *IRA2* and *BCY1* gene functions [101], which negatively regulate Ras-cAMP-PKA (Figure 1, Table 1). Furthermore, vacuolar acidity seems reduced in *ira2* mutant cells *in vivo* [11], also suggesting that *IRA2* is essential for proper v-ATPase function (Figure 2(a), Table 1). Thus it is not clear at this point if v-ATPase is acting upstream of or downstream of the Ras-cAMP-PKA pathway or if it participates in a complex feedback loop involving Ras-cAMP-PKA (Figure 2). Reciprocal regulation of PKA and v-ATPases has been proposed to reinforce PKA activation and v-ATPase assembly upon glucose sensing [14] but the roles of glucose and the Ras-cAMP-PKA pathway in v-ATPase assembly and disassembly clearly need further clarification.

As discussed earlier, part of the increased longevity and the maintenance of mitochondrial function achieved by vacuolar acidification could be linked to the uptake of neutral amino acids into the vacuole (Figure 2(b), [11]). Supporting this, overproduction of the vacuolar neutral amino acid antiporter Avt1 reduced age-induced mitochondrial dysfunction and extended life span. In addition, overexpression of the v-ATPase subunit Vma1 only partly suppressed age-induced mitochondrial dysfunction in cells lacking *AVT1* (Figure 2(b), [11]). In this respect it is interesting that v-ATPase and the proton-driven transport of lysosomal amino acids have been implicated in the activation of mammalian TORC1 in response to amino acids [15]. In yeast, TORC1 is activated by cytosolic leucine, the most frequently utilized (and neutral) amino acid (Figure 2(b), arrow iv), by a mechanism involving the cytoplasmic leucyl-tRNA synthetase LeuRS charged by leucine and the GTPase Gtr1 [13]. It is thus conceivable that the reduced v-ATPase-function and vacuolar proton-gradient in aging cells promotes both PKA and TORC1 activation via inefficient transport of neutral amino acids into the vacuole.

In summary, CR, via reduced glucose intake, may repress PKA activity through increased v-ATPase function and reduced cytosolic pH (Figure 2(b), arrow iii). Accordingly, maintained v-ATPase activity would extend life span via feedback regulation of both Ras-PKA- and TORC1 pathways, the latter via reducing cytosolic leucine levels.

Table 1: Common denominators of v-ATPase and peroxiredoxin functions in yeast which might impinge on calorie-restriction-mediated life span extension. For more details see the text. O/e: overexpression; Fe: iron; Δ: deletion mutant.

	Protein kinase A	H_2O_2 resistance	Fe-metabolism
Vacuolar ATPase	v-ATPase disassembly [101] and v-ATPase-driven vacuolar acidification [11] both inhibited in strains lacking *IRA2*	*vma2*Δ (V_1 domain) and *vma3*Δ (V_0) mutants very sensitive to H_2O_2 at permissive pH 5 [112]	O/e *VMA1* or *VPH2* suppressed age-induced loss of mitochondrial membrane potential [11]. Age-induced loss of mitochondrial DNA causes loss of membrane potential and defects in Fe/S-cluster biogenesis [113]
	v-ATPase activity regulates PKA activity upon glucose addition [14]	*vph1*Δ (V_0 vacuole) moderately sensitive to H_2O_2 at pH 5 [112]	v-ATPase inhibition by concanamycin A caused rapid loss of mitochondrial membrane potential [11]
			Reduced v-ATPase function suppresses defects associated with the loss of mitochondrial DNA and membrane potential [114]
			Aft1 is required for the survival of a strain lacking *VMA2* [115]
Peroxiredoxins	Tsa1 peroxidase function stimulated at low PKA activity (*gpa2*Δ, *gpr1*Δ, *cdc35-1*, o/e *PDE2*) [12]	*tsa1*Δ sensitive to H_2O_2 but not tert-butyl-OOH [116]	O/e *FRA1* suppressed slow growth and increased Aft1-dependent transcription in a strain lacking mitochondrial high-affinity Fe-transport (*mrs3*Δ *mrs4*Δ), in a *TSA1* dependent manner; Tsa1 interacts physically with Fra1 [117]
			Tsa1 and Tsa1Cys48 are required for aerobic growth of an *mrs3*Δ*mrs4*Δ strain [117]
	Tsa2 levels increased at low PKA activity (*ras2*Δ) [88]	*tsa2*Δ slightly resistant to H_2O_2 [12], *tsa1*Δ *tsa2* more sensitive than *tsa1*Δ [70]	*Tsa2*, as well as Tsa2C48, represses the expression of an Aft1-target (*FIT2*) in wt and *vma2*Δ, *Tsa2* binds to Fra1 in *vma2*Δ, increased *TSA2* levels in *vma2*Δ suppressed by Fe supplementation [115]

Similarly, amino acid-restriction could be expected to efficiently reduce TORC1 activity through increased v-ATPase function and Avt1 controlled amino acid transport into the vacuole (Figure 2(b), arrows ii & iv), thus also repressing Ras-cAMP-PKA activity (Figure 2(b), arrow iii). Future studies should address the roles of v-ATPase in feedback regulation of nutrient signaling pathways and in the cross-talk between the pathways.

6. A Link between Calorie Restriction, Vacuolar Acidification, and Peroxiredoxins in the Regulation of Aging

The compilation of data from different organisms identified vacuolar functions among several conserved genes essential for CR to extend life span [118]. In addition, a recent study examining the life spans of 166 yeast deletion mutants upon CR suggested that both loss of vacuolar pH control or antioxidant defences negatively affect life span during CR [46], suggesting that both processes are essential for CR life span extension. It is clear from the studies reviewed here that both Ras-PKA and TORC1 interact reciprocally with v-ATPase functions. Furthermore, the expression and activites of Prxs is regulated by nutrient signaling pathways during CR or changes in carbon source in the media [12, 88].

Regarding potential connections between v-ATPase and Prxs, the pH-regulated oligomerization and chaperone activity of a *Schistosoma mansoni* Prx *in vitro* [74] is certainly intriguing but the occurrence of Prx oligomerisation at acidic pH is currently unknown *in vivo*. As mentioned above, in starved yeast cells pulsed with glucose, cytosolic pH has been reported initially to reach as low as 5.3 [106], which is still significantly higher than the pH where oligomerisation of the *S. mansoni* Prx was observed *in vitro* (pH 4.2) [74]. Thus, it will be interesting to know whether Prxs from other organisms also oligomerize at acidic pH and at *in vivo* relevant values.

Another still intriguing connection between v-ATPase and oxidative stress, and hence the Prxs, is the observation that a yeast mutant lacking v-ATPase function (*vma2*Δ) is exquisitely sensitive to H_2O_2 [112] and less affected by other oxidants (Table 1) when grown under conditions that minimize the *vma* phenotype (i.e., acidic extracellular pH). Under these conditions *vma2*Δ cells also display elevated DCDF-DA staining, that might indicate elevated intracellular ROS, and increased protein carbonylation [112]. In addition, *vma2*Δ*tsa1*Δ mutants display a severe synthetic growth phenotype indicating that they share a function important in cell physiology [115].

Iron metabolism may be yet another connection between v-ATPases and Prxs. In cells lacking *VMA2*, microarray analyses revealed prominent induction of genes that are involved in iron metabolism under the control of the transcription factor Aft1 [112]. The link between v-ATPase and iron metabolism was further supported by the synthetic lethality of a strain lacking both *VMA2* and *AFT1* [115]. In yeast, iron is indirectly sensed through the amount of Fe/S-cluster proteins that are matured in the mitochondrial

matrix [119, 120], which itself is a function of cellular iron availability and of mitochondrial Fe/S-biogenesis. In a follow-up study, the *vma2*Δ mutant was, in accordance with a defect in mitochondrial Fe/S-cluster biogenesis, shown to contain total iron levels similar to the wild-type but, importantly, to display reduced activity of aconitase, a mitochondrial Fe-S cluster enzyme [115]. Importantly, adding a weak acid to wild-type cells which, like deficient v-ATPase function, is expected to acidify the cytosol, and mitochondria [106] was sufficent to induce Aft1 transcription [115]. These data indicate that v-ATPase-regulated pH homeostasis is crucial for mitochondrial Fe-S cluster biosynthesis/biogenesis. However, Fe-S clusters have been reported to become unstable at low pH [121, 122] indicating that v-ATPase-regulated pH homeostasis also may impinge on Fe/S-cluster stability. Interestingly, deficient mitochondrial Fe-S cluster biogenesis and growth arrest in daughters of replicatively aged yeast mother cells was earlier linked to the loss of mitochondrial DNA and mitochondrial membrane potential [113], suggesting that mitochondrial Fe-S clusters become instable with age as a result of mitochondrial DNA damage. Reciprocal crosstalk between mitochondria and v-ATPase regulated pH homeostasis is suggested by the observation that defects associated with the loss of mitochondrial DNA (e.g., slow growth and defective mitochondrial protein import) can be suppressed by loss of v-ATPase function [114] (Table 1). Taken together, these data raise the possibility that reduced vacuolar acidity in aging cells [11] may be an adaptation to suppress certain phenotypes associated with mitochondrial deficiency, but which on the contrary might exacerbate yet others, such as reduced mitochondrial Fe/S cluster biogenesis. Future studies are clearly necessary to identify mechanisms causing the loss of v-ATPase function with age as well as the intricate interplay between v-ATPase mediated pH homeostasis and mitochondrial function.

Is there now a link between Prxs and any of these phenotypes? Interestingly, *TSA2* transcription, but not the transcription of other antioxidant genes, is increased upon decreased v-ATPase function (in cells treated with concanamycin A or cells lacking *VMA2*, Table 1, [112, 115]). In line with a role in iron metabolism, increased *TSA2* transcription in a *vma2*Δ mutant could be suppressed by supplementing cells with iron. In addition, in cells lacking *TSA2* (*tsa2*Δ and *vma2tsa2*Δ), but not in peroxidase-negative mutants, expression of the Aft1-target gene *FIT2* is increased (4-fold and 2-fold, resp.), indicating that *TSA2* represses Aft1-activity in a manner independent of Tsa2 catalytic activity. Furthermore, Tsa1 and its peroxidatic cysteine appear necessary to support aerobic growth of cells lacking high affinity iron transport across the mitochondrial membrane by *mrs3mrs4* deficiency [117] (Table 1). Interestingly, Mrs3- and Mrs4-deficient cells require a repressor of the iron regulon, Fra1, for aerobic growth [117] and suppression of the aerobic growth defect of *mrs3mrs4* deficient cells by Fra1 required *TSA1* (Table 1). Taken together, repression of Aft1 transcription by Tsa2 and the interactions of both Tsa1 and Tsa2 with the iron regulatory protein Fra1 indicate a link between Prx and iron metabolism that will need to be elucidated at the molecular level.

Hence, it seems that both vacuolar pH control and Prx activity are linked to iron homeostasis and Fe/S cluster biogenesis and a further characterization of their impact on these vital processes appears important. More specifically, a better understanding of the role of Prxs in longevity requires more careful assessment of their described roles in peroxide scavenging and in signaling in aging cells. Since v-ATPases appear to impinge on mitochondrial Fe/S-cluster stability and both cytosolic Prxs in yeast appear to interact closely with iron metabolism, a better understanding of their roles in these processes might also help to unravel how Prxs and v-ATPases contribute to slow down the rate of aging during caloric restriction.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Missing Links in Antibody Assembly Control

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Fidelity of the humoral immune response requires that quiescent B lymphocytes display membrane bound immunoglobulin M (IgM) on B lymphocytes surface as part of the B cell receptor, whose function is to recognize an antigen. At the same time B lymphocytes should not secrete IgM until recognition of the antigen has occurred. The heavy chains of the secretory IgM have a C-terminal tail with a cysteine instead of a membrane anchor, which serves to covalently link the IgM subunits by disulfide bonds to form "pentamers" or "hexamers." By virtue of the same cysteine, unassembled secretory IgM subunits are recognized and retained (via mixed disulfide bonds) by members of the protein disulfide isomerase family, in particular ERp44. This so-called "thiol-mediated retention" bars assembly intermediates from prematurely leaving the cell and thereby exerts quality control on the humoral immune response. In this essay we discuss recent findings on how ERp44 governs such assembly control in a pH-dependent manner, shuttling between the cisGolgi and endoplasmic reticulum, and finally on how pERp1/MZB1, possibly as a co-chaperone of GRP94, may help to overrule the thiol-mediated retention in the activated B cell to give way to antibody secretion.

1. Introduction

In the arms race between pathogens and hosts, vertebrates developed a long-range weapon of high precision: the antibody. Antibodies are produced by activated B lymphocytes. Once they are secreted, the antibodies spread throughout the organism to recognize and bind to specific epitopes on pathogens. As such, the pathogens are stigmatized as a target for complement or phagocyte mediated attack and (if all is well) elimination. All weaponry is hazardous, because of the risk that it may "go off" at the wrong moment and aim at the wrong target. Thus, it comes as no surprise that the immune system is reined in by a wealth of safety measures (unfortunately, they are not always fail-proof as the prevalence of autoimmune diseases illustrates). Safety dictates that B lymphocytes should not secrete antibodies, unless there is a confirmed sighting of an antigen, that is, when there is a good match between the antigen and the particular antibody which the B lymphocyte happens to express on its surface. At that moment, the B lymphocyte commits to become a plasma cell, which arguably is one of the most prolific secretory cells in the metazoan kingdom as it releases in bulk the antibody that specifically targets that very antigen. How can it be that the same cell that first obstinately prevents antibodies from leaking out makes a volte-face in a matter of a few days and starts to spit out antibodies by the millions? Here we discuss recent findings that shed new light on this fascinating question.

2. The B Cell Receptor (Membrane Bound IgM) versus Secretory IgM

To ensure that the antibodies that are secreted recognize the exact same antigen as was originally sighted by or presented to the B lymphocyte, the B cell receptor (BCR) and the antibody are in fact two manifestations of the same molecular device. The core of the BCR is formed by two Ig-μ heavy chains (HC) that are covalently linked by a disulfide bond. Each HC is also covalently linked by a disulfide bond to a light chain (LC). Both the HC and the LC are composed of domains that all adopt a so-called Ig-fold, and each of these domains is stabilized by an intradomain disulfide bond. The HCs have one variable domain (V_H) at the N terminus and four constant domains (referred to as C_H1–C_H4 from N to C terminus), while the LCs have next to their N terminal

variable domain (V_L) a single constant domain (C_L). The C_L is juxtaposed to the C_H1 and the V_L to the V_H at the tips of the BCR (Figure 1). Together the V_H and V_L determine the specificity of the BCR in antigen recognition. An ingenious series of recombination events lead to the shuffling of the sequence of the V domains and ensure that the immune system can employ a vast repertoire (estimated at 10^7 different so-called idiotypes) of Igs, with each B lymphocyte displaying only one particular variant that matches a single particular antigenic epitope. The tetrameric (H_2L_2) IgM that forms the core of the BCR is anchored to the plasma membrane via transmembrane domains at the C-terminal part of the Ig-μ_m HCs downstream of C_H4 [1].

Secretory IgM consists of nearly identical H_2L_2 units (Figure 1). The LCs are indeed the same, but the secretory Ig-μ_s HCs differ from membrane bound Ig-μ_m HCs in their C-terminus, since through alternative splicing the last exons have been replaced. In place of a transmembrane domain, the C terminus of Ig-μ_s HC now displays a hydrophilic tail piece (TP) with one cysteine that is key both for preventing premature secretion and for the assembly of mature secretory IgM (see below). Once it commits to the plasma cell stage, the TP cysteine is used, in fact, to covalently link H_2L_2 "monomeric" units in polymeric secretory IgM by disulfide bonds [2]. As a result, plasma cells secrete IgM either as "hexamers" $(H_2L_2)_6$ or as "pentamers" $(H_2L_2)_5$J, in which a third antibody component, the J-chain, joins the "monomeric" units, again by disulfide bonds [1]. Altogether secretory IgM consists of entities that each exceeds 1 megadalton in molecular weight, as they are composed in the "pentameric" state of 21 polypeptides, bearing in total 51 N-glycans, and containing 98 intrachain and interchain disulfide bonds [1] (Figure 1).

3. Folding and Quality Control of IgM

Secretory proteins and proteins that are displayed on the plasma membrane are synthesized in the endoplasmic reticulum (ER) and the Igs are no exception. The nascent polypeptides obtain glycans and disulfide bonds in the ER as they fold with assistance of the array of ER resident chaperones and oxidoreductases [3]. In fact, the majority of those chaperones and foldases have been implicated in the maturation of antibodies as well as some folding assistants in the intermediate compartment between the ER and Golgi (ERGIC). To name the key players: the "classical" chaperones BiP (originally discovered as being bound to HC in cells lacking LC and hence named Ig binding protein and later found to be the ER resident HSP70) [4] and GRP94 (the ER resident HSP90) [5]; members of the protein disulfide isomerase (PDI) family (PDI itself, ERp72 [6], and–most relevant for this essay–ERp44 [7]; the lectins calnexin [8] and ERGIC-53 [9]; and, most recently, a plasma cell specific ER resident chaperone called pERp1 [10, 11] or MZB1 [12]. The chaperones interact with folding or assembly intermediates recognizing particular regions that are manifest to their immature state. Exposed hydrophobic patches are BiP and/or GRP94 targets; PDI family members recognize aberrant disulfide bonds or free cysteines; finally, calnexin (and calreticulin) recognizes monoglucosylated glycans [13–15]. As such, the nascent proteins are bound to the relay of chaperones until they reach their fully folded mature conformation.

Except for the lectins calnexin and ERGIC-53, the chaperones listed above are all soluble proteins that display a tetrapeptide KDEL (or KDEL-like) sequence at their C terminus. In case any of these chaperones either alone or in complex with a folding intermediate leaves from the folding promoting environment of the ER to stray along the secretory pathway, they will be captured by the KDEL receptors in the ERGIC or cisGolgi. From there the KDEL receptors with their cargo are shuttled back to the ER for further folding attempts [16, 17]. This retrieval system thus is instrumental to quality control of protein folding in the early secretory pathway. Along the same lines, assembly of LCs onto HCs is ensured. BiP binds to the unfolded C_H1 domain of the HC and facilitates its retention in the early secretory pathway, until LCs manage to displace BiP, and the heteromeric complex is "approved of" by the proximal ER quality control systems [18, 19].

4. Assembly Control of Secretory IgM and the Role of ERp44

Central to the effectiveness of the immune response is that unassembled IgM subunit should never be secreted. Indeed, nonpolymeric IgM would bind antigen but fail to fix complement. Thus, the plasma cell must orchestrate that correctly folded and assembled secretory H_2L_2 "monomeric" units oligomerize into mature secretory IgM. The task at hand is twofold; on the one hand polymerization must be promoted and polymers must be let go of by the ER quality control mechanisms; on the other hand unassembled "monomers" must be barred from traveling further along the secretory pathway and retrieved for further polymerization attempts (Figure 2).

IgM oligomerization is favored by the hexameric lectin ERGIC-53 [9], which captures fully folded and assembled "monomers" leaving the ER and, probably by organizing them in a planar way, may help the process of polymerization. Correctly assembled IgM polymers presumably then detach from ERGIC-53 in the Golgi and proceed along the secretory pathway. Release of cargo from ERGIC-53 in general seems to be facilitated by the progressively lower $[Ca^{++}]$ they encounter traveling from the ER to the Golgi [20]. In the case of IgM, moreover, the polymerization process itself could drive the detachment of the lectin ERGIC-53, because the glycan on the Ig-μ TP (Figure 1) that is recognized by ERGIC-53 in the context of IgM "monomers" [21] may become inaccessible in the polymer. Genetic defects in ERGIC-53 or in its partner protein MCFD2 lead to combined deficiency of factor V and factor VIII, since both clotting proteins rely on the ERGIC-53/MCFD2 complex to be actively transported out of the ER and then finally to be secreted into the blood stream [22, 23]. Yet, loss of ERGIC-53 or MCFD function does not result in an apparent impairment of IgM secretion (our unpublished results and [21]); hence ERGIC-53 may be

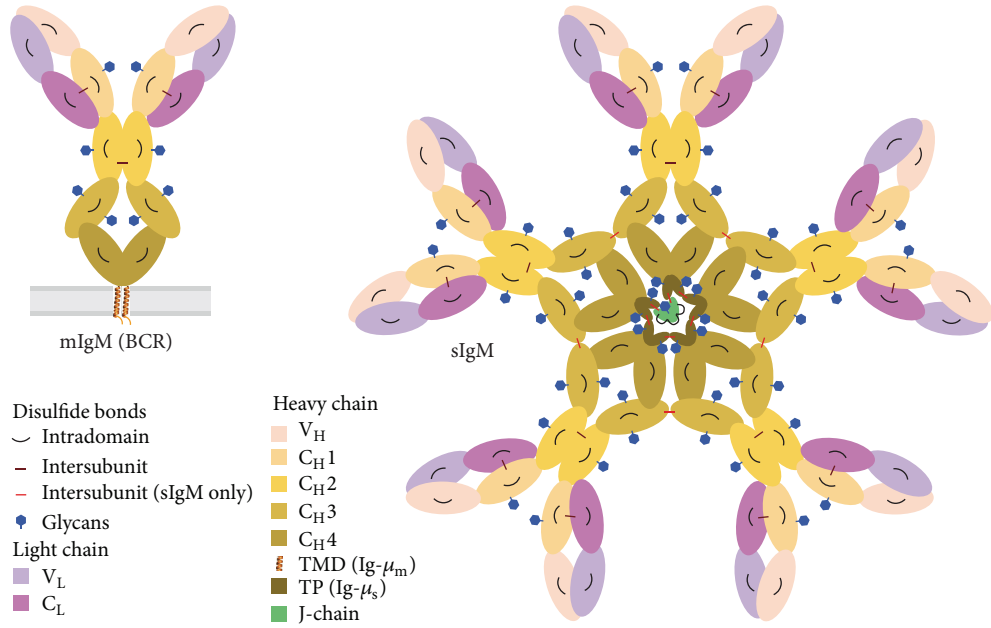
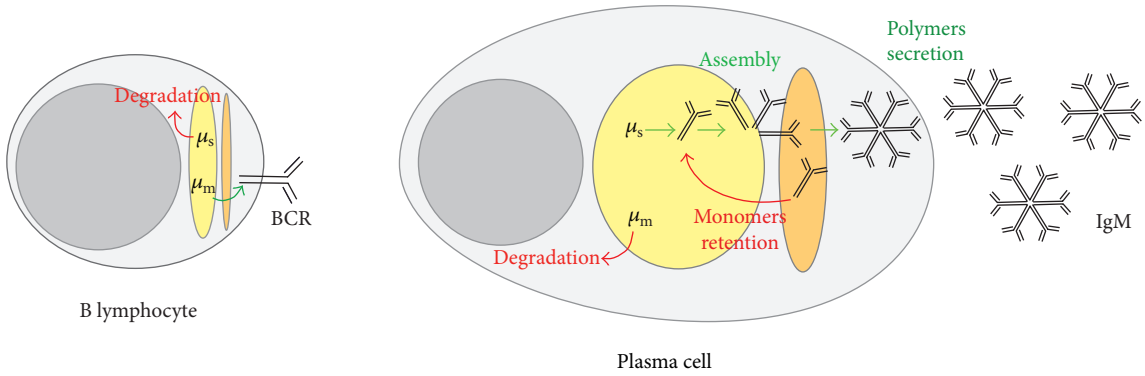


FIGURE 1: Schematic representation of membrane bound IgM and secretory IgM. B lymphocytes display membrane bound IgM (mIgM)—shown left—on their surface as the core part of the BCR. Once committed to the plasma cell stage they secrete secretory IgM (sIgM) in either "pentameric"—shown right—or "hexameric"—not shown—form. HCs and LCs consist of various Ig-fold domains V_H, C_H1–4; V_L, C_L that are color-coded as indicated. Glycans and intra- and intersubunit disulfide bonds between HCs, LCs, and J-chain are depicted. Note that the Ig-μ_m HC (in mIgM) differs from the Ig-μ_s HC (in sIgM) in their C-termini, having a transmembrane domain (TMD) or, respectively, a cysteine containing tail piece (TP) as indicated.



	B lymphocyte	Plasma cell
Rules that change	Produce μ_m (for membrane BCR)	Degrade μ_m
	Never assemble polymeric IgM	Assemble polymeric IgM
	Never secrete antibodies	Secrete antibodies at full gear
Common rules	Never secrete unassembled antibodies, degrade them instead	

FIGURE 2: Rules for IgM processing in B lymphocytes and plasma cells.B lymphocytes do not produce secretory antibodies. In B lymphocytes Ig-μ_m HC (μ_m) is synthesized in the ER; it is assembled in the early secretory pathway (in yellow) and then proceeds through the Golgi (orange) to be exposed on the plasma membrane as part of the BCR; Ig-μ_s HC (μ_s), instead, is retrotranslocated from the ER and degraded. Conversely, plasma cells are hardworking antibodies factories; they degrade μ_m but efficiently assemble μ_s into polymeric IgM and secrete them in bulk. Incompletely assembled IgM subunits are retained in the secretory pathway for another chance of being inserted into a polymer or to be finally degraded.

auxiliary to but cannot be essential for polymeric assembly of IgM.

Recent findings now also implicate ERp44, an ERGIC-53 partner, in patrolling IgM assembly. In the ERGIC-cisGolgi region, downstream of the "regular" quality control mechanisms in the ER, ERp44 governs a dedicated assembly control cycle for disulfide-linked polymers [9, 24]. ERp44 is a chaperone of the PDI family, composed of three thioredoxin-like domains (a, b, and b′) and a C-terminal tail which ends with an RDEL sequence. The C-terminal tail covers the active cysteine and the surrounding hydrophobic regions in domain a [25, 26]. ERp44 is localized more distally along the secretory pathway than other PDI-like proteins, being enriched in the ERGIC-cisGolgi region [9, 27]. By shuttling from the ER to the Golgi, ERp44 exploits the pH gradient in the early secretory pathway (pH 7.2 in the ER; pH 6.7 in the Golgi) to efficiently block the exit of unassembled subunits with exposed free thiols, which in the mature oligomer will be employed for inter-subunit disulfide bonds [24] (Figure 3).

At the more acidic pH of the Golgi the C-terminal tail opens, unveiling the active site cysteine and the surrounding hydrophobic region for interaction and formation of a mixed disulfide bond with the client proteins. The tail opening also leads to the exposure of the RDEL sequence to the KDEL receptors, which, in turn, allows the KDEL receptor/ERp44/cargo complex to be brought back to the ER (Figure 3, inset (a)). In the ER, instead, the higher pH stabilizes the tail in the closed conformation, which precludes ERp44 from binding its substrates too early during the quality control process (Figure 3, inset (b)). The pH-driven cycle likely involves protonation of the active site cysteine in the more acidic cisGolgi and deprotonation in the more alkaline ER. In the deprotonated thiolate state, the active site cysteine supports a network of electrostatic interactions that keep the tail associated with the surroundings of the active site and thereby keeps it in the closed conformation. In the protonated state these interactions are weakened such that the tail dissociates and ERp44 adopts an open conformation [24].

Thus, ERp44 is central to prevent secretion of unassembled immunoglobulin IgM subunits [7, 9]. Upon retrieval to the ER, the intermediates get another chance of being correctly assembled or—if the assembly attempts are ultimately unsuccessful—become degraded [28]. ERp44 activity not only concerns retrieval of IgM assembly intermediates, but likely patrols disulfide-linked assembly in general. For instance, ERp44 also retrieves unassembled adiponectin from the cisGolgi to the ER in adipocytes [29, 30] and exerts quality control on proper disulfide bond formation of the serotonin transporter protein (SERT) [31]. Moreover, ERp44 is responsible for keeping some partner proteins retained at their appropriate intracellular location in the early secretory pathway such as Ero1α [7, 32], SUMF1 [33], and peroxiredoxin 4 (PRX4) [34].

5. Missing Links in the IgM Assembly Model

The pH regulation of ERp44 may explain how the assembly process of IgM is patrolled by virtue of missing (disulfide) links. Yet, despite the elucidation of this elegant assembly control, there are several missing links in our understanding of how B cells finally commit to IgM secretion. For one, little is known about the mechanisms that promote the incorporation of J chains into polymers that as a result become "pentamers" and how the plasma cell balances its "hexamer" and "pentamer" output [35]. Then, several aspects of the ERp44 cycle are still unclear. One question is how clients are let go of in the ER, in particular how the mixed disulfide bond between ERp44 and its client is reduced (Figure 3, inset (b)). Likewise, the oxidative source that allows the formation of mixed disulfide bonds with clients in the cisGolgi remains to be identified (Figure 3, inset (a)). The active sites of the majority of PDI-like proteins have two cysteines that form a disulfide bond, which then is donated to clients. Having only a single active site cysteine, ERp44 cannot deliver the oxidative equivalent for a mixed disulfide with clients. Perhaps, Ero1α, ERp44's foremost partner [25], provides the oxidative equivalents, as it is also responsible (in part) for reoxidizing PDI [36, 37]. Another partner of ERp44, PRX4, similarly may play such a role. For instance, ERp44 could form a tandem with Ero1α or PRX4 but release these partners once they catalyze formation of mixed disulfide bond of ERp44 with a client protein. Another scenario could be envisaged in which the oxidative equivalents are provided in the form of a disulfide bond between two ERp44 proteins using the free (active site) cysteines. In that case, the clients may displace one of the ERp44 molecules forming a mixed disulfide with the other. The free ERp44 then can team up with another free ERp44, whereupon formation of the disulfide that links the ERp44 homodimer may be catalyzed by PDI, Ero1α, or the like. Indeed, a substantial fraction of ERp44 is present in the cell as a disulfide linked dimer [7].

Arguably the most intriguing missing link in our appreciation of IgM secretion control is what eventually will overrule the thiol-mediated retention. The unstimulated B lymphocyte already makes some Ig-μ_s HC but secretes little if any antibody (Figure 2). Instead, all Ig-μ_s HCs are retrotranslocated into the cytosol to be degraded by the proteasome [28, 38]. Apparently, there is a switch from a nonassembly competent to an assembly competent state in the course of the B to plasma cell differentiation process, which in fact involves an overhaul of the complete cellular makeup. In the course of a few days, cells first stock up on metabolic prowess and then gradually enlarge the secretory pathway to a most impressive capacity before they actually initiate bulk antibody secretion around two days after activation [39]. The full-blown plasma cell then secretes a record load of IgM, which amounts to the equivalent of its own mass on a per day basis. After a week or less of this massive secretion, however, the majority of plasma cells die, while few will commit to long-term survival to sustain B cell memory [1].

Dormant B lymphocytes are not exceptional in being barred from secreting IgM, since also other cells that are transfected with constructs for all secretory IgM components fail to efficiently secrete it [9, 21]. Instead, plasma cells are exclusive in becoming efficient antibody secretors. Thus, the key to the "assembly switch" must lie in the reprogramming of the B lymphocyte in the first days of the differentiation

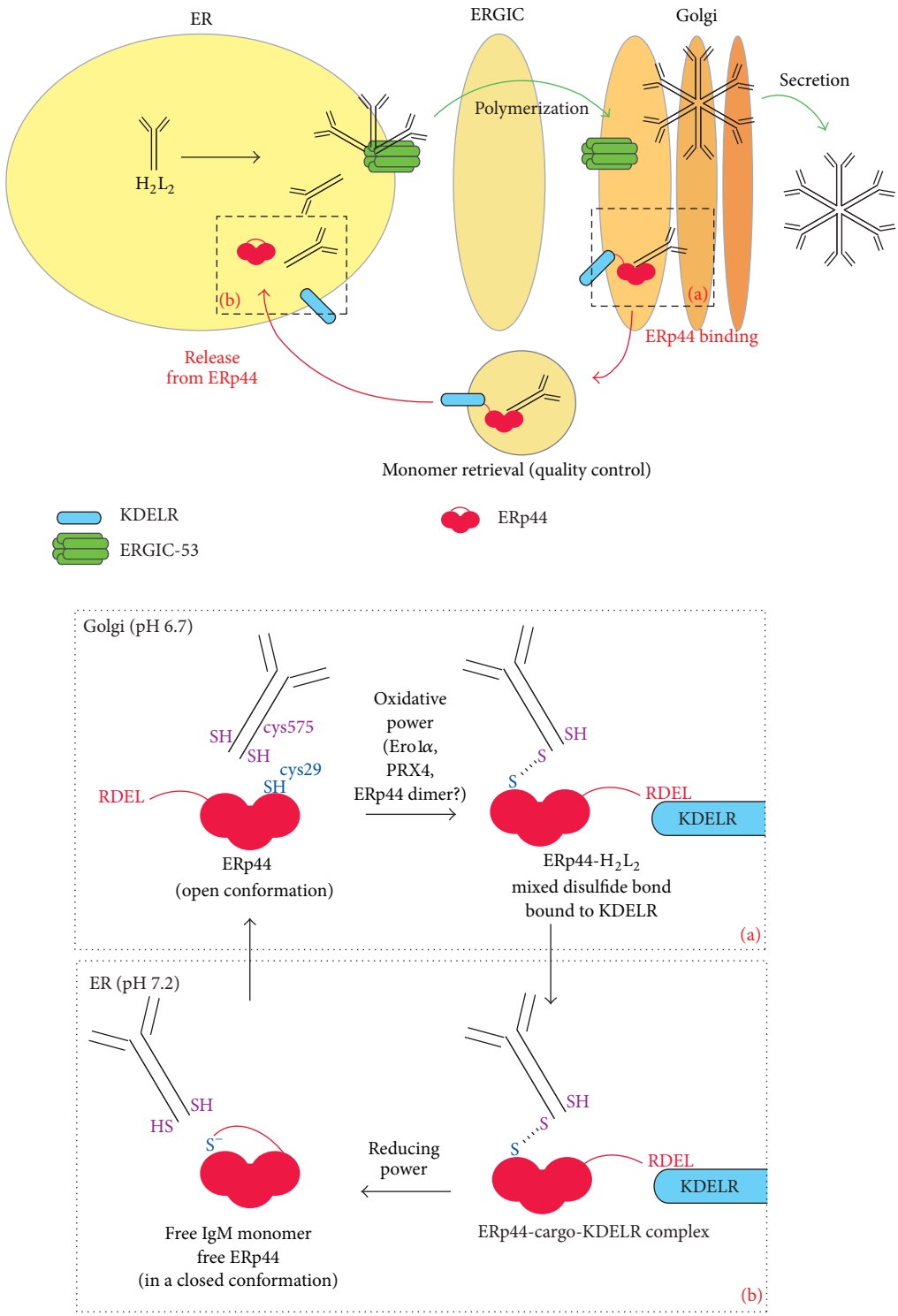


FIGURE 3: ERp44 quality control and IgM polymerization. IgM monomers are assembled into polymers in the early secretory pathway with the help of the hexameric lectin ERGIC-53. IgM polymers are released from ERGIC-53 in the Golgi and can then be secreted. In the Golgi, ERp44—in an open conformation thanks to the slightly acidic pH—interacts with unassembled IgM subunits exposing the C-terminal cysteine 575 in the SH conformation (inset (a)): the covalent binding between ERp44's active site cysteine 29 and the IgM subunits requires an oxidative power whose source is still unknown. The interaction with the cargo favors the exposure of ERp44's C-terminal RDEL for binding to the KDEL receptor and the complex KDEL receptor-ERp44-cargo (IgM H_2L_2 "monomer") is then transported back to the ER. Here, a reducing power disentangles the disulfide bond between ERp44 and the IgM "monomer." The neutral pH in the ER, which also inhibits the KDEL receptor activity, now stabilizes ERp44 in a more closed conformation (inset (b)). Keeping ERp44 in a closed conformation in the ER likely is important to avoid that ERp44 interacts with subunits that productively take part in the polymerization process.

process. One could argue that promoting polymerization simply involves mitigating ERp44 activity. Yet, the retention of unassembled IgM is unabated in plasma cells, as only fully assembled IgM polymers are secreted. In accordance, ERp44 is upregulated in the course of B cell differentiation coordinately with all other folding factors in the early secretory pathway, including ERGIC-53, Ero1α, and PRX4 [9, 39, 40]. Moreover, redox conditions in the ER do not seem to alter dramatically during differentiation, as the glutathione GSH/GSSG balance essentially remains the same [41]. Likewise, several proteins involved in redox homeostasis increase during B cell differentiation, but they do so commensurate with the expansion of the secretory machinery, apparently to match the growing need for disulfide bond formation [39, 40].

6. pERp1/MZB1: An "Assembly Switch"?

One candidate that may embody the "assembly switch" is pERp1 [10, 11] also known as MZB1 [12]. This protein is exclusively expressed in the ER of B cells and is highly upregulated in the course of the differentiation process to reach levels that are equivalent to the most abundantly expressed ER proteins, such as BiP and GRP94 [11]. Its expression levels are the highest in marginal zone B cells [12], in accordance with their being the most prolific IgM secretors [42]. Importantly, knockdown of pERp1/MZB1 slows down IgM polymerization and thus leads to reduced IgM secretion [11, 12].

Further support for the notion that pERp1/MZB1 is key for IgM assembly comes from a recent study on Kaposi-associated herpesvirus (KSHV). Herpesviridae are notorious for their misleading of the immune system. Upholding herpes family values, an estimated one-third of the KSHV genome indeed is dedicated to immune evasion [43], and as it turns out, this includes the K4.2 immediate early gene. Namely, the K4.2 gene product associates with pERp1/MZB1 and thereby somehow sabotages its function, resulting in a markedly reduced efficiency of IgM secretion from plasma cells [44].

As opposed to KSHV driven shipwrecking of pERp1/MZB1, another pathologic condition involves uncontrolled pERp1/MZB1 expression. MicroRNAs (miRs) contribute to the regulation of gene expression through annealing with target mRNAs, causing their degradation or translational inhibition. A recent report now implicates pERp1/MZB1 as the most prominent target of miR-185 [45], while it had been shown already that reduced levels of miR-185 cause autoantibody production [46, 47]. These findings suggest that miR-185 insufficiency leads to uncontrolled pERp1/MZB1 levels, which in turn gives way to unchecked IgM polymerization and hence unwarranted secretion of antibodies, including autoantibodies. This miR-185 is haploinsufficient in the vast majority of individuals with a $\sim 3 * 10^6$ bp deletion in chromosome 22 at the q11.2 location. This 22q11.2 deletion syndrome is a relatively prevalent (1 in 2000–4000 live births) genetic disorder that can lead to a wide range of symptoms including congenital heart disease, renal and/or skeletal abnormalities, and learning difficulties, owing to the haploidy of the ~30–40 genes in the affected region [48]. Importantly, symptoms of the 22q11.2 deletion syndrome include an increased prevalence of autoimmune disorders and B cell defects [47] that may well stem (at least in part) from lowered miR-185 levels and concomitant uncontrolled pERp1/MZB1 function.

All these findings indeed point at pERp1/MZB1 as key for IgM assembly. Yet, how pERp1/MZB1 would assist IgM polymerization remains unclear. With the idea in mind that thiol-mediated retention should be counteracted, it was explored whether pERp1/MZB1 could fulfill a role as thiolreductase, but evidence for such activity was meager [11] to nonexistent [12]. Therefore, pERp1/MZB1 most likely derives its putative proassembly effect from a nonthiol related activity. Homology searches already revealed that pERp1/MZB1 is not related to any of the known chaperone families [11]. Instead, pERp1 has several distant family members in a variety of organisms ranging from plants to humans [12].

After evaluating sequence homologies anew, we now report that pERp1 is part of the saposin-like protein family with, as its closest relatives the canopy homolog proteins CNPY1-4; an alignment is shown in Figure 4. The CNPY proteins and pERp1/MZB1 share a common arrangement of cysteines, indicative of a conserved disulfide bonded structure, an N-terminal signal peptide, and a KDEL-like C-terminal tetrapeptide. Note that the alignment suggests that CNPY1 lacks the N-terminal sequence contained in other family members; however, CNPY-like encoding regions are present in the genomic locus upstream of the annotated CNPY1 initiation (data not shown) and in homologs in other species [49]. Altogether, the shared sequence elements strongly suggest a common residency and related activities in the early secretory pathway for pERp1/MZB1 and the CNPY family members. Along these lines pERp1/MZB1 may well be renamed CNPY5.

Mammalian CNPY1 has not been studied but in zebrafish it associates with the fibroblast growth factor (FGF) receptor. This activity is essential for proper FGF signaling in the developing brain of the fish [49]. CNPY2 was recently reported to enhance expression levels of the LDL receptor in hepatocytes in an FGF21 dependent manner [50]. CNPY3 and 4 were identified as PRAT4A and [51], PRAT4B, respectively [52]. CNPY3 is important for Toll-like receptor (TLR) expression levels [51] except for those of TLR3 [53], while such an effect for CNPY4 has been confirmed for TLR4 only [52].

Several cochaperones of the cytosolic chaperone HSP90 have been identified [54], but for its ER resident paralog, GRP94, the existence of cochaperones for long remained elusive [55]. Excitingly, CNPY3 appears to act as a cochaperone of GRP94 [53]. As such, it assists the folding of TLRs by enhancing or modifying the GRP94 chaperone function, although at present it is unclear at what stage in the folding or dimerization of TLRs the GRP94/CNPY3 tandem comes into play [56]. Altogether it is tempting to speculate that CNPY family members act as cochaperones of GRP94 with CNPY1 assisting FGF receptor folding, CNPY2 LDL receptor maturation, and that pERp1/MZB1 may drive IgM polymerization by virtue of its assistance to or modulation of GRP94 function. In line with this scenario, pERp1/MZB1 interacts

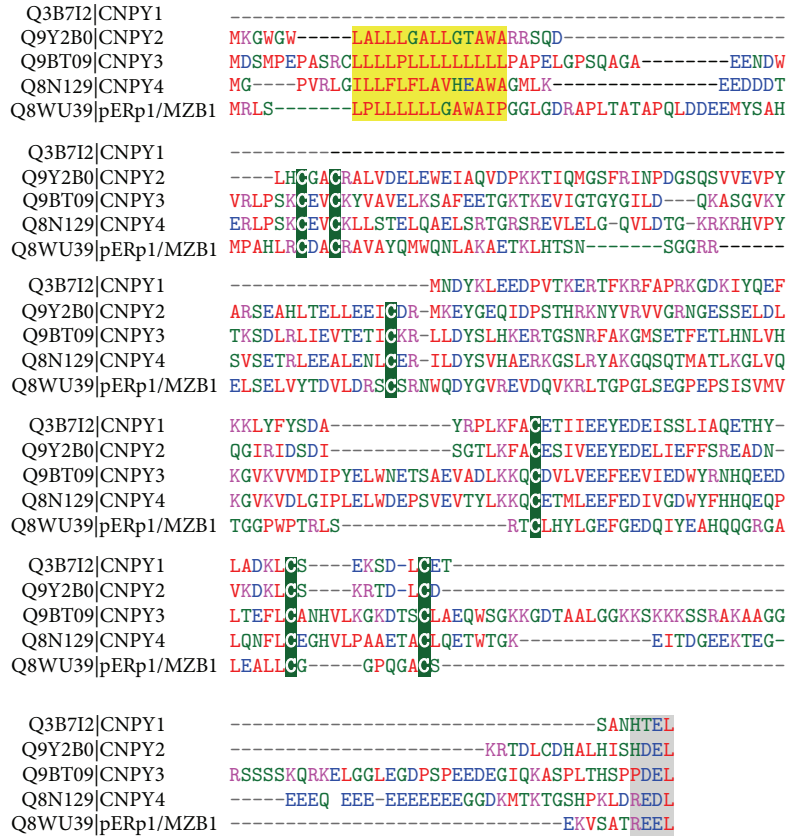


Figure 4: Alignment of pERp1/MZB1 and members of the CNPY protein family. The alignment of the five human protein sequences with UniProt identifiers as indicated was generated with the ClustalW algorithm and manually curated. The signal peptide encoding residues are highlighted in yellow, the C-terminal KDEL-like tetrapeptides are highlighted in grey, and the conserved cysteines in green. Standard color coding for the residues is as follows: alkaline in pink; acidic in blue; other hydrophilic in green; and hydrophobic in red.

with GRP94 [10, 12]. Moreover, pERp1/MZB1 promotes cell surface expression of integrins, which also are GRP94 clients [12]. Finally, it is noteworthy that pERp1/MZB1 interacts with the PDI family member ERp57 as well [12]. Perhaps, the GRP94-pERp1/MZB1 tandem recruits ERp57 to catalyze formation of the IgM inter-subunit disulfide bonds.

7. Concluding Remarks

In all Ig secreting vertebrates (from cartilaginous fish onwards) the first wave of the antibody response appears to involve polymeric IgM, whether "tetrameric" (in teleost fish), "pentameric", or "hexameric" [57]. Polymeric IgM has a higher valency than "monomeric" IgM, which compensates for the low affinity of this first wave antibody response. Another driving force for the primary response to obligatorily involve polymeric IgM may well be the avoidance of leakiness. Premature secretion of antibodies on the one hand can lead to autoimmune effects if B cells happen to produce antibodies that cross-react with self-antigens. On the other hand, they may inadvertently lead to a prophylactic effect by competing with the BCR for scarce antigens and thereby ultimately prevent full scale B cell activation and memory. ERp44 and its rigorous role in thiol-mediated retention seem to effectively preclude the leakiness, but this categorical retention, in turn, necessitated the invention of an escape valve when the B cells become a plasma cell. A tightly controlled switch to polymerization seems to fulfill that role. We argue that pERp1/MZB1, whether in conjunction with GRP94 or not, serves the B cell in switching from a nonsecretory to a secretory phenotype.

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Review Article

The Role of S-Nitrosylation and S-Glutathionylation of Protein Disulphide Isomerase in Protein Misfolding and Neurodegeneration

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Neurodegenerative diseases involve the progressive loss of neurons, and a pathological hallmark is the presence of abnormal inclusions containing misfolded proteins. Although the precise molecular mechanisms triggering neurodegeneration remain unclear, endoplasmic reticulum (ER) stress, elevated oxidative and nitrosative stress, and protein misfolding are important features in pathogenesis. Protein disulphide isomerase (PDI) is the prototype of a family of molecular chaperones and foldases upregulated during ER stress that are increasingly implicated in neurodegenerative diseases. PDI catalyzes the rearrangement and formation of disulphide bonds, thus facilitating protein folding, and in neurodegeneration may act to ameliorate the burden of protein misfolding. However, an aberrant posttranslational modification of PDI, S-nitrosylation, inhibits its protective function in these conditions. S-nitrosylation is a redox-mediated modification that regulates protein function by covalent addition of nitric oxide- (NO-) containing groups to cysteine residues. Here, we discuss the evidence for abnormal S-nitrosylation of PDI (SNO-PDI) in neurodegeneration and how this may be linked to another aberrant modification of PDI, S-glutathionylation. Understanding the role of aberrant S-nitrosylation/S-glutathionylation of PDI in the pathogenesis of neurodegenerative diseases may provide insights into novel therapeutic interventions in the future.

1. Introduction

Neurodegenerative diseases share several common pathological characteristics, including the aberrant aggregation of misfolded proteins, leading to the formation of abnormal protein inclusions [1]. These diseases are also frequently classified as protein conformational disorders in which protein aggregation occurs due to the exposure of hydrophobic regions [2]. The most common neurodegenerative diseases include Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Creutzfeldt-Jakob disease (CJD), and Huntington's disease (HD). These diseases differ according to the specific group of neurons targeted and the type of misfolded proteins that aggregate. In AD, the accumulation of aggregated proteins occurs in cortical regions and involves both β-amyloid (βA), which forms extracellular amyloid plaques, and tau, which is hyperphosphorylated and forms intracellular neurofibrillary tangles (NFT) [3, 4]. PD involves the formation of Lewy bodies (LB) containing misfolded α-synuclein [5], and in HD aggregated Huntington protein with expanded polyglutamine repeats forms inclusions in the nucleus [6]. Similarly, in ALS, cytoplasmic inclusions contain copper/zinc (CuZn) superoxide dismutase 1 (SOD1) [7–9], TAR DNA binding protein 43 (TDP-43) [10–13], or fused in sarcoma/translated in liposarcoma (FUS/TLS) [14]. Recently, a hexanucleotide repeat expansion in an intronic region of the chromosome 9 open reading frame 72 (C9orf72) gene, encoding a gene of unknown function, was linked to the greatest proportion of familial ALS cases [15, 16]. For AD, PD, and ALS, 90–95% of cases arise sporadically, while the remainder are familial in nature. Genetic mutations in Amyloid Precursor Protein (APP), leads to increased accumulation of A-β and fibril formation [17–20], and Presenilin 1, 2 (PS 1, 2), which regulates APP processing via gamma

secretase [21–23], causes rare familial cases of AD [24]. Similarly, some forms of autosomal dominant familial PD is caused by α-synuclein mutations [25] leading to the aggregation of α-synuclein into insoluble fibrils, which are the primary components of LB [26], while mutations in PINK1, Parkin, and DJ-1 cause autosomal recessive PD cases [27]. However, in contrast to these conditions, HD is early onset and entirely genetic in nature.

The causal factors underlying the pathogenesis of sporadic neurodegenerative diseases remain poorly understood. However, due to the typical late onset of these disorders, neurodegeneration can be conceptualized as pathology that arises during the normal aging process, involving increases in oxidative stress and the production of free radicals which damage cells by decreasing antioxidant defenses. In AD, increased free radical accumulation and elevated levels of oxidative and nitrosative stress are associated with alterations in A-β metabolism [28, 29]. Meanwhile, in PD, nitrosative stress is associated with impairment of the mitochondrial respiratory chain, leading to energy deficiency and cell death [30]. In addition, oxidative and nitrosative stress are associated with endoplasmic reticulum (ER) stress, through the accumulation of misfolded proteins in the ER, and upregulation of molecular chaperones in the protein disulphide isomerase (PDI) family [31]. PDI possesses both general protein chaperone and disulphide interchange activity, thus facilitating the formation of native disulphide bonds in proteins. It also facilitates the degradation of these proteins via ER-associated degradation (ERAD), whereby irreparably misfolded proteins are targeted for retrotranslocation to the cytoplasm, where they undergo polyubiquitination and subsequent degradation by the proteasome [32–35]. There is now sufficient evidence that in conditions of elevated nitrosative stress, PDI undergoes an aberrant posttranslational modification known as S-nitrosylation, which inhibits its enzymatic activity [36]. Hence, in late onset neurodegenerative disease, there is a decrease in cellular defences and a corresponding increase in oxidative and nitrosative damage to lipids, proteins, DNA, and RNA [37, 38].

In this review, we will begin by examining the role of nitrosative stress, redox potential, and S-nitrosylation/S-glutathionylation of proteins linked to neurodegeneration. The structure and function of PDI family members will be discussed, and the importance of PDI in neurodegenerative disease will be highlighted. We will examine the evidence that PDI is aberrantly S-nitrosylated and discuss the functional significance of this modification in neurodegeneration. Finally, we speculate that PDI may also be S-glutathionylated in neurodegenerative disease.

2. Nitrosative Stress

Reactive nitrogen and oxygen species (RNS and ROS), primarily superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), or nitric oxide (NO), are highly reactive molecules that normally function at low levels as mediators of intracellular signalling processes in mammalian cells [36, 39]. However, RNS and ROS can accumulate in cells under pathological conditions, triggering nitrosative or oxidative stress. This leads to numerous detrimental effects on cellular function including posttranslational modifications of proteins, lipid peroxidation, DNA, damage, and dysregulation of redox signalling [28, 37, 38, 40]. Nitrosative or oxidative stress results when there is an imbalance between the production of RNS/ROS and cellular antioxidant defence mechanisms such ascorbic acid, glutathione (GSH), or enzymes including superoxide dismutases, catalases, and glutathione peroxidases. GSH is a particularly important antioxidant as it is the most abundant cellular thiol-containing molecule; the ratio of reduced GSH to its oxidized form (GSSG) makes a major contribution to cellular redox potential and homeostasis [28, 29, 41]. However, the thiol/disulfide systems, which include GSH/GSSG, and plasma cysteine/cystine (Cys/CySS) pools are not necessarily in equilibrium and may respond differentially to specific stressors [42]. Nitrosative or oxidative stress may be induced by familial mutations, exogenous toxins (xenobiotics, pesticides), or via normal aging processes such as alterations in mitochondrial respiration [31, 43]. Neurons are particularly vulnerable to the effects of RNS/ROS due to a relative deficiency in antioxidant enzymes glutathione peroxidase (GPx) and catalase (Cat), compared to other cell types, and their higher metabolic demands which generate RNS/ROS from mitochondrial metabolism [38, 39, 43, 44].

RNS are derived primarily from O_2^- and NO, a small, diffusible inter- and intracellular messenger that normally mediates many intracellular signalling pathways [29, 31, 45, 46]. NO is generated by NO synthases (NOS) that use oxygen (O_2) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to convert L-arginine to L-citrulline [47]. NOS is constitutively expressed in several isoforms in the central nervous system (CNS): endothelial NOS (eNOS), inducible NOS (iNOS), neuronal NOS (nNOS), and an isoform expressed in the inner mitochondrial membrane (mtNOS) [48–50]. The covalent addition of NO to a cysteine thiol or thiolate anion on specific proteins, to form an S-nitrosothiol (SNO) group, is a process termed "S-nitrosylation" [36, 51–56].

3. S-Nitrosylation

In recent years, S-nitrosylation has been increasingly implicated in many physiological and pathological conditions [36]. Under normal conditions, S-nitrosylation is a reversible posttranslational modification analogous to acetylation and phosphorylation that regulates protein activity [55, 57]. The SNO-group can be removed in these situations by denitrosylation enzymes, primarily S-glutathione reductase (GSNOR; alcohol dehydrogenase III) in conjunction with GSH and NADH as an electron donor [58, 59]. However, reduced oxidoreductase thioredoxin (TRX) [60, 61] can oxidize S-nitrosoglutathione (GSNO) to release GSH and NO [62, 63]. Recombinant human PDI can denitrosylate GSNO [64] and *in vitro* SOD1 can modify the stability of S-nitrosothiols by enhancing the decomposition of GSNO, resulting in production of NO [65], possibly by its reduced metal ions [66].

S-nitrosylation is both a reversible and irreversible process [67]. Under pathological conditions, S-nitrosylation of specific proteins is an abnormal, irreversible process and is linked to protein misfolding, ER stress, mitochondrial

dysfunction, synaptic degeneration, and cell death [36]. A well-recognized mechanism for NO production in neurodegenerative diseases is activation of N-methyl-D-Aspartate receptors (NMDAr) [68, 69]. Activation of NMDArs generates ROS and results in calcium (Ca^{2+}) influx into the cell [31, 70–72], which in turn activates nNOS to produce NO [50]. S-nitrosylation may also lead to NO-independent oxidation of proteins via ROS, producing reversible modifications in the form of intramolecular/mixed disulphide bonds. One of the proposed pathways for the further oxidation of cysteines is through the hydrolysis of sulfenic acid (SOH), which then may be susceptible to irreversible oxidation from accumulating ROS leading to stable sulfinic ($-SO_2H$) or sulfonic ($-SO_3H$) acid formation [73–75]. However, $-SO_2H$ can be reduced back to the free thiol group if the enzyme sulfiredoxin is induced and this can occur in neurons due to activation of NMDAr by increased synaptic activity [76]. In addition, S-nitrosylation can reversibly influence further posttranslation modifications of cysteine residues. When there are two proximal cysteine residues, S-nitrosylation of one of these can facilitate disulphide bond formation [77–79]. Under conditions of excessive nitrosative stress, however, S-nitrosylation inhibits the formation of disulphide bonds [67, 75]. Another pathological mechanism linked to S-nitrosylation has also been implicated in ALS. Cells expressing familial ALS mutants, SOD^{A4V} and SOD^{G37R}, have increased denitrosylation activity of GSNO in comparison to wild type (WT) SOD1 [80]. This deficiency in S-nitrosylation is especially elevated in mitochondria of mutant SOD1 cells [81].

Whilst most proteins contain multiple cysteine residues, the features underlying the specificity for S-nitrosylation are not fully defined, but appear to rely on tertiary rather than primary structure. Previous studies have suggested that the formation of S-nitrosylated proteins (SNO proteins) requires a cysteine flanked by a proximal acid-base motif, hydrophobic content, low pKa, and high exposure of sulfur atoms [67, 82]. However, a recent bioinformatics study predicted that the known SNO-Cys sites in proteins are more heterogenous than this, although the presence of a charged residue in close proximity to NO-Cys and another oppositely charged residue within a larger region was a common feature [82]. The stability of the resulting SNO-group depends upon the local environment of the cysteine residues, but studies of the dissociation energies of the S–N bond suggest that there is a wide variation, with this bond remaining stable theoretically from seconds to years [83, 84].

Up to one thousand SNO proteins have now been identified [85] including many proteins linked to neurodegenerative diseases [36, 77, 86–89]. For instance, S-nitrosylation of dynamin-related protein (Drp1) (SNO-Drp), found in postmortem brains of AD cases, is associated with β-A formation and subsequent activation of mitochondrial fission [77, 87]. In sporadic and familial PD, S-nitrosylated Parkin (SNO-Parkin) has reduced E3 ligase function, leading to proteasomal dysfunction [90]. Similarly, proteins involved in apoptosis (XIAP/Caspase 3, GAPDH-Siah), antioxidant activity (Prx2), the phosphatase pathway (PTEN), neuroinflammation (COX2), and autophagy (JNK1 and IKKβ) are also S-nitrosylated (for comprehensive review see [36]). Furthermore, SNO-proteins may alter cellular redox homeostasis through an interaction with GSH and therefore may influence other post-translational modifications, such as S-glutathionylation [36, 41]. Some proteins, such as NMDAr, are S-nitrosylated under both normal and pathological conditions [36]. S-nitrosylation/denitrosylation of NMDAr is important in physiological cellular signalling processes [52, 53, 91], but overactivation is associated with an increased production of SNO-proteins and neurodegeneration [31]. However, it should be noted that S-nitrosylation of NMDAr at Cys399 is protective by deactivation of the receptor, thus preventing glutamate excitotoxicity [53, 67, 78, 91].

4. S-Glutathionylation

S-glutathionylation is another posttranslational modification that has been implicated in the regulation of diverse proteins involved in energy metabolism, signalling pathways, Ca^{2+} homeostasis, antioxidant enzymatic activity, and protein folding [92] (for a comprehensive review see [41]). S-glutathionylation is induced by RNS/ROS and involves the formation of a disulfide between GSH and a cysteine residue [41]. As reduced GSH is the most abundant cellular thiol, it plays an important role in S-glutathionylation [41], although protein thiols represent a similar redox pool, and therefore may also be critical in providing antioxidant protection against oxidative stress [93]. S-nitrosylated cysteines can be converted to S-glutathionylated cysteines, supporting the premise that products of nitrosative stress induce S-glutathionylation [41]. However, the exact identity of the metabolites that act as proximal donors in this reaction remain to be elucidated [41] and it is unclear whether SNO proteins are intermediates for S-glutathionylation *in vivo*. Under oxidizing conditions, S-glutathionylation is reversible via the release of GSH from cysteine residues by thiol-disulphide oxidoreductase enzymes (TDOR). TDOR enzymes include TRX, which reduces intra- and intermolecular disulphide bonds, and glutaredoxin (GRX) which reduces protein-GSH bonds [94–96]. TRX and GRX catalyze the reduction of disulphide bonds and reactivate proteins that have undergone oxidation from sulfhydryl groups [95, 96]. Alterations in the ratio of GSH/GSSG and conditions that promote RNS/ROS production result in cysteine modifications that are precursors to the formation of mixed disulphides with GSH [95, 97, 98]. However, the role of S-glutathionylation during nitrosative and oxidative stress has not been completely defined. Glutathionylation at physiological levels may therefore represent a mechanism whereby cysteine residues faced with oxidation are protected from irreversible damage. The reduction of GSH-protein disulphide by GRX is essential in this process as it maintains the cellular availability of GSH and acts in concert with TRX to maintain the cellular thiol status [95].

S-glutathionylation has been implicated in neurodegeneration [95, 99–101]. The ratio of GSH/GSSG decreases in brains of aged rats [102], and accumulation of S-glutathionylated p53 in the inferior parietal lobule of AD patients

has also been reported [101]. In PD models, administration of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which causes damage to dopaminergic neurons, caused an early decrease in the levels of GSH, inhibition of mitochondrial complex 1, and dopaminergic cell loss [103]. Furthermore, increases in GSH, GRX, and GSH reductase were detected in the brains of transgenic HD mice models (R6) [104, 105]. S-glutathionylation of SOD1 isolated from human erythrocytes at Cys111 promoted SOD1 monomer formation and subsequent aggregation [106]. Hence, alterations in S-glutathionylation and redox potential are important mediators of protein misfolding, and aberrant disulphide bond formation is implicated in this process.

5. ER Stress and Neurodegeneration

The major cellular location for protein disulphide bond formation is the ER. The highly oxidizing environment of this compartment (GSH : GSSG ratio~3 : 1) is necessary for formation of disulphide bonds and is in stark contrast to the reducing environment of the cytosol (GSH : GSSG ratio~100 : 1) [41, 92, 107]. The ER environment, therefore, is highly sensitive to changes in nitrosative and oxidative stress [31, 36].

ER stress is increasingly implicated as a pathogenic mechanism in neurodegenerative diseases [108–114]. ER stress occurs when misfolded proteins accumulate within the ER lumen, triggering the unfolded protein response (UPR) [115]. The UPR is a set of signalling pathways that initially aim to restore homeostasis by: (1) reducing protein synthesis and translocation, attenuating further accumulation of unfolded proteins in the ER, (2) activation of ER-resident chaperones including PDI to increase the protein folding capacity of the ER, and (3) induction of ERAD. The UPR activates three ER stress sensor proteins: inositol requiring kinase 1 (IRE1 α/β), double-stranded RNA-activated protein kinase-(PKR-) like ER kinase (PERK), and activating transcription factor 6 (ATF6), which transduce signals to the nucleus and cytosol [115, 116]. However, if homeostasis cannot be restored, apoptosis is triggered [115, 117]. Prolonged UPR activation linked to RNS or ROS triggers apoptosis through C/EBP homologous protein (CHOP), caspase 4, c-Jun, and c-Jun N-terminal kinase (JNK) [41, 118, 119].

PDI family members fulfil crucial roles in regulating ER stress by maintaining native protein conformation and facilitating protein degradation [120]. The remainder of this review will focus on the PDI family and the effect of S-nitrosylation/S-glutathionylation on PDI and its functional role in neurodegeneration.

6. PDI Family Members

There are currently 21 identified members of the PDI family [32, 120–125], which share several features in common; at least one domain with a TRX fold, the presence of a signal sequence, and ER localization due to the presence of an KDEL or other ER retention signal [32, 120, 126]. Whilst PDI family members contain a TRX domain, they essentially differ from TRX due to their higher redox potentials, substrate binding domains, and their ability to display both isomerase and chaperone activities, which renders them more efficient than TRX at forming/reforming disulphide bonds [127, 128]. Whilst PDI family members primarily mediate protein folding, other functions have also been ascribed to them, including regulation of Ca^{2+} homeostasis [129, 130] and ERAD, thus ameliorating protein misfolding within the ER [33–35].

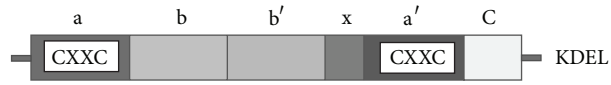

Figure 1: Domains of PDIA1. TRX-like domains representing catalytic active domains a a′. The b domain and b′ are catalytically inactive. The linker region is responsible for binding to the substrate. The C terminal is followed by an ER retrieval signal KDEL.

PDI disulphide-isomerase activity catalyzes the rearrangement of nonnative (incorrectly formed) disulphide bonds on nascent proteins, which would otherwise result in the formation of a misfolded structure. This activity is mediated through catalysis of thiol disulphide exchange (isomerization), whereby non-native disulphide bonds are initially reduced, and then oxidized to form the native structure [131–133]. Disulphide formation and stability are facilitated by the redox conditions of the ER [31]. Thus, active-site cysteines shift between two redox states: oxidation and the formation of disulphide bonds and reduction leading to the formation of thiols with free sulfhydryls [134]. In addition, PDI also has general chaperone activity which is independent of its disulphide interchange function [135–137]. This chaperone activity does not require its catalytic domains or active sites [138, 139].

PDI (PDIA1), the prototype of the PDI family, is a 55 kDa protein with two catalytically inactive TRX domains (b and b′), inserted between two TRX-like catalytic domains (a and a′), and an acidic C terminal domain with an ER-retention motif (KDEL). PDIA1 contains a CXXC catalytically active motif (Figure 1). All domains of PDI are required for efficient catalysis of disulphide bond formation and rearrangement [32, 120, 140]. The structure of yeast PDI has revealed that the binding of PDI to misfolded protein substrates is facilitated by two of the active cysteines positioned on opposite sides of the molecule [140, 141]. The noncatalytic b′ domain is situated on the base and is the major site for binding of substrates [141], although other domains also contribute to this process. The b-b combination of noncatalytic domains is present only in PDIA1, PDIA2 (PDIp), PDIA3 (ERp57), and PDIA4 (ERp72) family members [142–146]. PDIA1 has the broadest substrate specificity of the PDI family members examined to date [144].

PDIA2 is primarily expressed in pancreatic cells and dopaminergic neurons [146–148]. The domain structure of PDIA2 is similar to PDIA1, with a CXXC motif in the homologous a, a′ domains, intervening b, b′ domains, a x-linker region, and an N-terminal ER sequence [149]. PDIA2 also contains a KEEL motif at the C-terminus, an ER retention signal analogous to KDEL [150]. Similar to PDI, PDIA2 can

interact with protein substrates with and without cysteine residues [148, 151], suggesting that PDIA2 may act as a chaperone independent of catalyzing disulphide bond formation [147]. However, although its domain organization is similar to PDI, its physiological role remains unclear.

PDIA3 is the second most abundant soluble protein after PDIA1 found in the ER [120]. It contains a protein sequence homologous to PDIA1, with similarities in domain architecture but differences in substrate binding [152]. Whilst PDIA3 is an oxidoreductase with thiol-dependent reductase activity [153], it is different to the other PDI family members in that it acts primarily on glycosylated proteins by associating noncovalently with the lectin chaperones calnexin and calreticulin [154]. The catalytic properties differ from PDIA1 and the redox potential of PDIA3 is also lower than PDIA1 [155, 156]. PDIA3, like PDIA1, has two CXXC motifs at the conserved active sites and four similar TRX-like domains (a-b-b′-a′) [153, 156]. The C-terminus of PDIA3 has an ER retention signal with a sequence similar to that of PDIA1 [153] and a nuclear localisation signal near the C terminal with a high affinity for importin [128, 157, 158]. In addition, PDIA3 and PDIA1 differ in terms of substrate binding specificity due to differences in homology in their b′ domains. The binding domain of PDIA3 is enriched in lysine and arginine residues, so that PDIA3 binds to proteins containing negatively charged P domains, such as those found in calreticulin [142, 158]. The oxidative and catalytic property of PDIA3 and PDIA1 both rely on a charged glutamic acid and a pair of lysine residues found behind the active CXXC site [120].

Some PDI family members possess more than two CXXC active sites. PDIr, Erp46, and PDIA4, also known as Ca^{2+} binding protein (CaBP2) [159], all have three active sites [121, 160–163], and ERdJ5 contains four active sites [164]. PDIA4 is similar to PDIA1 in its catalytic domains but has lower sequence similarity in the other domains. It can also act as a substitute for PDIA3 in folding specific proteins, but it does not bind to glycoproteins [165]. Other PDI gene family members include DNAJC10, ERP27, ERP29 (ERP28), ERP44, PDIA5, PDIA6, PDILT, and TXNDC5 (for comprehensive review please refer to [125]). However, this review will focus on PDIA1, PDIA2, PDIA3, and PDIA4 as these are the only PDI family members to date that are reported to undergo S-nitrosylation.

7. The Presence of PDI in Non-ER Compartments

Whilst PDI family members are primarily considered to be ER-localized, they are also present in other cellular locations, including the nucleus, cytoplasm, cell surface, and extracellular space [128]. Few proteins linked to neurodegeneration are present in the ER, so it is possible that PDI plays an important role in these locations. In the ER, PDI must be maintained in a balance between its oxidized and reduced states to facilitate disulphide bond formation [166, 167]. However, in non-ER compartments, PDI family members have an increased ability to catalyze the reduction of disulphide bonds compared to the ER [168]. The mechanism of transit of PDI from the ER remains unknown, and because of the presence of the KDEL retention signal, observations of non-ER localized PDI have previously been questioned [128]. However, other primarily ER-localized proteins that possess a KDEL motif, such as calreticulin and binding immunoglobulin protein (BiP), are also secreted and located in the nucleus, cytoplasm and cell surface [169–176].

PDI in the cytosol has been postulated to act as a cofactor with insulin-degrading enzyme (IDE) during insulin metabolism, while acting in concert with reduced GSH to catalyze disulphide bond cleavage [177]. There is also evidence that PDI redistributes away from its ER location into the cytoplasm in pathological conditions. ER stress causes the redistribution of PDIA1 and PDIA3 from the ER to the cytosol [178], consistent with the notion that PDI in locations other than the ER is neuroprotective. Furthermore, one study demonstrated that overexpression of reticulon-4A (NOGO A) triggered the redistribution of PDI from the ER into vesicular-type structures localized in an undefined cellular compartment, both *in vitro* and *in vivo*, which occurred in the absence of the UPR [179]. Deletion of NOGO A, B from ALS mouse models, involving transgenic overexpression of mutant $SOD1^{G93A}$, led to earlier onset and increased disease progression, indicating that reticulons mediate PDI function and redistribution in neurodegeneration [179]. A more recent study, using human neuroblastoma SH-SY5Y cells overexpressing reticulon protein 1C (RTN-1C), demonstrated that redistribution of PDI away from the ER into vesicular structures led to a consequent increase in the enzymatic activity of PDI and a decrease in S-nitrosylation [180].

PDI has also been detected at the cell membrane, where a role in NO signalling has been described. S-nitrosylated extracellular proteins transfer NO to the cytosol via the reducing activity of cell surface PDI [181, 182]. During this process, cell-surface PDI also undergoes thiol modification [183]. Furthermore, PDIA3 interacts with prion proteins (PrP) at the cell surface and may play a key role in PrP accumulation [184]. In addition, PDIA1 and PDIA3 have been detected in the nucleus, where they are posited to anchor DNA loops to the nuclear matrix [128, 185, 186]. PDI-like activity has also been detected in mitochondria, although PDIA1 has not been identified in this compartment [187], and it is possible that Mia 40 contributes to this activity [188, 189].

PDIA1 and PDIA3 have also been detected at mitochondrial-associated ER membranes, where, remarkably, they may regulate apoptosis signalling [190]. The expression of polyglutamine expanded Huntington protein led to PDIA1 and PDIA3 accumulation in this location, where it triggered mitochondrial outer membrane permeabilization through activation of proapoptotic BCL-2 family members, triggering apoptosis [190]. Hence, whilst PDI functions protectively through its chaperone and isomerase activities [191], it can also trigger pro-apoptotic mechanisms [190]. While this process has not yet been fully defined, the novel proapoptotic function of PDI may represent a new link between protein misfolding and cell death.

8. Role of PDI in Neurodegeneration

There is now substantial evidence linking PDI family members to protein misfolding in neurodegeneration. PDIA1 is upregulated in AD brain tissues [192], PDIA3 forms a complex with calreticulin and A-β peptides in patients' CSF [193], and NFTs are immunopositive for PDI [194, 195]. Similarly, in cellular models of PD, treatment of dopaminergic neurons with 6-hydroxydopamine (6-OHDA) induces ER stress, oxidation, and aggregation of PDIA3 [196]. PDIA2 is upregulated in SH-SY5Y human neuroblastoma treated with either 1-methy-4-phenyl-pyridinium (MPP+) or proteasome inhibitor lactacystin while immunoreactivity to PDIA2 has also been detected in LB in postmortem brains of PD patients [146]. Furthermore, the a′ domain of PDIA1 inhibits α-synuclein fibril formation [197], and coexpression of PDIA1 decreased synphilin-1 positive LB formation in the cytoplasm [75]. PDIA1 was upregulated in the brains of Creutzfeldt-Jakob disease (CJD) patients [198], while PDIA1 and PDIA3 were upregulated in prion disease in scrapie infected rodents [199]. Pharmacological inhibition of PDIA3 using bacitracin increased the accumulation of aggregated PrP, also suggesting that PDI is not functional in prion disease [184]. Furthermore, upregulation of PDIA1 and PDIA3 was associated with mitochondrial dysfunction in cells expressing misfolded PrP [199]. The detection of mitochondrial apoptosis triggered by PDIA1 and PDIA3 in HD models [190] also highlights the intrinsic link between PDI upregulation and mitochondrial dysregulation in neurodegeneration [199].

There is also increasing evidence for an important role for PDI in ALS. PDIA1 is upregulated and is a component of TDP-43 and FUS-positive cytoplasmic inclusions in motor neurons of sporadic ALS patients [200, 201]. Additionally, PDIA1 is a risk factor for the development of ALS [202]. PDIA1 also colocalizes with mutant SOD1-positive inclusions in cell culture and transgenic SOD1 rodents [89, 203, 204]. Overexpression of PDIA1 decreases the formation of mutant SOD1 inclusions whereas knockdown of PDI using siRNA increases the proportion of inclusions [89]. Furthermore, a synthetic mimic of the PDIA1 active site; (±)-trans-1,2-bis (mercaptoacetamido)cyclohexane (BMC), is protective against mutant SOD1 aggregation in cell culture [89]. SOD1 contains four cysteine residues, and non-native disulphide bonds between Cys6 and Cys111 have been implicated in mutant SOD1 aggregation [205]. Conversely, upregulation of PDIA1 in microglia in $SOD1^{G93A}$ mice was associated with increased levels of NADPH oxidase (NOX), superoxide, and tumour necrosis factor-α. Pharmacological inhibition and knockdown of PDIA1 using siRNA decreased superoxide and NOX activation in microglia, therefore providing further evidence for a potential neurotoxic role of PDIA1 [206].

PDI is therefore upregulated during UPR activation and is part of a cellular protective mechanism that prevents protein misfolding and aggregation in neurodegeneration. PDI family members are especially vulnerable to oxidative and nitrosative-linked posttranslational modifications due to the highly oxidizing environment of the ER and the presence of cysteine residues in the PDI catalytic regions. Irreversible S-nitrosylation of PDI (SNO-PDI) may therefore ameliorate its protective function in neurodegenerative disorders and thus contribute to disease.

9. SNO-PDI and Neurodegeneration

PDI is S-nitrosylated by endogenous nNOS in both its TRX domains leading to a significant reduction in isomerase and chaperone activity [75]. Also, induction of SNO-PDI using NO donor S-nitrosocysteine (SNOC) completely abrogates the catalytic activity of PDI, resulting in neuronal cell death [207].

SNO-PDI has been detected in postmortem brain tissue of sporadic PD and AD patients [75] and lumbar spinal cord tissues of ALS patients and $SOD1^{G93A}$ mice [89]. This was linked to excessive production of NO or exposure to exogenous agents such as rotenone [75]. PDI was shown to be modified in the cysteine thiol groups in the C-terminal CXXC motif, leading to the accumulation of polyubiquitinated proteins and activation of the UPR [75]. SNO-PDI formation is associated with synphilin misfolding in PD [31] and mitochondrial mediated apoptosis in prion infection [199]. SNO-PDI is also found in cultured astrocytes after ischemia/reperfusion-induced iNOS production, leading to increases in ubiquitinated aggregates that colocalize with SOD1 [7].

One potential physiological mechanism of SNO-PDI production involves pathological hyperactivation of NMDAr [31] and inhibition of mitochondria leading to the generation of ROS, nNOS, and NO [31, 70, 71]. Exposure of cortical neurons to NMDA produces SNO-PDI, leading to an increase in polyubiquitinated proteins and apoptosis after 24 hrs of treatment. Furthermore, overexpression of WT PDI leads to a decrease in polyubiquitination and apoptosis, suggesting that PDI may provide protection against excitotoxicity from excessive stimulation of NMDA receptors [75]. Additionally, treatment with Rotenone, a mitochondrial complex inhibitor, produces elevated levels of SNO-PDI [75], suggesting that mitochondria are another source of NO or cytosolic nNOS [31]. NO disrupts Ca^{2+} homeostasis, potentially via S-nitrosylation of the ER Ca^{2+} channel ryanodine receptor, and induction of ER stress [57, 208]. ER-resident proteins are particularly vulnerable to S-nitrosylation and as such a positive feedback mechanism would create a scenario whereby excessive RNS/ROS increasingly deactivates protective ER-resident chaperones such as PDI, prolonging UPR activation, leading to increases in ROS/RNS generation eventually resulting in cell death [31]. ER dysfunction due to excessive oxidative/nitrosative stress may, thus, lead to the S-nitrosylation of PDI in neurodegenerative disease [31]. However, PDI family members PDIA1, PDIA3, and PDIA4 can be S-nitrosylated independently of UPR induction [209]. Alternatively, PDI located at the cell surface may also promote production of SNO proteins. It has been previously suggested that extracellular SNO proteins may transfer NO to the cytoplasm via the reducing activity of cell surface PDI [181, 182]. According to this theory, reduced NO may readily penetrate the plasma membrane, leading to SNO production [128] (Figure 2). Hence, the formation of SNO-PDI results in the abrogation of the normally protective isomerase/chaperone

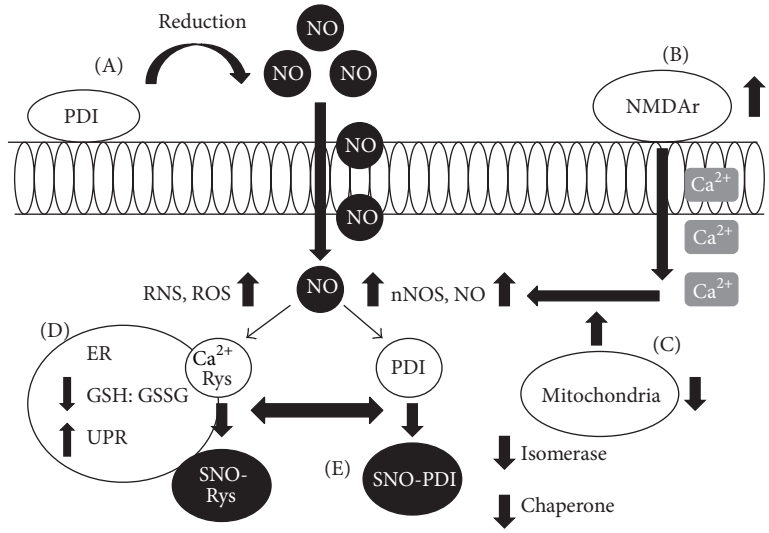

Figure 2: Cell surface PDI, NO, and SNO-PDI. (A) Cell surface PDI reduces NO from extracellular SNO proteins (SNO-P) and in the process undergoes thiol modification. (B) Hyperactivation of the NMDAr leads to an intracellular influx of Ca^{2+} ions (NMDAr may also undergo reversible S-nitrosylation to ameliorate excessive activity). (C) Inhibition of mitochondria contributes to an increase in intracellular NO which is potentially oxidized by O_2 leading to an increase in NO, nNOS, ROS, and RNS. (D) Increases in RNS/ROS alters the ER redox environment, and NO S-nitrosylates Ca^{2+} ryanodine (Ryn) receptor leading to a disruption in Ca^{2+} homeostasis. (E) ER-resident proteins such as PDI are vulnerable to S-nitrosylation, deactivating its isomerase and chaperone activity, leading to accumulation of misfolded proteins, ER stress, and UPR induction.

activity of PDI, which may contribute to protein misfolding and production of SNO proteins. This suggests that SNO-PDI may be a common pathological mechanism contributing to neurodegenerative diseases.

10. S-Glutathionylation and PDI

A link between S-glutathionylated PDI and neurodegenerative disease has not yet been established [210]. However, cysteine residues in the a and a′ domains of PDI make it a potential target for S-glutathionylation [211].

PDI has been shown to be S-glutathionylated at two of its four active cysteine sites (Cys53, Cys56 or Cys397, Cys400) [92]. S-glutathionylation was induced in these cells by treatment with anticancer agent O_2–[2,4-dinitro-5-(*N*-methyl-*N*-4-carboxyphenylamino) phenyl]1–(*N*,*N* dimethylamino)diazen-1-ium-1,2-diolate (PABA/NO), which led to a dose-dependent increase in intracellular NO [210], triggering UPR induction and cell death [92]. S-glutathionylation of PDI has been demonstrated in human leukemia (HL60) and ovarian cancer cells (SKOV3) inhibiting its isomerase function [205]. In addition, S-glutathionylation of PDI abrogates its chaperone activity and prevents binding to oestrogen receptor alpha (ERα) [212]. The PDI-ERα interaction may protect ERα from oxidation and ensure its native protein conformation [213]. However, aberrant S-glutathionylation of PDI leads to destabilisation of the receptor and dysregulatation of ERα signaling. This may subsequently mediate cell death via activation of the UPR and reduced ERα stability [212]. However, although PABA/NO treatment increased levels of intracellular NO, it did not lead to S-nitrosylation of PDI [210]. There are two pools of S-nitrosylated proteins, GSH stable and GSH labile proteins, with the latter pool being readily subject to conversion to S-glutathionylated products [41]. Therefore, the lack of SNO proteins after PABA/NO treatment may be due to conversion of SNO proteins to S-glutathionylated proteins [210] (Figure 3). This notion therefore provides a link between S-nitrosylation and S-glutathionylation, although the exact relationship between these modifications remains unknown [41].

S-glutathionylation of PDI was proposed to be an upstream signalling event triggering misfolded protein accumulation and UPR induction [210, 211]. As PDI may regulate redox potential at the cell surface [182, 214], it therefore may facilitate cell adhesion [215], antigen processing [216], and glioma cell invasion [217]. S-glutathionylation of cell surface proteins alters extracellular and intracellular redox homeostasis [210]. Hence, irreversible S-glutathionylation/S-nitrosylation of cell surface PDI could alter redox potential, leading to amelioration of the protective chaperone/isomerase functions of PDI. This mechanism may therefore contribute to the excessive production of SNO and S-glutathionylated proteins observed in neurodegenerative disease.

11. Conclusion

PDIs are a large family of chaperones and foldases which have complex yet still inadequately described functions with emerging roles in neurodegenerative diseases. Whilst S-nitrosylation plays a normal physiological role in signalling pathways, aberrant modification is triggered during

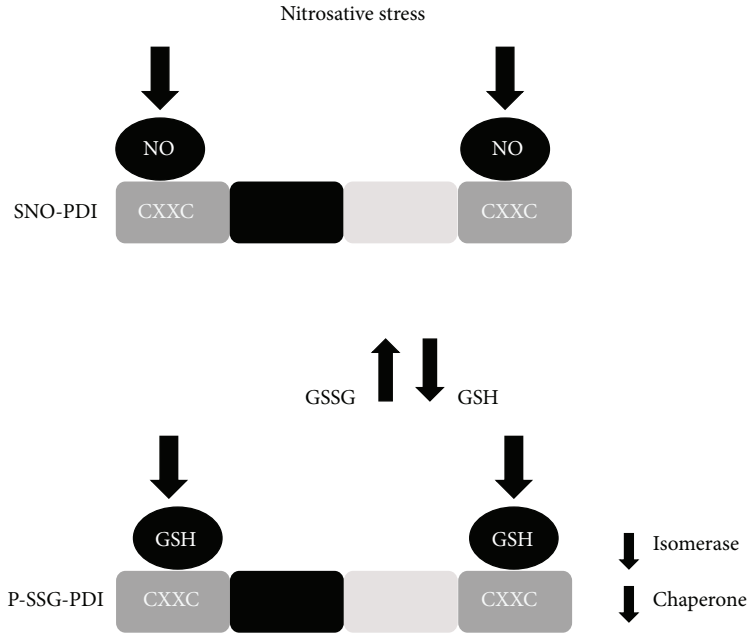

Figure 3: S-glutathionylation of PDI. Nitrosative stress from an exogenous agent (PABA/NO) increases intracellular NO and leads to the production of SNO-PDI. However, this may result in a decrease in GSSG/GSH ratio and increases in the free cellular pool of GSH. GSH then binds to the catalytic (a, a′) domains of PDI, resulting in S-glutathionylation (P-SSG) of its cysteine residues and attenuation of its protective isomerase and chaperone activity.

conditions of elevated nitrosative and oxidative stress. Accumulating evidence suggests that SNO-PDI plays a role in the pathogenesis of neurodegenerative diseases such as AD, PD, and ALS, and this may exacerbate neurodegeneration via a number of mechanisms. However, most of the available reports are correlative in nature and therefore more direct approaches examining the contribution of S-nitrosylation of PDI family members to neurodegeneration are warranted. S-nitrosylation is also linked to another previously described modification of PDI, S-glutathionylation, although the S-glutathionylation of PDI and its role in neurodegenerative diseases have not been elucidated. Whilst PDI family members are conventionally regarded as being ER localized, they are also present and catalytically active in several other cellular locations, which is likely to be particularly important in disease as few proteins associated with neurodegeneration are found in the ER. Finally, cell surface PDI, which reduces NO allowing it to pass through the plasma membrane, may lead to the production of SNO proteins and therefore also contribute to the pathogenesis of neurodegenerative diseases. The broad involvement of PDIs in human neurodegenerative diseases highlights the need for a better understanding of how they become inactivated by posttranslational modification, which is crucial to evaluate their use as possible targets for disease intervention.

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Research Article

Cysteine-10 on 17β-Hydroxysteroid Dehydrogenase 1 Has Stabilizing Interactions in the Cofactor Binding Region and Renders Sensitivity to Sulfhydryl Modifying Chemicals

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17β-Hydroxysteroid dehydrogenase type 1 (17β-HSD1) catalyzes the conversion of estrone to the potent estrogen estradiol. 17β-HSD1 is highly expressed in breast and ovary tissues and represents a prognostic marker for the tumor progression and survival of patients with breast cancer and other estrogen-dependent tumors. Therefore, the enzyme is considered a promising drug target against estrogen-dependent cancers. For the development of novel inhibitors, an improved understanding of the structure-function relationships is essential. In the present study, we examined the role of a cysteine residue, Cys^{10}, in the Rossmann-fold NADPH binding region, for 17β-HSD1 function and tested the sensitivity towards sulfhydryl modifying chemicals. 3D structure modeling revealed important interactions of Cys^{10} with residues involved in the stabilization of amino acids of the NADPH binding pocket. Analysis of enzyme activity revealed that 17β-HSD1 was irreversibly inhibited by the sulfhydryl modifying agents N-ethylmaleimide (NEM) and dithiocarbamates. Preincubation with increasing concentrations of NADPH protected 17β-HSD1 from inhibition by these chemicals. Cys^{10}Ser mutant 17β-HSD1 was partially protected from inhibition by NEM and dithiocarbamates, emphasizing the importance of Cys^{10} in the cofactor binding region. Substitution of Cys^{10} with serine resulted in a decreased protein half-life, without significantly altering kinetic properties. Despite the fact that Cys^{10} on 17β-HSD1 seems to have limited potential as a target for new enzyme inhibitors, the present study provides new insight into the structure-function relationships of this enzyme.

1. Introduction

17β-Hydroxysteroid dehydrogenase (17β-HSD) enzymes are involved in the interconversion of inactive and active sex-steroid hormones, thereby playing an essential role in the intracrine regulation of estrogen-, androgen-, and progesterone-dependent physiological functions [1]. 17β-HSD1 is responsible for the conversion of estrone to estradiol, the most potent natural estrogen. The highest expression of this enzyme is found in breast and ovarian tissue. Importantly, 17β-HSD1 expression showed a negative correlation with breast cancer progression and was identified as an independent prognostic marker for the disease-free and overall survival of patients with breast cancer [2, 3]. Elevated expression of 17β-HSD1 was observed in endometrial cancer [4] and nonsmall cell lung cancer [5]. Furthermore, the expression ratio of 17β-HSD1 and 17β-HSD2 was found to be a predictor of the response to tamoxifen in postmenopausal breast cancer patients [6].

The administration of specific 17β-HSD1 inhibitors led to significantly decreased tumor growth in breast cancer cell xenograft tumor mouse models [7, 8] and reversed estrogen-dependent endometrial hyperplasia in transgenic mice [9], indicating that this enzyme is a promising drug target against estrogen-dependent diseases such as endometriosis and endometrial cancer as well as breast and ovarian tumors.

Several classes of chemicals inhibiting 17β-HSD1 were tested *in vitro*, including steroid-like molecules, nonsteroidal compounds, and chimeric molecules, acting on both the active center and the cofactor binding site of the enzyme [8, 10–16]. However, only a limited number of inhibitors have been tested *in vivo* so far and further research is needed. In order to develop potent and selective 17β-HSD1 inhibitors, a profound understanding of the structure-function relationships of the enzyme is essential.

17β-HSD1 belongs to the short-chain dehydrogenase/reductase (SDR) family and contains the conserved Rossmann-fold for nucleotide binding, the catalytic triad with residues Ser^{142}, Tyr^{155}, and Lys^{159} [17] and a dimerization region. Lys^{149} is a critical residue for the discrimination between C-18 and C-19 steroid substrates [18].

Targeting of functionally essential cysteine residues in proteins is suggested as a promising approach for the design of new types of pharmaceutical agents [19]. 17β-HSD1 contains six cysteines. In a previous study, we demonstrated that the structurally related enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) contains a cysteine residue at position 90, analogous to Cys^{10} on 17β-HSD1 [20]. Mutation of Cys^{90} on 11β-HSD2 led to almost complete inactivation of the enzyme. Therefore, we hypothesized that Cys^{10} on 17β-HSD1 may represent a target site for novel inhibitors. In the present study, we applied 3D structure modeling and enzyme activity measurements to investigate the biochemical properties of Cys^{10}Ser mutant 17β-HSD1 and to compare its sensitivity towards sulfhydryl modifying agents with that of wild-type 17β-HSD1.

2. Materials and Methods

Cell culture media were purchased from Invitrogen (Carlsbad, CA) and [2,4,6,7-^{3}H]-estrone from Amersham Pharmacia (Piscataway, NJ, USA). All other chemicals were from Fluka AG (Buchs, Switzerland) and of the highest grade available.

2.1. Construction of Expression Plasmids and Site-Directed Mutagenesis. In order to facilitate detection, an octahistidine tag was added to the C-terminus of human 17β-HSD1 by PCR amplification using oligonucleotide primers containing the tag-coding sequence (forward primer 5′-TAAACCCTGAGGAGGTGGCGGAGGTCTTC-3′, reverse primer 5′-TGCTCTAGAAGCTTAATGATGATGATGATGATGATGATGCTGCGGGGCGGCCGGAGGATCG-3′). The activity of the tagged 17β-HSD1 was indistinguishable from that of the untagged enzyme. The substitution of Cys^{10} to serine was introduced into the C-terminal histidine-tagged 17β-HSD1 cDNA in Bluescript vector by site-directed mutagenesis using the forward primer 5′-TCATCACCGGCTCTTCCTCG-3′ and the reverse primer 5′-GAGGAAGAGCCGGTGATGAG-3′ according to the Quick Change mutagenesis kit (Stratagene, Amsterdam, The Netherlands), followed by subsequent recloning into pcDNA3.1 expression plasmid.

2.2. Cell Culture, Transfection, and Western Blotting. HEK-293 cells were grown to 60–70% confluence in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum, 4.5 g/L glucose, 50 units/mL penicillin/streptomycin, and 2 mM glutamine, followed by transfection using the calcium phosphate precipitation method. The transfection efficiency was 32 ± 3%. To examine the expression levels of the overexpressed proteins, we performed SDS-PAGE (30 μg of total cellular proteins loaded per lane) followed by immunoblotting and detection of the histidine-tagged proteins using tetra-His antibody (Qiagen GmbH, Hilden, Germany; Cat. No. 11561526). As a loading control β-Actin was visualized using an anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA; Cat. No. B1204).

2.3. 17β-HSD1 Activity Measurements. To determine 17β-HSD1 activity, HEK-293 cells were transfected by the calcium phosphate precipitation method. Cells were harvested 48 h after transfection, washed twice with phosphate-buffered saline (PBS), and centrifuged for 4 min at 150 ×g. Supernatants were removed and the cell pellets were quick-frozen in a dry ice-ethanol bath and stored at −80°C. The reductase activity of 17β-HSD1 was measured by incubation of cell lysates for 10 min at 37°C in the presence of 200 nM radiolabeled estrone and 400 μM NADPH in a reaction buffer containing 20% glycerol, 1 mM EDTA, and 50 mM potassium phosphate, pH 7.4. Inhibitors were diluted from stock solutions in dimethylsulfoxide (DMSO) and immediately used in the assays. The DMSO concentration did not exceed 0.1% and had no effect on the enzyme activities.

For the enzyme activity assay in intact cells, HEK-293 cells were grown in 10 cm culture dishes, transfected with plasmids for histidine-tagged wild-type 17β-HSD1 or Cys^{10}Ser mutant, detached 24 h after transfection and distributed in 96-well plates at a density of 10,000 cells per well. After 16 h, cells were incubated in serum- and steroid-free medium, and the conversion of radiolabeled estrone to estradiol was determined upon incubation for 30 min at 37°C in a total volume of 50 μL containing 200 nM estrone. The reaction was stopped by adding methanol containing 2 mM unlabeled estrone and estradiol, followed by separation of steroids by TLC and scintillation counting.

For determination of enzyme kinetics, estrone concentrations ranging from 10 to 800 nM were used. Data (mean ± SD) were obtained from three independent experiments and were calculated using the Data Analysis Toolbox (Elsevier MDL, Allschwil, Switzerland).

2.4. Analysis of the Protein Stability of Wild-Type 17β-HSD1 and Mutant $Cys^{10}Ser$. The stability of wild-type and mutant 17β-HSD1 protein was analyzed basically as described previously [21]. Briefly, to investigate the protein half-life of wild-type 17β-HSD1 and mutant Cys^{10}Ser, HEK-293 cells grown in six-well plates were transfected with plasmids for mutant and wild-type enzyme, washed once with PBS 24 h after transfection, and incubated with fresh medium containing 50 μg/mL cycloheximide. At 0, 12, 24, and 48 h, aliquots of cells were harvested, followed by immunodetection using

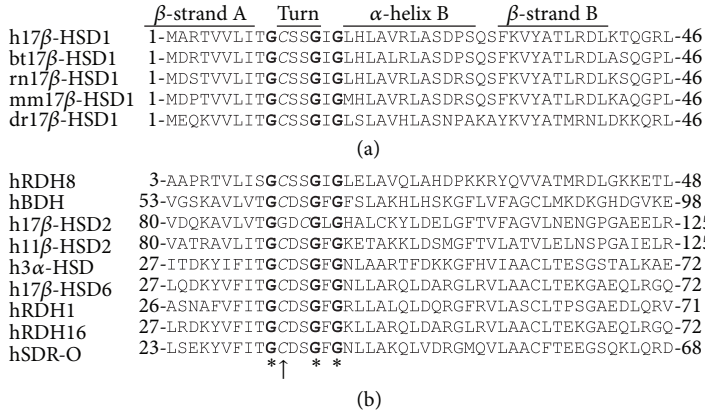

Figure 1: Sequence alignment of the cofactor binding domain of 17β-HSD1. Peptide sequences of human (h), bovine (bt), rat (rn), mouse (mm), and zebrafish (Danio rerio, dr) 17β-HSD1 (SDR28C1) are shown in the *upper panel* and those of the related human SDR enzymes all-trans retinol dehydrogenase RDH8 (SDR28C2), hydroxybutyrate dehydrogenase BDH (SDR9C1), 17β-HSD2 (SDR9C2), 11β-HSD2 (SDR9C3), 3α-HSD (SDR9C4), 11-cis retinol dehydrogenase (SDR9C5), 17β-HSD6 (SDR9C6), retinol dehydrogenase SDR-O (SDR9C7), and retinol dehydrogenase RDH16 (SDR9C8) in the *lower panel*. The locations of β-strand A through β-strand B are indicated above the alignment. The position of the conserved Cys^{10} on 17β-HSD1 is indicated by an *arrow* and the conserved glycine residues of the Rossmann-fold by asterisks (*in bold*).

tetra-His antibody. Longer incubations with cycloheximide were inappropriate due to toxic effects in HEK-293 cells.

Alternatively, cells transfected with plasmids for mutant and wild-type enzyme were washed once 16 h after transfection, followed by incubation in leucine-free DMEM (MP Biomedicals, Illkirch, France) for 45 min to deplete endogenous leucine. The medium was then replaced by 1 mL of leucine-free DMEM supplemented with 20 μCi/mL L-leucine-[3,4,5-^{3}H(N)], followed by incubation for 3 h. The labeling was terminated by addition of 5 mM unlabeled leucine, washing twice with DMEM, and incubation in DMEM. At 0, 12, 24, and 48 h, aliquots of cells were snap-frozen prior to purification of histidine-tagged proteins by using a Ni-NTA agarose kit according to the manufacturer (Qiagen AG, Hombrechtikon, Switzerland). After elution, proteins were subjected to SDS-PAGE, the gels were dried and exposed to tritium-sensitive screens (TR uncoated BaFBr:Eu^{2+} screens) for 16 h, followed by analysis using a Cyclone Phosphor-Imager (PerkinElmer Life and Analytical Sciences, Shelton, CT, USA).

2.5. Multiple Sequence Alignment. The protein sequences of 17β-HSD1 from different species and different human SDRs were compared using the ClustalW algorithm, run on http://www.ebi.ac.uk/, with default program parameters.

2.6. Structural Modeling. To investigate the effect on the structure of 17β-HSD1 of mutating Cys^{10} to Ser, we extracted 1FDT from the Protein Data Bank (PDB). 1FDT contains human 17β-HSD1 cocrystalized with estradiol and $NADP^+$ [22]. Cys^{10} was converted to serine using the Biopolymer option in the Insight II software package. The structure of the Ser^{10} mutant 17β-HSD1 was refined with Discover 3 for 10,000 iterations, using a distant dependent dielectric constant of 2.

3. Results

3.1. Multiple Sequence Alignments and 3D Structure Modeling. The analysis of the peptide sequence of human 17β-HSD1 using the Clustal W algorithm [23] revealed that residue Cys^{10} is highly conserved among species, including rodents and zebrafish (Figure 1), suggesting a role for this residue in the stability and/or function of the protein. Cys^{10} is located between the first two of three highly conserved glycine residues of the Rossmann-fold nucleotide binding domain. Interestingly, the cysteine and the two downstream serine residues also are present in all-trans retinol dehydrogenase RDH8, which like 17β-HSD1 belongs to the SDR28C family. Moreover, the cysteine residue at this position is conserved in all members of the SDR9C family, except for 17β-HSD2, which has a cysteine residue downstream by two positions.

To begin to understand the role of Cys^{10} for 17β-HSD1 function, we analyzed the interactions of Cys^{10} with adjacent amino acids in 17β-HSD1 cocrystalized with estradiol and $NADP^+$ [22]. The crystal structure of 1FDT reveals that Cys^{10} has van der Waals contacts with Ile^7, Gly^9, Gly^{15}, Ala^{34}, and Thr^{35}. Gly^9, Gly^{15}, and Thr^{35} directly stabilize the binding of the cofactor $NADP^+$ (Figure 2(a)). The numerous close contacts of Cys^{10} with amino acids in the NADP(H)-binding site indicate that substitution of the sulfhydryl group with a larger chemical would disrupt this site and probably alter NADP(H) binding and catalytic activity.

To begin to study the function of this cysteine residue, we substituted Cys^{10} with serine, the biochemically most similar amino acid. As revealed by the 3D model (Figure 2(b)), mutating Cys^{10} to serine does not lead to significant changes in contacts between Ser^{10} and Ile^7, Gly^9 and Ala^{34}. However, all three contacts between Ser^{10} and Gly^{15} are shorter than between Cys^{10} and Gly^{15}, and Gly^{15} is more distant from a phosphate oxygen on $NADP^+$ (Figure 2(b)). Also, Ser^{10} is a

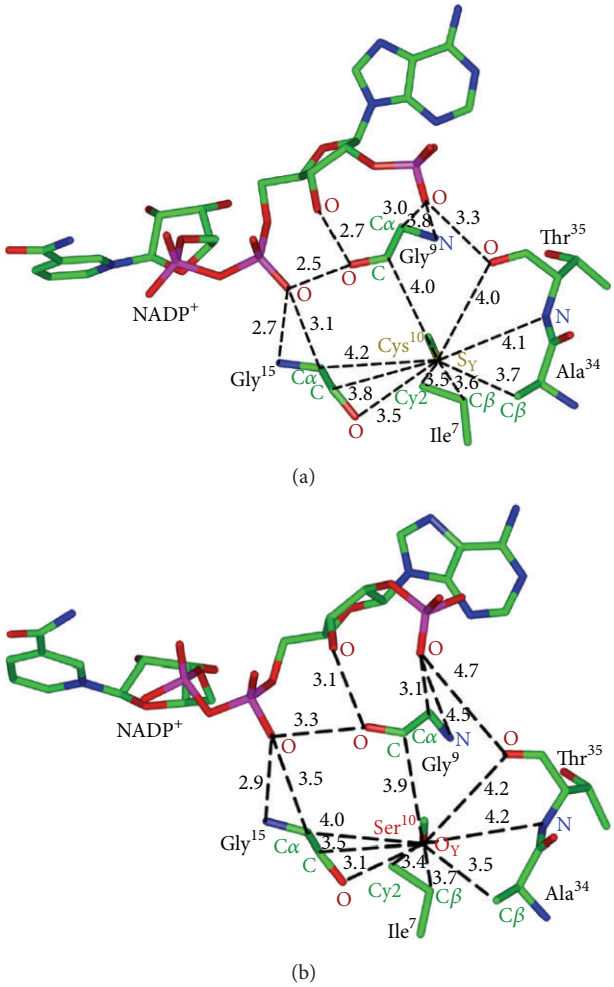

FIGURE 2: Position of Cys^{10} in wild-type 17β-HSD1 and of Ser^{10} in mutant 17β-HSD1. Cys^{10} has stabilizing interactions with neighboring residues involved directly in the binding of cofactor NADPH. *A*, wild-type 17β-HSD1. Cys^{10} has van der Waals contacts with Ile^{7}, Gly^{9}, Gly^{15}, Ala^{34}, and Thr^{35}. Gly^{9}, Gly^{15}, and Thr^{35} contact $NADP^{+}$. *B*, $Cys^{10}Ser$ mutant 17β-HSD1. Ser^{10} has van der Waals contacts with Ile^{7}, Gly^{9}, Gly^{15}, Ala^{34}, and Thr^{35}. The backbone nitrogen on Gly^{9} and the backbone oxygen on Thr^{35} no longer contact the oxygen on the adenosine phosphate.

little more distant from the backbone oxygen on Thr^{35}, and Thr^{35} is 4.7 Å from the oxygen on the adenosine phosphate. The backbone nitrogen on Gly^{9} is 4.5 Å from the same phosphate oxygen on $NADP^{+}$ (Figure 2(b)).

3.2. Inhibition of 17β-HSD1 by Sulfhydryl Modifying Agents. In a previous study, sulfhydryl modifying chemicals were found to exert potent inhibitory effects on 11β-HSD2 and substitution of Cys^{90} by serine abolished enzymatic activity [20]. To test the hypothesis that Cys^{10} on 17β-HSD1 has similar essential stabilizing interactions in the NADPH binding region, we assessed the inhibitory potential of the cysteine modifying agent N-ethylmaleimide (NEM) and of dithiocarbamate chemicals. NEM appeared to be a weak

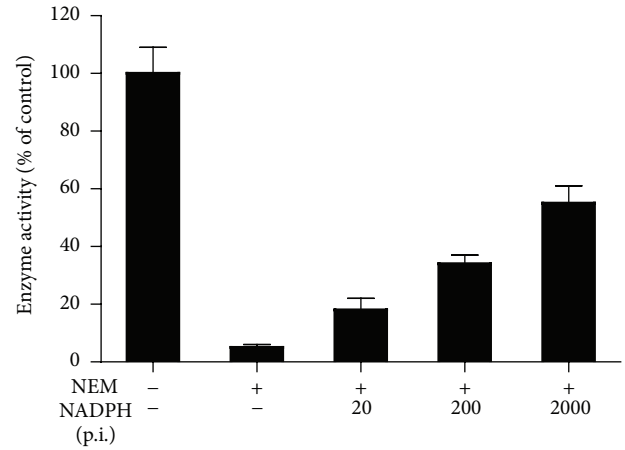

FIGURE 3: Preincubation with NADPH protects 17β-HSD1 from inhibition by NEM. Lysates of HEK-293 cells expressing 17β-HSD1 were preincubated for 15 min with saline or various concentrations of NADPH as indicated, prior to the addition of estrone (200 nM), NADPH (400 μM), and the sulfhydryl modifying agent N-ethylmaleimide (NEM, 60 μM). The estradiol produced was determined after 10 min of incubation. Data (mean ± SD) were normalized to the control in the absence of NEM and were obtained from at least three independent experiments measured in triplicate.

inhibitor with an IC_{50} of 22 ± 6 μM upon simultaneous incubation of lysates of 17β-HSD1 expressing HEK-293 cells with 200 nM estrone and increasing concentrations of NEM. In line with an irreversible mode of inhibition, preincubation with NEM resulted in a more pronounced inhibitory effect in a time-dependent manner. Preincubation of lysates with 20 μM NEM for 1 h prior to the addition of estrone completely abolished 17β-HSD1 activity (data not shown).

Next, we tested dithiocarbamate chemicals for inhibition of 17β-HSD1. In contrast to the previously observed potent inhibition of 11β-HSD2, dithiocarbamates turned out to have rather modest inhibitory effects on 17β-HSD1. The IC_{50} values for thiram (21 ± 4 μM), disulfiram (8 ± 1 μM), maneb (25 ± 3 μM), and zineb (24 ± 3 μM) upon simultaneous incubation of the 17β-HSD1 enzyme preparation with substrate and inhibitor were about two orders of magnitude higher than those obtained for 11β-HSD2, indicating that 17β-HSD1 is much less prone to inhibition by sulfhydryl modification.

Importantly, as shown in Figure 3, preincubation with NADPH for 15 min protected from inhibition by NEM, an effect which was concentration dependent. Preincubation with NADPH also protected from inhibition by dithiocarbamates (data not shown), suggesting that binding of NADPH prevents the covalent modification of Cys^{10} in the cofactor binding region.

3.3. Substitution of Cys^{10} with Serine Leads to Decreased Protein Stability. To study the role of Cys^{10} for 17β-HSD1 function, we generated mutant $Cys^{10}Ser$ and compared the expression of histidine-tagged wild-type and mutant enzymes upon transient expression in HEK-293 cells, which lack endogenous 17β-HSD1 expression. Despite comparable

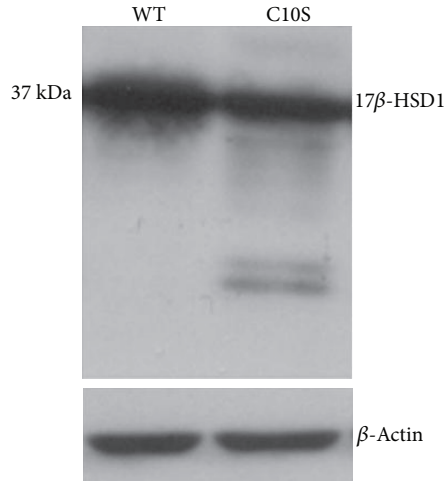

Figure 4: Western blot analysis of the protein expression of histidine-tagged wild-type 17β-HSD1 and mutant Cys^{10}Ser. A representative experiment from four independent transfections is shown.

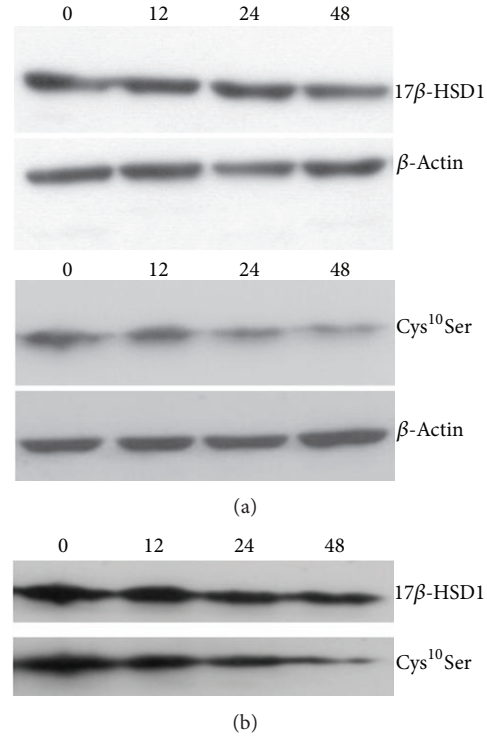

Figure 5: Stability of 17β-HSD1 and Cys^{10}Ser mutant. C-terminal histidine-tagged wild-type 17β-HSD1 and Cys^{10}Ser mutant enzymes were expressed in HEK-293 cells. *A*, 24 h posttransfection cells were incubated with 50 μg/mL cycloheximide to inhibit *de novo* protein synthesis. After 0, 12, 24, and 48 h cells were harvested and the amount of expressed protein was analyzed semiquantitatively using anti-histidine antibody. β-actin served as control. *B*, the protein half-life of 17β-HSD1 and Cys^{10}Ser mutant was estimated by pulse-chase experiments, labeling cellular proteins with tritiated L-leucine, followed by washing and incubation in leucine-free medium for different times. After purification of histidine-tagged proteins by Ni-NTA agarose, proteins were separated by SDS-PAGE and gels were dried and exposed to tritium-sensitive screens for detection of radioactivity by phosphor-imaging. Representative experiments are shown.

transfection efficiency, mutant Cys^{10}Ser was expressed at approximately twofold lower levels than wild-type 17β-HSD1 (Figure 4). Moreover, several low-molecular weight bands were detected, indicating a lower stability of the Cys^{10}Ser mutant protein.

Next, we performed experiments in transfected HEK-293 cells using cycloheximide in order to block *de novo* protein synthesis and estimate protein half-life. The signal detected for histidine-tagged 17β-HSD1 protein was not significantly decreased 48 h after blocking translation with cycloheximide (Figure 5(a)), indicating a protein half-life greater than 48 h. Longer incubations are not appropriate due to the cytotoxicity of cycloheximide. In contrast, mutant Cys^{10}Ser was less stable and the protein half-life of the mutant enzyme was estimated to be 26 ± 7 h (mean ± SD). A pulse-chase experiment using ^{3}H-leucine labeling confirmed the decreased stability of the mutant protein (estimated protein half-life of 15 ± 6 h) compared with the wild-type 17β-HSD1 (estimated protein half-life of 36 ± 10 h) (Figure 5(b)). The differences in the protein half-life estimation by the pulse-chase and cycloheximide methods may be explained by the incomplete inhibition of protein synthesis in the latter approach.

3.4. Mutant Cys^{10}Ser Retains Catalytic Activity and Is Protected from Inhibition by Sulfhydryl Modifying Agents. A comparison of the enzyme activities of lysates expressing wild-type 17β-HSD1 or mutant Cys^{10}Ser showed approximately 50% lower activity for the mutant (data not shown). However, after correction for protein expression using anti-histidine-tag antibody there was no significant difference between wild-type and mutant enzymes. The analysis of enzyme kinetics revealed a slightly higher apparent K_m but no significant change in the maximal velocity (V_{max}) for estrone reduction (Table 1). In intact cells, the activity of mutant Cys^{10}Ser was indistinguishable from that of the wild-type enzyme (data not shown).

Table 1: Enzyme kinetics of wild-type 17β-HSD1 and Cys^{10}Ser mutant. The reduction of various concentrations of estrone (10 to 800 nM) to estradiol was measured in cell lysates from transfected HEK-293 cells in the presence of 400 μM NADPH. The data were normalized according to protein expression and represent mean ± SD from four independent experiments measured in triplicate.

	V_{max} (nmol × h^{-1} × mg^{-1})	K_m (nM)
17β-HSD1 WT	110 ± 5	43 ± 6
17β-HSD1 Cys^{10}Ser	124 ± 3	65 ± 3

Next, we compared the effect of different dithiocarbamate chemicals on the activity of wild-type 17β-HSD1 and mutant Cys^{10}Ser. Mutant Cys^{10}Ser was protected from inhibition by all of the dithiocarbamates tested (Figure 6). Similarly, the cysteine-modifying agent NEM inhibited the activity of the

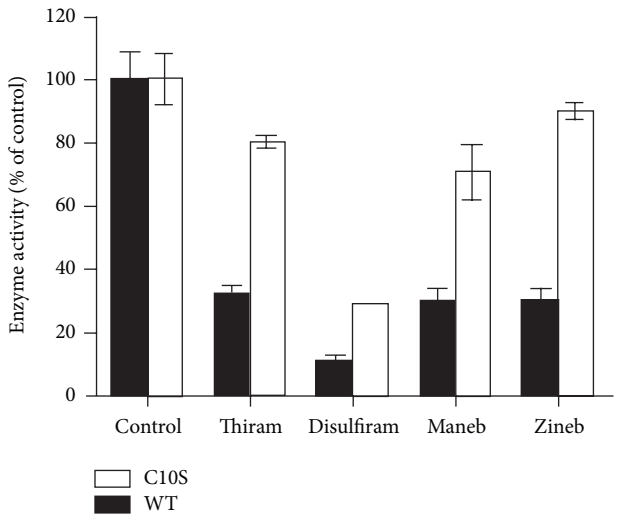

FIGURE 6: Inhibition of 17β-HSD1 and Cys^{10}Ser mutant by dithiocarbamate chemicals. The conversion of estrone (200 nM) to estradiol in the presence of 400 μM NADPH and various dithiocarbamates (80 μM final concentration) was measured in lysates of transfected HEK-293 cells. Black bars, wild-type 17β-HSD1 enzyme (WT); white bars, Cys^{10}Ser mutant 17β-HSD1 (C10S). The amount of total protein in the reaction was equalized to exclude differences due to unspecific binding of the inhibitors to cellular proteins.

wild-type enzyme approximately two times stronger than that of mutant Cys^{10}Ser, confirming the role of this cysteine for 17β-HSD1 function (data not shown).

4. Discussion

Analysis of the 17β-HSD1 protein sequences from different species revealed that the cysteine at position 10 in the human enzyme is highly conserved. Protein sequence alignment further revealed that a cysteine at this position in the Rossmann-fold nucleotide binding region is conserved in the SDR subfamilies SDR28C and SDR9C. The 3D structure of 17β-HSD1 predicts several van der Waals contacts between Cys^{10} and residues involved directly in the binding of NADPH. The 3D model of Ser^{10} mutant 17β-HSD1 finds that although most contacts are conserved there are changes that may be significant. For example, Ser^{10} is closer to Gly^{15}, and Gly^{15} is more distant from a phosphate oxygen on NADP$^+$. Also, in the Ser^{10} mutant 17β-HSD1, the backbone oxygen on Thr^{35} and backbone nitrogen on Gly^9 move to 4.7 Å and 4.5 Å, respectively, from the oxygen on the adenosine phosphate. In wild-type 17β-HSD1, the backbone oxygen on Thr^{35} and backbone nitrogen on Gly^9 are 3.3 Å and 3.8 Å, respectively, from this oxygen on NADP$^+$. The loss of these two stabilizing contacts could reduce the affinity for NADP(H) in the Ser^{10} mutant 17β-HSD1. Another possible contribution to lower stability of NADP(H) in the Ser^{10} mutant may be the different chemical properties of the thiol group on Cys^{10} and alcohol on Ser^{10}. For instance, only the thiol group is predicted to be partially deprotonated at neutral pH, which could better stabilize the site around Cys^{10}. K_m values for NADPH will need to be determined in follow-on studies to address this question.

Nevertheless, our prediction is supported by results from Huang et al. who demonstrated that insertion of a positively charged lysine residue in the neighborhood of Cys^{10} and Ser^{12} led to a more than 20-fold increase in the preference of 17β-HSD1 for NADP(H) against NAD(H) [24]. In a previous study, we found that the related SDR enzyme 11β-HSD2 also contains a cysteine residue at the position corresponding to Cys^{10} on 17β-HSD1 in the cofactor binding region [20]. The 11β-HSD2 Cys^{90}Ser mutant almost completely lost its enzymatic activity, due to impaired protein folding and mislocalization of the mutant protein.

Interestingly, mutations of the analogous residue Cys^{69} in the human (R)-3-hydroxybutyrate dehydrogenase led to a slight increase in the apparent K_m for both NADP(H) and NAD(H) [25]. The Cys^{69}Ser mutant of the (R)-3-hydroxybutyrate dehydrogenase, analogous to 17β-HSD1 Cys^{10}Ser, showed a twofold lower apparent V_{max} compared with the wild-type enzyme. The slight increase in the apparent K_m of 17β-HSD1 mutant Cys^{10}Ser for estrone, observed in our measurements in cell lysates, indicates a decreased affinity for the substrate as a result of disturbed interactions with the cofactor NADPH. A careful structural comparison between the interactions of residues with the cofactor and substrate in 17β-HSD1, 11β-HSD2, and (R)-3-hydroxybutyrate dehydrogenase should offer an explanation for the different effects of the modification of the analogous cysteine residues of the three SDRs. In human retinol dehydrogenase, the function of the analogous Cys^{38} is not studied in detail; however, Boerman and Napoli showed that the protein contains cysteine residues in close proximity, which are essential for the catalytic activity [26].

In our experiments, the preincubation with increasing concentrations of NADPH was able to protect 17β-HSD1 from inhibition by the sulfhydryl modifying agents NEM and dithiocarbamates, suggesting an indirect role of Cys^{10} in stabilizing the binding of the cofactor.

Dithiocarbamates and NEM were able to inhibit the activity of 17β-HSD1, although at concentrations significantly higher than those needed for inhibition of 11β-HSD2. The high sensitivity of 11β-HSD2 toward sulfhydryl modifying chemicals was recently shown to be dependent on the presence of a cysteine residue in the substrate binding region [27]. An analogous cysteine residue is absent in the substrate binding region of 17β-HSD1. Our western blot experiments detected low-molecular bands for the 17β-HSD1 Cys^{10}Ser mutant, suggesting increased degradation of the mutant enzyme. The more rapid degradation is likely due to changes in the protein conformation and thus exposure of amino acid residues normally buried inside the protein, followed by activation of the proteasome. A limitation of the present study includes that wild-type and mutant enzymes were overexpressed and expression levels are higher than endogenous levels. Thus, the estimated half-life of the proteins may be different in an endogenous situation. Nevertheless, the results demonstrate a reduced stability of the mutant compared with the wild-type enzyme.

The present work provides novel information on the structure-activity relationship of 17β-HSD1 and reveals that Cys^{10} is involved in essential stabilizing interactions in the cofactor binding region. Furthermore, we showed that Cys^{10} is the target for sulfhydryl modifying agents. Future studies using protease digestion of purified 17β-HSD1 wild-type and Cys^{10}Ser mutant proteins should provide further mechanistic insight into the stabilizing interactions of this residue.

Conflict of Interests

The authors declare that they have no conflict of competing financial interests.

Acknowledgment

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Review Article

The Mitochondrial Disulfide Relay System: Roles in Oxidative Protein Folding and Beyond

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Disulfide bond formation drives protein import of most proteins of the mitochondrial intermembrane space (IMS). The main components of this disulfide relay machinery are the oxidoreductase Mia40 and the sulfhydryl oxidase Erv1/ALR. Their precise functions have been elucidated in molecular detail for the yeast and human enzymes *in vitro* and in intact cells. However, we still lack knowledge on how Mia40 and Erv1/ALR impact cellular and organism physiology and whether they have functions beyond their role in disulfide bond formation. Here we summarize the principles of oxidation-dependent protein import mediated by the mitochondrial disulfide relay. We proceed by discussing recently described functions of Mia40 in the hypoxia response and of ALR in influencing mitochondrial morphology and its importance for tissue development and embryogenesis. We also include a discussion of the still mysterious function of Erv1/ALR in liver regeneration.

1. Introduction

Because almost all proteins in eukaryotic cells are synthesized by cytosolic ribosomes, protein translocation across membranes is critical for organelle biogenesis. The invention of organelle-specific targeting systems in the cytosol was instrumental to facilitate correct translocation events and to avoid mistargeting. These pathways are usually complemented by machineries in the organelle lumen which provide driving force and ensure directionality. For example, in the endoplasmic reticulum (ER) and the mitochondrial matrix members of the Hsp70 family of chaperones bind incoming substrates and thereby prevent their backsliding (ratchet-like mechanism) [1]. A similar mechanism is employed for protein import into the mitochondrial intermembrane space (IMS). Here formation of inter- and intramolecular disulfide bonds by the essential mitochondrial disulfide relay is critical for translocation across the mitochondrial outer membrane [2–6]. In this review, we will discuss the disulfide relay and its components, compare and contrast the machineries in yeast and human cells, and discuss additional potentially nonoxidative functions of disulfide relay components in human cells.

2. Substrates of the Mitochondrial Disulfide Relay

Most proteins that are imported into mitochondria contain either a mitochondrial targeting signal (MTS) or internal targeting sequences [4, 7, 8]. They are thereby targeted into the mitochondrial matrix or to the two mitochondrial membranes. In contrast, only few of the precursors of IMS proteins carry the so-called bipartite presequences consisting of an MTS and a hydrophobic sorting region [8, 9]. The import of the majority of soluble IMS proteins is facilitated by the mitochondrial disulfide relay system in a process that is linked to the oxidative folding of the proteins [3, 10] (Figure 1). Most of the so far identified disulfide relay substrates belong to the families of twin-CX_3C proteins or twin-CX_9C proteins (C, cysteine; X, any amino acid) (Figure 1(a)). The members of both families are small proteins with most of them having a size of around 10 kDa. They share a common simple core structure that consists of two antiparallel alpha helices arranged in a helix-loop-helix motif [11]. Each helix contains two cysteines that are separated by either three or nine amino acids for members of the twin-CX_3C or twin-CX_9C families, respectively [11–16]. Twin-CX_3C or

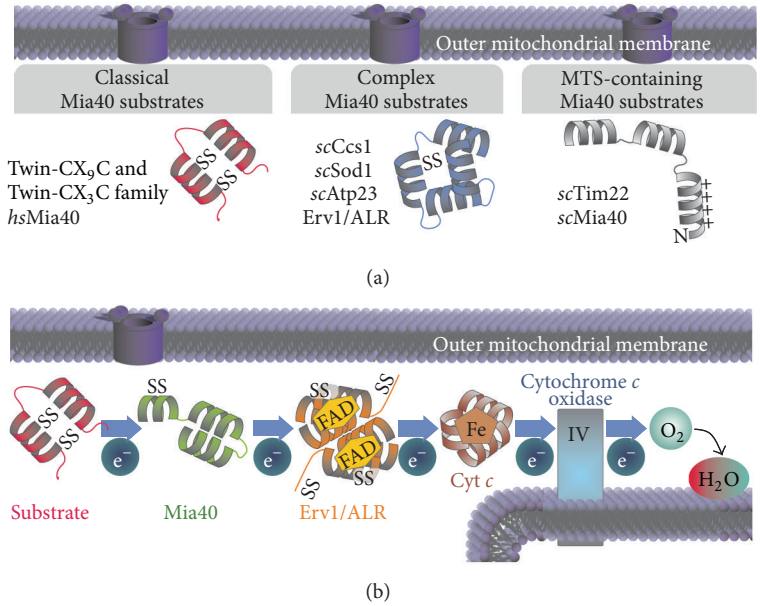

Figure 1: Substrates and general outline of the mitochondrial disulfide relay. (a) Mia40 substrates can be classified into three groups: (1) members of the twin-CX_9C and twin-CX_3C family, respectively. Members of both families rely on four cysteines localized within two α-helices for proper import. (2) The proteins *sc*Ccs1, *sc*Sod1, *sc*Atp23, and Erv1/ALR form a second group of substrates with more complex folds and disulfide patterns. So far no common signal for the interaction with Mia40 has been identified in these proteins. (3) The two MTS-containing Mia40 substrates Tim22 and *sc*Mia40 are imported in a membrane potential-dependent manner and require Mia40 for proper folding only. (b) General outline of oxidative folding in the IMS. During substrate oxidation electrons are transferred from the substrate to Mia40. To reoxidize Mia40 electrons are transferred further via ALR to cytochrome *c* (Cyt *c*) and then to cytochrome *c* oxidase. Molecular oxygen (O_2) is used as final electron acceptor to finally yield water (H_2O).

twin-CX_9C proteins fulfill diverse functions within the IMS. They serve as chaperones for newly imported proteins, are involved in metal transfer and insertion during respiratory chain biogenesis, or are part of mature respiratory chain complexes [13, 17–22]. In human and yeast cells exist a total of five proteins that belong to the twin-CX_3C family. Conversely, the twin-CX_9C family appears to be significantly larger in mammalian cells, and in addition numerous proteins exist that do not adhere exactly to the nine amino acid-wide spacing (and instead have, e.g., CX_8C or $CX_{10}C$ motifs). So far more than 30 twin-CX_9C family members have been identified in human cells, and some of them have been confirmed to be disulfide relay substrates [23, 24].

In addition to twin-CX_3C and twin-CX_9C proteins several more complex substrates exist that rely on the mitochondrial disulfide relay for oxidation (Figure 1(a)). In yeast the import of the dually localized copper chaperone for superoxide dismutase 1 (Ccs1) and in part also that of superoxide dismutase 1 (Sod1) depends on the mitochondrial disulfide relay [25–27]. Likewise, import and oxidation of the sulfhydryl oxidase Erv1 which itself is part of the mitochondrial disulfide relay (see below) are driven by the disulfide relay system [28]. Further substrates are the mitochondrial protease Atp23 and the inner membrane protein Tim22 [29, 30]. The latter protein contains a bipartite presequence and thus requires oxidation only for folding but not for mitochondrial import. Because so far a systematic identification of interaction partners and substrates of the mitochondrial disulfide relay system is lacking in yeast and mammalian cells, we do not know how large the group of disulfide-containing IMS proteins is. It is likely that it will be significantly larger than previously anticipated as the recently solved partial IMS proteome contains numerous proteins that are dually localized between cytosol and IMS, but lack MTS, and might therefore be disulfide relay substrates [31].

3. The Mechanism of Oxidation-Dependent Protein Import by the Mitochondrial Disulfide Relay System

All proteins of the IMS are synthesized by cytosolic ribosomes [32] (Figure 2). However, only very few of them contain a classical MTS or internal targeting signals that guide them to mitochondria. Instead, most IMS proteins contain conserved cysteine patterns or other still ill-defined motifs that are recognized by the IMS-localized mitochondrial disulfide relay but likely also by so far not identified cytosolic factors [4, 10]. Such factors could ensure targeting of disulfide relay substrates to mitochondria for posttranslational import and maintain them in an import-competent unfolded state. In addition, disulfide relay substrates have to be kept in a reduced state in the cytosol. This is facilitated by cytosolic glutaredoxins in human cells and the thioredoxin system in yeast [33, 34] and potentially by the presence of zinc ions that can complex reduced cysteines [35, 36]. In yeast,

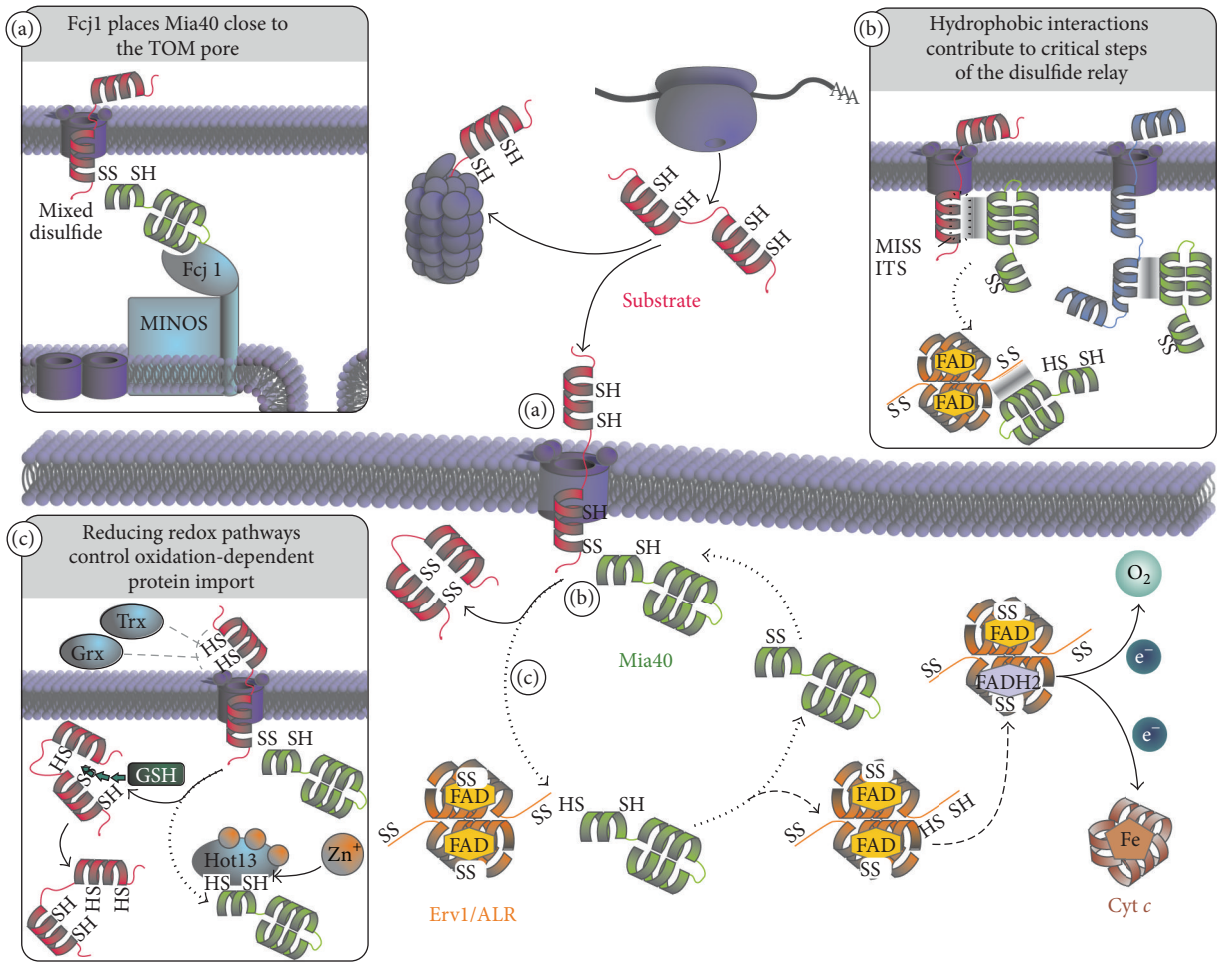

Figure 2: The mitochondrial disulfide relay system facilitates protein import and folding into the IMS. Classical substrates of the twin-CX_3C and twin-CX_9C families are translated on cytosolic ribosomes. In part, these proteins are degraded by the proteasome, while the majority becomes posttranslationally imported into the IMS through the TOM pore. Noncovalent and covalent interactions between Mia40 and the substrate are necessary for translocation and oxidative folding of the substrate. Immediately after the first cysteine of the substrate translocates a mixed disulfide between Mia40 and substrate is formed. The substrate becomes oxidized by resolving the mixed disulfide complex. Reduced Mia40 is then reoxidized by the flexible N-terminal domain of one subunit of Erv1/ALR, allowing another round of substrate oxidation. Within the Erv1/ALR homodimer electrons are transferred from the N-terminal cysteines of one subunit to the C-terminal cysteines of the other subunit from where they are shuttled to the prosthetic FAD molecule. Erv1/ALR then passes electrons onto cytochrome *c*—and further to cytochrome *c* oxidase and oxygen yielding H_2O as product. Alternatively, electrons can be transferred from the FAD directly onto oxygen thus forming H_2O_2. (a) The MINOS complex is important for the organization of the IMS in yeast. Both the arrangement of the cristae and the close proximity of the TOM and TIM pore are mediated by MINOS. Fcj1 binds to the MINOS complex and also interacts with Mia40, thereby placing Mia40 close to the TOM pore. (b) Hydrophobic interactions between the hydrophobic groove of Mia40 and twin-CX_3C and twin-CX_9C proteins are necessary for substrate recognition by Mia40. The same hydrophobic patch on Mia40 also mediates its interaction with the N-terminal domain of ALR. Moreover, the hydrophobic groove of Mia40 also equips the protein with a holdase-like function that can serve in importing cysteine-less substrates. (c) Several redox control pathways facilitate efficient oxidative import and folding of substrates. In the cytosol substrate cysteines are maintained in their reduced state mainly by thioredoxins (Trx) and glutaredoxins (Grx) in yeast and human cells, respectively. During Mia40-dependent oxidation reduced glutathione (GSH) exhibits a proofreading function by reducing wrongly oxidized substrates and resolving trapped intermediates of substrate and Mia40. Upon becoming reduced, the cysteines of Mia40 are prone to bind zinc ions, thereby interfering with reoxidation. The zinc chelating protein Hot13 keeps Mia40 zinc free.

the amounts of import-competent substrates are also controlled by the cytosolic proteasome system that degrades substantial amounts of newly synthesized disulfide relay system substrates before they can be imported [37]. At present it remains unclear whether such a degradation pathway is also found in mammalian cells and under which conditions it may serve in adapting amounts of imported IMS proteins.

Translocation of IMS proteins takes place across the translocase of the outer membrane (TOM). Upon exposure of a recognition motif termed MISS or ITS (for mitochondrial intermembrane space sorting and IMS-targeting signal, resp.) disulfide relay substrates are recognized by the protein Mia40 (for mitochondrial IMS import and assembly; in mammalian cells also CHCHD4) [14, 38], which thereby serves

both as import receptor and chaperone and oxidoreductase [23, 29, 39] (Figure 2, insets (a) and (b)). Mia40 consists of a structural helix-loop-helix motif with two stabilizing disulfide bonds that form hydrophobic substrate recognition and binding groove and a redox-active CPC motif that is positioned in a flexible helix which hovers over the substrate binding site [40, 41]. Yeast and human Mia40 share high homology, except for an N-terminal extension in yeast Mia40 that contains a bipartite presequence which is lacking in the human protein. Human Mia40 appears in two different splice variants (CHCHD4.1 and CHCHD4.2) [42, 43]. They are completely identical except for the very N-terminal part of the protein. The isoform 1 does contain an additional cysteine at position four; however, whether the isoforms exhibit different functionality or substrate specificity is not known. Like for ALR the import and folding of human Mia40 depend on the disulfide relay system [44]. In contrast, yeast Mia40 requires the disulfide relay system only for oxidative folding [45]. In yeast, Mia40 is positioned close to the *trans*-side of the TOM complex by its interaction with Fcj1 (for formation of cristae junction; in human cells mitofilin) [46] (Figure 2, inset (a)). Fcj1 is part of the MINOS complex which organizes the topology of the cristae in the inner mitochondrial membrane [46–50].

MISS/ITS motifs have been well defined for classical twin-CX_3C and twin-CX_9C proteins but their nature has to be clarified for the growing class of nonclassical substrates like Atp23. It contains hydrophobic residues, and in most cases, a single cysteine residue, that are positioned on the same side of an alpha helix [14, 38] (Figure 2, inset (b)). After recognition of the MISS/ITS signal by the hydrophobic binding groove of Mia40, the thiolate anion of a cysteine in the substrate performs a nucleophilic attack on the oxidized CPC motif of Mia40 which results in the formation of an intermolecular disulfide bond [2, 39]. This disulfide bond together with the hydrophobic interactions between substrate and Mia40 prevents the backsliding of the incompletely translocated substrate into the cytosol, thus coupling import to oxidative protein folding [51]. Consequently, mutation of critical cysteines in Mia40 substrates also results in very low amounts of these substrates in the IMS [2]. This indicates that hydrophobic interactions with Mia40 might be sufficient to drive IMS import at least of some proteins that neither contain classical MTS nor cysteines to interact with Mia40. Furthermore, Mia40 might also contribute to protein folding as it is capable of stabilizing cysteine-free unfolded proteins and prevents their aggregation [29] (Figure 2, insert (b)).

The intermolecular disulfide bond between substrate and Mia40 is resolved by another nucleophilic attack of a thiolate anion in the substrate leaving an oxidized substrate molecule and a reduced Mia40 molecule [39]. Thus, for this import mechanism to work Mia40 substrates have to contain at least two cysteines. For the introduction of more than one disulfide bond, multiple oxidized Mia40 molecules or molecular oxygen are necessary. It has been suggested that Mia40 can act more efficiently in the introduction of multiple disulfide bonds by forming a ternary complex with its substrate and the essential protein Erv1 (in mammalian cells augmenter of liver regeneration (ALR), growth factor erv1-like (Gfer1), hepatopoietin or *hs*Erv1) [52, 53]. The very rapid introduction of disulfide bonds after the formation of the initial intermolecular disulfide bond is supported by the fact that *in vivo* no semioxidized intermediates of substrate proteins can be observed [23].

The sulfhydryl oxidase Erv1/ALR acts to reoxidize the CPC motif in Mia40 [39]. It is a homodimeric protein in which each subunit consists of two domains [54]. The first—N-terminal domain—contains a redox-active CXXC motif and serves as a "shuttle arm" that mediates the electron transfer from Mia40 to a CXXC motif in the C-terminal core domain of Erv1/ALR [39, 55]. To this end, this mainly unstructured domain interacts with the substrate-binding groove of Mia40 [56, 57]. Consequently, overexpression of Erv1/ALR in intact cells delays oxidative protein folding and IMS import because the N-terminal arm blocks substrate binding to Mia40 [23]. Both Mia40 and Erv1/ALR are perfectly adapted to this critical interaction of shuttle arm and hydrophobic groove. In a heterologous yeast system human ALR or human Mia40 when expressed individually could to a large part complement their yeast counterparts. However, only if human Mia40 and human ALR were concomitantly used to substitute the respective yeast proteins full complementation was ensured [44]. After Mia40 reoxidation the shuttle arm of Erv1/ALR swings over to the core domain of the second subunit of Erv1/ALR (intersubunit electron transfer) and becomes reoxidized by the core CXXC motif [55]. This core CXXC motif is reoxidized by the redox cofactor of Erv1/ALR-flavin adenine dinucleotide (FAD) by the formation of a charge-transfer complex [58]. The FAD is held in place by the very compact four-helix bundle structure of Erv1/ALR [54, 59, 60]. The dimer of Erv1/ALR is stabilized by hydrophobic interactions and in mammalian cells additionally also by disulfide bonds [39, 61]. Finally, the reduced FAD cofactor is reoxidized by either transferring electrons directly onto molecular oxygen which gives rise to the production of hydrogen peroxide or alternatively by transferring electrons to cytochrome *c* [39, 62, 63]. To which extent both pathways are utilized in intact cells remains unclear although *in vitro* cytochrome *c* appears to be the preferred electron acceptor [39, 58, 62, 64, 65]. At least in yeast cells Erv1 can transfer electrons also onto an anaerobic electron acceptor [66]. The identity of this acceptor and whether it is conserved in mammalian cells remains unclear.

In addition to Mia40 and Erv1/ALR further factors modulate oxidative folding in the IMS—the protein helper of Tim protein (Hot13, in mammalian cells RCHY1) and the local glutathione pool (Figure 2, insert (c)). Hot13 is a cysteine-rich protein that is capable of chelating zinc ions [67]. Although low amounts of zinc ions can facilitate mitochondrial import *in vitro*, too high amounts hamper substrate oxidation and Mia40 reoxidation by binding to reduced cysteines [36, 67, 68]. It has thus been proposed that Hot13 keeps the CPC motif of Mia40 in a zinc-free state thereby accelerating oxidation-dependent protein import. *In vitro* substrate oxidation appears to yield side products with nonnative disulfides or substrates that are trapped in their mixed disulfide complex with Mia40 [39]. Formation of these products is avoided by the presence of reduced glutathione.

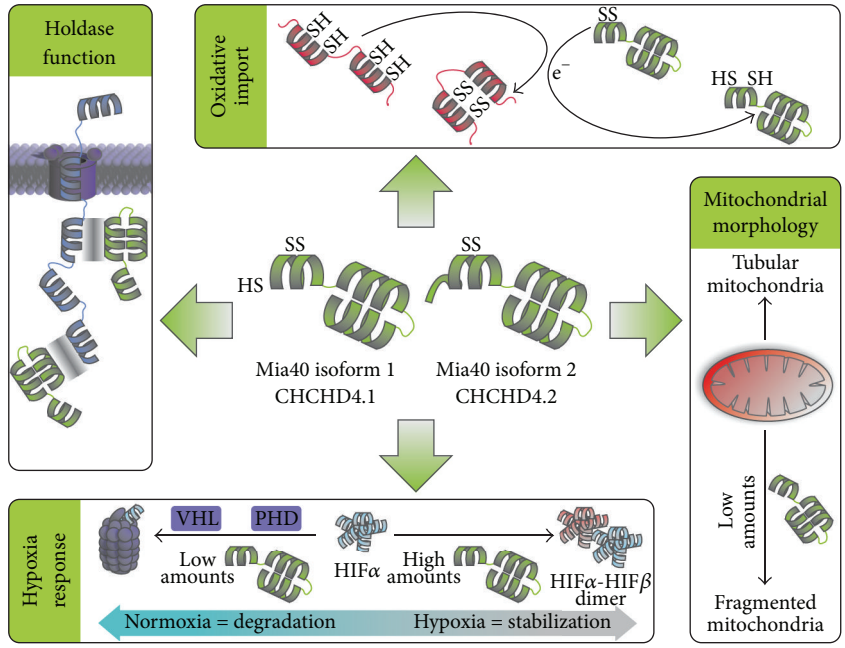

FIGURE 3: Functions of human Mia40. The protein exists in two different isoforms. Differences in function between the isoforms are not known. *Oxidative Import*. Mia40 is the main receptor for import and oxidation of twin-CX_9C and twin-CX_3C proteins in the IMS. *Holdase Function*. Mia40 shields hydrophobic patches on proteins imported into the IMS, thus allowing proper folding of these proteins. *Mitochondrial Morphology*. Reduced amounts of Mia40 lead to increased mitochondrial fission and the formation of a more fragmented mitochondrial network. *Hypoxia Response*. Under normoxic conditions the protein HIF1α is constantly degraded. This degradation is mediated by hydroxylation by prolyl hydroxylase domain enzymes (PHD) and ubiquitination by the Hippel-Lindau E3 ligase (VHL). Upon hypoxia oxygen as substrate of PHD is missing resulting in impaired HIF1α degradation and increased stability. If not degraded HIF1α can dimerize with constitutively expressed HIF1β and induce the hypoxia response. Silencing Mia40 using RNAi prevents stabilization of HIF1α under hypoxic conditions. In contrast increased amounts of Mia40 as can also be found in certain tumors result in increased stabilization of HIF1α.

Also in intact cells reduced glutathione seems to be beneficial for oxidation-dependent protein import: on the one hand by contributing to the reduced redox state of Mia40 substrates in the mammalian cytosol, and on the other hand by accelerating oxidative protein folding by a still unresolved mechanism [23]. Like zinc ions glutathione might be a two-edged sword. The IMS glutathione pool in yeast and mammalian cells has been measured to be as reducing as the one in the cytosol [23, 69]. The IMS glutathione redox potential is thereby in the range of the redox potential of Mia40 substrates, raising the question of how these substrates can be oxidized and maintained in an oxidized state [35, 65, 69–73]. Although this point has not been addressed experimentally, it is likely that the thermodynamically feasible reduction of Mia40 substrates is kinetically prevented, for example, hampering the equilibration between protein thiols and glutathione. In principle glutathione can affect IMS proteins *in vivo* as the CPC motif of Mia40 is affected by glutathione in intact cells [23, 69]. Consequently, Mia40 is maintained in a partially reduced state in yeast cells [69]. The reduced part of molecules might well be involved in either isomerisation or reduction reactions like oxidoreductases in other systems that facilitate oxidative protein folding. However, such a novel role of Mia40 has not been shown.

Besides their function in oxidative protein folding in mitochondria Mia40 and Erv1/ALR also function in potentially unrelated (nonmitochondrial) pathways. Mia40 was shown to be critical for mitochondrial dynamics and the hypoxia response [43] (Figure 3). For Erv1/ALR, a plethora of different cellular and physiological functions were described (Figure 4). Erv1/ALR influences fusion and fission processes of mitochondria [74–77]; it is important for the development of certain organs during embryogenesis [78, 79] and functions as mitogen to enhance regenerative capacities of liver tissue [80–82]. These functions will be discussed in the following.

4. Physiological Impact of Mia40 and Erv1/ALR

4.1. A Function of Mia40 in Hypoxia. Mia40 is not only necessary for proper assembly of the respiratory chain but is also involved in the stabilization of hypoxia inducing factor 1α (HIF1α) [43, 74] (Figure 3). In the presence of high amounts of oxygen HIF1α is continuously degraded by the proteasome after hydroxylation by oxygen-dependent prolyl hydroxylase domain (PHD) enzymes and subsequent ubiquitinylation by the E3 ligase VHL (von Hippel-Lindau)

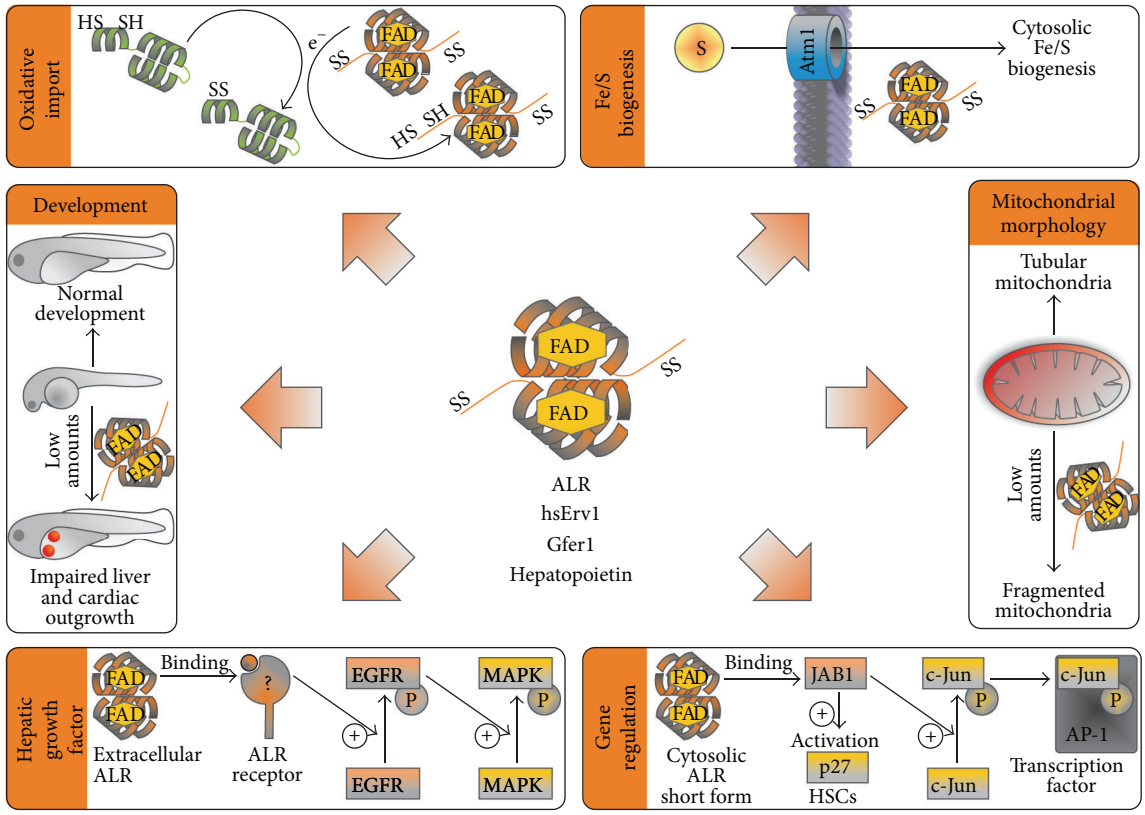

Figure 4: Functions of Erv1/ALR. *Oxidative Import*. Erv1/ALR reoxidizes Mia40. *Fe/S Biogenesis*. In yeast Erv1/ALR is required for the biogenesis of cytosolic Fe/S proteins but not for mitochondrial Fe/S proteins. *Development*. Erv1/ALR is expressed during development. Knockdown or chemical inhibition of Erv1/ALR leads to impaired development of organs such as liver and hamper cardiac outgrowth. *Mitochondrial Morphology*. Erv1/ALR is important for mitochondrial morphology in undifferentiated cells. Knockdown of Erv1/ALR in mouse embryonic stem cells leads to mitochondrial fragmentation and increased levels of Drp1 (dynamin-related protein 1). *Hepatic Growth Factor*. Extracellular Erv1/ALR increases regenerative capacities of liver tissue. Extracellular Erv1/ALR can bind to a so far unknown receptor. Upon binding tyrosine-phosphorylation of the epidermal growth factor receptor (EGFR) is enhanced which promotes phosphorylation and activation of mitogen activated protein kinase (MAPK). *Gene Regulation*. A truncated cytosolic form of Erv1/ALR can bind to Jun activating binding protein 1 (JAB1). Binding to JAB1 increases JAB1-mediated phosphorylation of c-Jun. Upon phosphorylation c-Jun can form a complex with AP-1 complex (activator protein 1). In hematopoietic stem cells (HSC) sequestering of JAB1 by ALR prevents binding of JAB1 to p27(kip).

[83–85]. Under low oxygen conditions PHDs lack oxygen and fail to completely hydroxylate HIF1α. Moreover, reactive oxygen species take part in the stabilization process by further inhibiting PHD [86, 87]. The stabilization of HIF1α by low oxygen concentrations can be mimicked by incubating cells with iron chelators as PHD activity depends on an iron cofactor [88–90].

The modulation of Mia40 levels affects HIF1α stabilization at low oxygen concentration but not by treatment with iron chelators [43]. Upon depletion of Mia40 using siRNA-mediated knockdown HIF1α failed to accumulate under low oxygen conditions, while Mia40 overexpression enhanced HIF-1α stabilization under hypoxic conditions. Since the hypoxia response is critical for tumor growth Mia40 depletion effectively inhibited tumor growth and angiogenesis *in vivo* [43]. In line with these findings in human cancer, increased Mia40 expression was found to correlate with the signature of hypoxia gene expression [43]. Whether the described effect of Mia40 on the stabilization of HIF1α arises from a direct interaction or is indirectly mediated by an impaired respiratory chain remains unclear and is an exciting question for future research.

4.2. Physiological Functions of Erv1/ALR—in Mitochondria and the Cytosol? Human patients with a homozygous mutation in Erv1/ALR exhibit respiratory-chain deficiency, myopathy, congenital cataract, sensorineural hearing loss, and delayed development [59, 91] (Figure 4). In zebrafish the formation of heart and liver is impaired upon chemical inhibition or silencing of Erv1/ALR [78, 79]. In addition, chemical inhibition of Erv1/ALR induces apoptosis in human embryonic stem cells [79]. Likewise, silencing of Erv1/ALR in mouse embryonic stem cells results in caspase-induced apoptosis as well as in excessive fragmentation of mitochondria and elimination of damaged mitochondria through mitophagy [76, 77]. Taken together these data underline the importance of Erv1/ALR for mitochondrial functionality especially during development.

Most of those physiological effects of Erv1/ALR likely derive directly or indirectly from its role in the mitochondrial disulfide relay (Figures 2 and 4). However, they might also be linked to a role in the biogenesis of cytosolic iron sulfur proteins which has been described for yeast Erv1 [92]. Moreover, they may derive from a so far unappreciated function of Erv1/ALR in the cytosol where overexpressed and tagged Erv1/ALR could be detected in some studies [92]. Unfortunately, overexpression of IMS proteins without bipartite MTS frequently results in mislocalization to the cytosol [23]. It thus remains unclear whether endogenous Erv1/ALR also is dually localized. Besides full length Erv1/ALR a shorter isoform consisting only of the C-terminal core domain has been described to exist in the cytosol and nucleus of mammalian cells and to be secreted as a growth factor [93, 94]. The existence of this isoform has been confirmed by immunoblotting of human cell lysate against endogenous ALR although specificity controls using siRNA-mediated knockdown were lacking in these studies.

4.3. A Role for Erv1/ALR in Liver Regeneration. Erv1/ALR has been described to enhance the regenerative capacities of liver tissue [95–97]. For this role of Erv1/ALR two different mechanisms were proposed. In one model Erv1/ALR acts extracellularly as mitogen [81]. Several studies describe the regeneration-enhancing abilities of Erv1/ALR on damaged liver tissue after application of the purified C-terminal domain of human or rat Erv1/ALR [80]. The C-terminal domain can be cross-linked to a 60 kDa protein which is probably located at the cellular surface [81]. The putative Erv1/ALR receptor does not interact with other mitogenic factors such as epidermal growth factor (EGF), transforming growth factor α (TGF-α), or insulin [81]. Binding of Erv1/ALR to its receptor triggers EGF-receptor phosphorylation which then results in activation of the mitogen-activated protein kinase (MAPK) signaling cascade [82].

In a second model cytosolic Erv1/ALR acts independently of the MAPK pathway. Cytosolic Erv1/ALR thereby interacts with Jun-activating domain-binding protein 1 (JAB1) which promotes phosphorylation of c-Jun and therefore formation of the c-Jun/activator protein-1 (AP-1) transcription factor complex [98]. C-Jun is part of the cytosolic COP9 signalosome that has also been shown to interact with Erv1/ALR [99]. The interaction between Erv1/ALR and JAB1 depends on the presence of the CXXC motif in the C-terminal core domain of Erv1/ALR because mutation of the motif to CXXS prevented phosphorylation of c-Jun [100]. The studies addressing the cytosolic function of Erv1/ALR were all either performed *in vitro* using recombinant proteins or by overexpressing Erv1/ALR. As already stated above overexpression of IMS proteins leads to cytosolic or nuclear mislocalization [23]. This is especially true for Erv1/ALR which becomes imported and folded more slowly than classical substrates of the disulfide relay.

The interaction of Erv1/ALR with JAB1 is not limited to liver cells. Knockdown of Erv1/ALR in hematopoietic stem cells leads to an increased inhibition of the cyclin-dependent kinase inhibitor p27(kip) by JAB1 while overexpression of ALR leads to a decreased inhibition of p27(kip), probably because JAB1 is sequestered by ALR [101]. Furthermore, it was shown that the quiescence promoting properties of ALR in HSC are dependent on Camk4 (Ca^{2+}/calmodulin-dependent protein kinase 4). HSCs isolated from Camk4$^{-/-}$ mice possessed reduced levels of ALR and p27(kip) and were deficient in proliferation, which could be restored by ectopic expression of ALR [102].

A complementing explanation for the enhancement of liver regeneration is that Erv1/ALR treatment decreases cytotoxicity of natural killer cells and decreases IFN-γ (interferon-gamma) levels [103, 104]. Alternatively, it has been proposed that extracellularly administrated Erv1/ALR enhances liver regeneration by inducing anti apoptotic gene expression, thereby improving cell survival [105]. However, this anti apoptotic effect does not seem to be limited only to hepatocytes because in human lymphocytes recombinant Erv1/ALR also inhibited apoptosis [106]. Recently, it was shown that in primary hepatocytes the increased expression and synthesis of ALR after liver damage is regulated by the transcription factor Nrf2 [107]. This indicates that the regenerative abilities of ALR are not only achieved by extracellular treatment of damaged cells but might constitute physiological relevant cellular survival mechanisms.

4.4. The Disulfide Relay System—Open Questions. Mia40 and Erv1/ALR are well characterized regarding their functions as oxidoreductase and sulfhydryl oxidase of the mitochondrial disulfide relay, respectively. Still several open questions remain (Figure 5): first, despite the identification of many human twin-CX_9C proteins by *in silico* approaches, a concise identification of disulfide relay substrates is still lacking. This becomes especially important because in recent years several proteins with complex structures have been identified as Mia40 substrates. Since these substrates do not adhere to classical cysteine patterns, they cannot be predicted by *in silico* approaches. This might indicate that the substrate range of the disulfide relay is much wider than previously anticipated. It might also include targets for thiol-dependent redox regulation that cycle between oxidized and reduced states and consequently adapt their activities.

Secondly, while we understand oxidative protein folding in the IMS in detail, little is known about the cytosolic processes that take place before translocation across the outer membrane. In previous studies, cytosolic factors were identified which facilitate the import of MTS-containing proteins. However, most disulfide relay substrates lack such targeting information and thus appear like cytosolic proteins. It will therefore be exciting to identify factors that interact with IMS proteins after their translation and guide them to mitochondria or mediate their degradation.

Thirdly, the role of Mia40 and Erv1/ALR in processes that appear not directly linked to mitochondria such as hypoxia and liver regeneration is still mechanistically ill-defined. It especially remains unclear whether the functions of both proteins in each case are connected to their function in the disulfide relay or if they operate by completely different mechanisms. We think that it will be exciting to establish

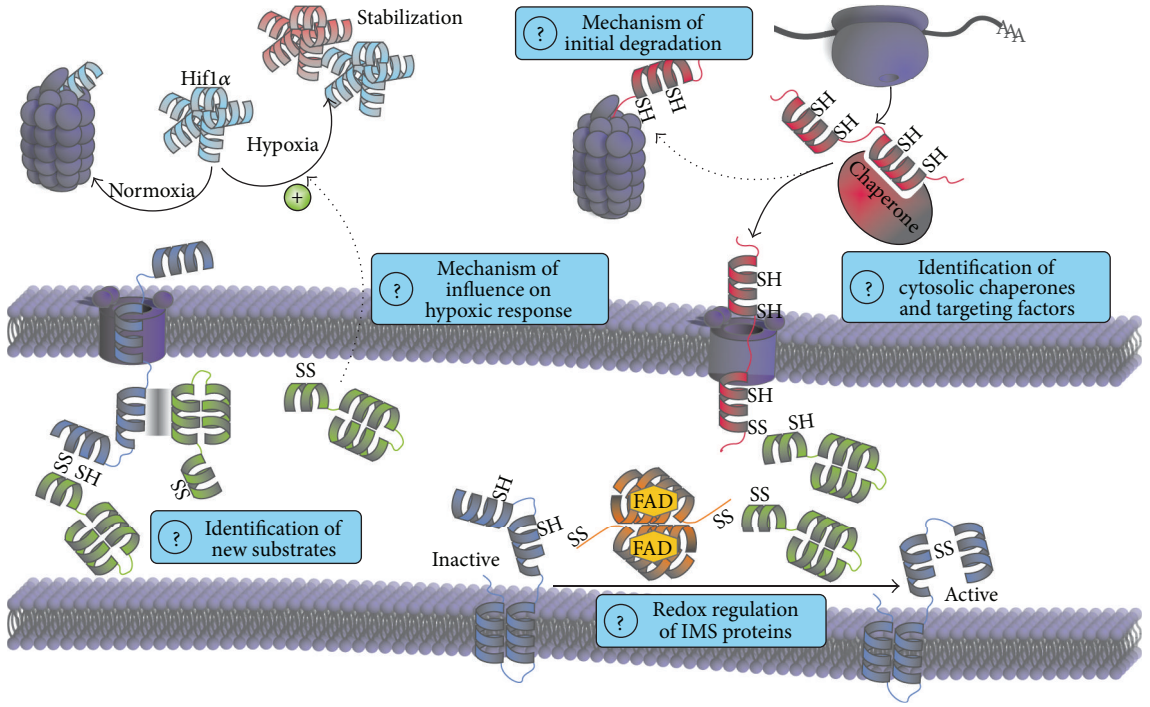

FIGURE 5: Open questions. *Hypoxia.* Why Mia40 is required for the stabilization of HIF1α under hypoxic conditions is unknown. It will be interesting to reveal the underlying mechanism and if the influence of Mia40 is direct or indirect. *New Substrates.* During the last years proteins were found to be dependent on Mia40 that do not share the same motifs as the classical twin-CX_9C and twin-CX_3C substrates. They are either dependent on the function of Mia40 as oxidoreductase or holdase. *Initial Degradation and Cytosolic Chaperones/Targeting Factors.* In yeast a portion of IMS proteins is directly degraded after translation. However, it is not known if this takes place in other organisms, how it is regulated, and how it works at the molecular level. Moreover, it remains unclear how proteins after translation are kept import competent and targeted to mitochondria. *Redox Regulation.* The mitochondrial disulfide relay is known to introduce structural disulfide bonds which are required for protein stability. However, it is not known if Mia40 or Erv1/ALR can regulate the activity of IMS proteins by reversible oxidation or reduction.

in detail the molecular mechanisms that underlie these potentially extra-mitochondrial functions and thus link the biochemistry of thiol oxidation with its physiological impact.

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Review Article

Destroy and Exploit: Catalyzed Removal of Hydroperoxides from the Endoplasmic Reticulum

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Peroxidases are enzymes that reduce hydroperoxide substrates. In many cases, hydroperoxide reduction is coupled to the formation of a disulfide bond, which is transferred onto specific acceptor molecules, the so-called reducing substrates. As such, peroxidases control the spatiotemporal distribution of diffusible second messengers such as hydrogen peroxide (H_2O_2) and generate new disulfides. Members of two families of peroxidases, peroxiredoxins (Prxs) and glutathione peroxidases (GPxs), reside in different subcellular compartments or are secreted from cells. This review discusses the properties and physiological roles of PrxIV, GPx7, and GPx8 in the endoplasmic reticulum (ER) of higher eukaryotic cells where H_2O_2 and—possibly—lipid hydroperoxides are regularly produced. Different peroxide sources and reducing substrates for ER peroxidases are critically evaluated. Peroxidase-catalyzed detoxification of hydroperoxides coupled to the productive use of disulfides, for instance, in the ER-associated process of oxidative protein folding, appears to emerge as a common theme. Nonetheless, *in vitro* and *in vivo* studies have demonstrated that individual peroxidases serve specific, nonoverlapping roles in ER physiology.

1. Introduction

Hydrogen peroxide (H_2O_2) is an intracellular metabolite, which serves important roles as a second messenger in redox signaling [1]. However, since elevated levels of H_2O_2 (and of other reactive oxygen species, ROS) can damage proteins, nucleic acids, and lipids by peroxidation, temporal and spatial limitation of H_2O_2 levels is critically important. Thus, half-life and spatial distribution of H_2O_2 in the cell are tightly regulated by nonenzymatic antioxidants as well as by specific scavenging enzymes, including the so-called peroxidases of the peroxiredoxin (Prx) or glutathione peroxidase (GPx) families [2]. Prx and GPx isoforms reside in different subcellular compartments where they catalyze the reduction of H_2O_2 to H_2O [2]. The most relevant producers of intracellular ROS/H_2O_2 are the transmembrane enzyme complexes of the nicotinamide adenine dinucleotide oxidase (NOX) family, various enzymes and the respiratory chain in mitochondria, peroxisomal enzymes, and sulfhydryl oxidases in the endoplasmic reticulum (ER) [3–7]. Due to the presence of specific aquaporin channels in cellular membranes, the local diffusion of H_2O_2 is usually not restricted by organelle boundaries [8, 9].

There are a total of six isoforms of Prx in mammals, all of which form distinct types of antiparallel homooligomers [10]. H_2O_2-mediated oxidation of the active site peroxidatic cysteine (C_P) to a cysteine sulfenic acid is a common feature of Prxs. However, only so-called 2-Cys Prxs possess a resolving cysteine (C_R), which attacks the C_P sulfenic acid, leading to the formation of a C_R–C_P disulfide bond. In typical 2-Cys Prxs, the C_R–C_P disulfide connects antiparallel dimers, whereas in atypical 2-Cys Prxs, it forms intramolecularly. In order to complete the catalytic cycle, these disulfide bonds are reduced by a thioredoxin-type oxidoreductase [10–12]. In contrast, 1-Cys Prxs (such as human PrxVI) lack a C_R and instead form a mixed disulfide heterodimer with π glutathione S-transferase, which catalyzes the glutathione-driven reductive regeneration of the Prx [13, 14].

A remarkable feature of Prxs is their susceptibility to oxidative inactivation. Thus, C_P sulfenic acid can react with

a second molecule of H_2O_2, which gives rise to C_P sulfinic acid. This leads to Prx inactivation, stabilization of decameric over dimeric configuration, and, in some cases, to an increase in chaperone activity [15–17]. At least in cytoplasmic and mitochondrial typical 2-Cys Prxs, sulfinic acid formation can be reversed by the action of sulfiredoxin at the expense of ATP [18, 19]. Under highly oxidizing conditions, C_P sulfinic acid can further and irreversibly react with a third molecule of H_2O_2 to form C_P sulfonic acid [15].

The GPx family is phylogenetically unrelated to Prxs but shares the ability to reduce hydroperoxide substrates [2]. A total of eight mammalian GPxs are known. They are subclassified into two groups according to the amino acid tetrad in their catalytic center. In SecGPxs (human GPx1–4 and 6) or CysGPxs (GPx5, 7, and 8), the common constituents Gln, Trp, and Asn are supplemented with a peroxidatic selenocysteine (Sec) or Cys, respectively [20]. Furthermore, GPxs differ with regard to their oligomeric state, with GPx1–3, 5, and 6 constituting homotetramers and GPx4, 7, and 8 monomers [21].

Upon hydroperoxide-mediated oxidation of the active-site selenocysteine, SecGPxs typically react with two molecules of glutathione (GSH) yielding glutathione disulfide (GSSG), which historically accounted for the generalized family name glutathione peroxidases [2, 21]. However, the use of GSH as reductant is not a common feature of GPxs nor is it strictly conserved within the SecGPx subgroup [2, 21–25]. In invertebrates and plants, monomeric CysGPxs harbor a C_R and exhibit an identical reaction mechanism as atypical 2-Cys Prxs (see above) [20, 26, 27]. In contrast, no typical C_R is present in the human monomeric CysGPxs GPx7 and 8.

The ER serves many distinct cellular functions [28]. One of these is chaperone-mediated folding of nascent polypeptide chains, which often involves the introduction of disulfide bonds via oxidation of two adjacent cysteines. This process termed oxidative protein folding is driven by a number of distinct pathways, the most conserved of which involves the sulfhydryl oxidase endoplasmic oxidoreductin 1 (Ero1) as disulfide donor [29]. Since Ero1 can utilize molecular oxygen (O_2) as terminal electron acceptor, it generates stoichiometric amounts of H_2O_2 for every disulfide bond produced, as demonstrated *in vitro* [30]. In addition, H_2O_2 sources other than the paralogs Ero1α and Ero1β exist within the mammalian ER. Although initially assigned to phagocytic cells only, more recent findings have shown that NOX family members are expressed in various cell types [3] where they produce H_2O_2 at different subcellular sites including the ER [31–33]. Likewise, the secreted quiescinsulfhydryl oxidases were identified as producers of H_2O_2 [34], although these enzymes function in the extracellular space [35] and their contribution to intracellular oxidative protein folding is uncertain [36, 37]. It has also been suggested that ROS produced by mitochondrial respiration could impact on disulfide-bond formation in secretory compartments including the ER [38]. Leakage of the mitochondrial electron transport chain, predominantly at complex III, releases superoxide and H_2O_2 into the intermembrane space of mitochondria [39, 40]. The close apposition of ER and mitochondria [41] could enable these ROS to contribute to ER-associated oxidative protein folding.

This review will focus on PrxIV, GPx7, and GPx8, which reside in the ER of vertebrates, lancelets, ascidians, and—in case of PrxIV—echinoderms and arthropods [42]. As detailed further below, all ER-resident peroxidases can use protein disulfide isomerases (PDIs; the "thioredoxins of the ER") as reducing substrates, allowing them to exploit the oxidizing power of ER peroxide sources for oxidative protein folding. However, reducing substrates other than PDIs may also participate in the reaction cycle of ER peroxidases.

2. H_2O_2 in the ER: Bulk Metabolite or Locally Restricted Messenger?

Reliable detection of the cellular distribution of H_2O_2 is a challenging task. The recent development of genetically encoded sensors, which can be expressed in different subcellular compartments, significantly facilitated the monitoring of spatial and temporal changes in H_2O_2/ROS concentration [43]. For instance, targeted expression of the yellow fluorescent protein-based, ratiometric, and H_2O_2-sensitive HyPer sensor was used to record the oxidizing environment in the mammalian ER [33, 44–46]. On the basis of the predominantly oxidized state of ER-localized HyPer ($HyPer_{ER}$) and the predominantly reduced state of HyPer on the cytoplasmic surface of the ER, a high $[H_2O_2]_{ER}$, which is strictly confined to the lumen of the organelle, has been inferred [44]. Several lines of evidence argue against this interpretation though. First, as detailed in the following paragraph, numerous examples for signaling roles of ER-derived H_2O_2 are known, which suggest analogy to the critical involvement of Nox-derived H_2O_2 in receptor tyrosine kinase (RTK) signal transduction at the cell surface [47–50] (Figure 1). Second, the presence of peroxidases in the ER lumen (see below) appears incompatible with a high steady-state $[H_2O_2]_{ER}$. Third, the demonstration of aquaporin 8-facilitated entry of H_2O_2 into the ER [8] suggests that aquaporin 8 can also facilitate exit of ER-derived H_2O_2 (see also Figure 1). Forth, since the ratiometric readout of HyPer is based on the formation of an intramolecular disulfide bond [51], oxidation of HyPer in the ER could be catalyzed by resident oxidoreductases independently of H_2O_2. Consistent with this assumption, no effect on $HyPer_{ER}$ oxidation was observed upon overexpression of PrxIV or of ER-targeted catalase in pancreatic beta-cells [46]. The increased oxidation of $HyPer_{ER}$ observed in response to higher levels of Ero1α [44, 52] can therefore reflect both enhanced oxidation of PDIs and a rise in $[H_2O_2]_{ER}$. Thus, the Ero1α-induced increase in oxidation of $HyPer_{ER}$ can only be partially reversed by addition of the H_2O_2 scavenger butylated hydroxyanisole (our unpublished observations). Conversely, increased oxidation of $HyPer_{ER}$ in response to NOX4 induction is blunted by coexpression of catalase in the ER [33].

The role of H_2O_2 as signaling molecule typically manifests in the formation of short-lived microdomains of elevated $[H_2O_2]$ [49, 53]. For instance, ligand binding to RTKs at the

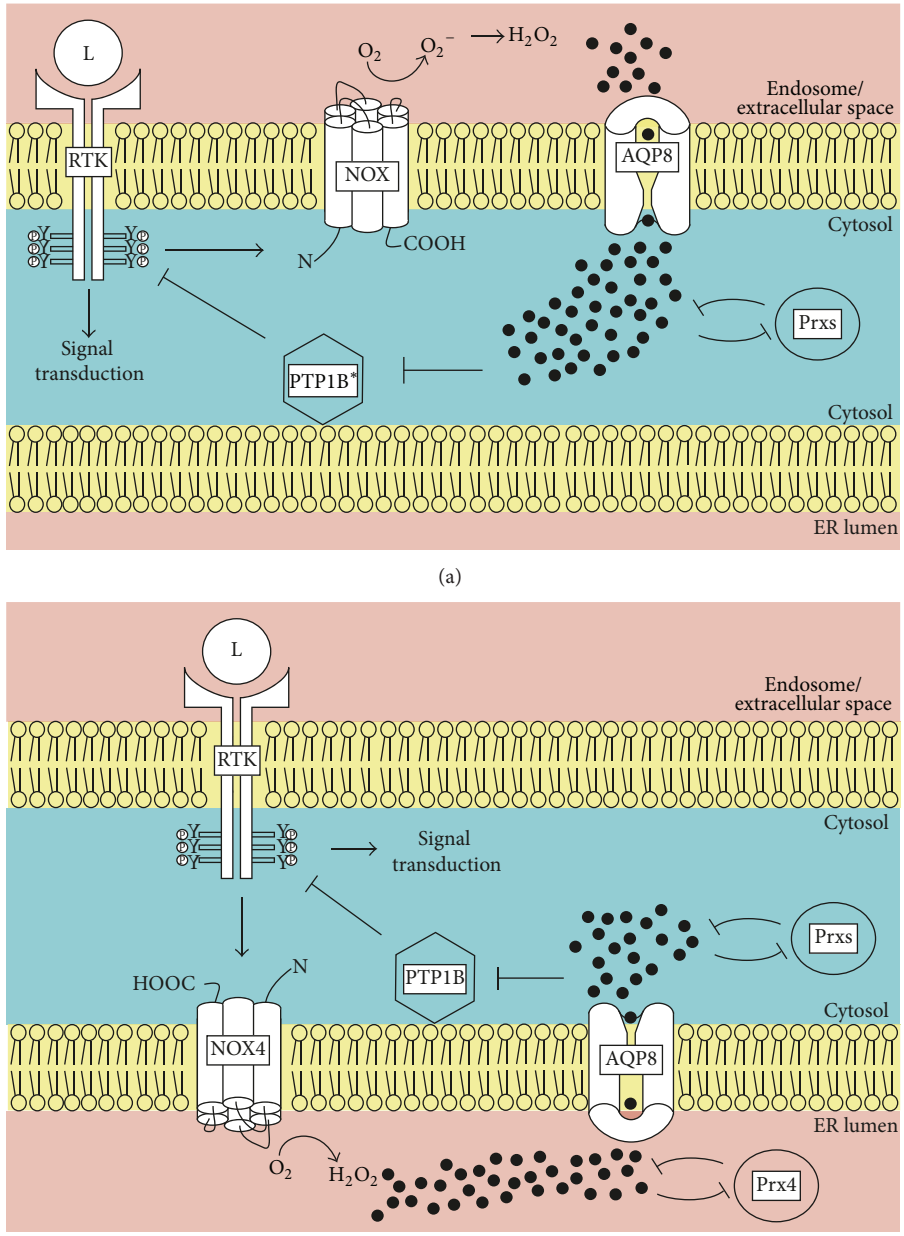

FIGURE 1: RTK signaling involves NOX-derived H_2O_2 as second messenger. (a) Binding of ligand (L) to receptor tyrosine kinases (RTK) on the cell surface activates NADPH oxidases (NOX) and leads to the generation of extracellular or, following endocytosis, endosomal superoxide (O_2^-), which can be dismutated to H_2O_2 (black filled circles). Upon aquaporin 8 (AQP8)-facilitated diffusion across the plasma/endosomal membrane, H_2O_2 locally inactivates the intracellular negative regulators phosphotyrosine phosphatases (PTPs) and peroxiredoxins (Prxs), which prolongs RTK signal transduction. This step mostly, but not exclusively (as depicted by an asterisk), involves the endoplasmic reticulum (ER)-associated PTP1B. Spatial restriction of H_2O_2 is achieved by cytosolic ROS scavengers like Prxs. (b) An ER-centered route of RTK-mediated signal transduction involves NOX4 in the ER membrane and PTP1B. In this context, ER-luminal buildup of H_2O_2 is controlled by ER-resident PrxIV.

cell surface such as platelet-derived growth factor receptor, epidermal growth factor receptor (EGFR), or insulin receptor stimulates the local production of H_2O_2 via crosstalk with NOX enzymes [47, 49, 54, 55]. This leads to oxidative inactivation of protein tyrosine phosphatases (PTPs), which prolongs RTK signaling until cytosolic ROS scavengers such as Prxs have cleared H_2O_2 [56–60] (Figure 1(a)). At least in certain contexts, such H_2O_2-dependent signal amplification is mediated by ER-resident NOX4 and PTP1B [31] (Figure 1(b)). Thus, activated EGFR is internalized into endosomes and transported close to the ER [61] where its PTP1B-dependent dephosphorylation is negatively regulated

by NOX4-derived H_2O_2 [31]. In the case of the granulocyte-colony stimulating factor receptor pathway, also ER-resident PrxIV (see next section) can modulate the signaling amplitude [62] (Figure 1(b)).

NOX4-initiated signal transduction is linked to the adaptive/apoptotic output of the ER stress response—a conglomeration of ER-derived signaling cascades known as the unfolded protein response (UPR) [63]. In the context of atherosclerosis, oxysterol-stimulated smooth muscle cell apoptosis depends on NOX4, which is upregulated through the ER stress sensor Ire1α to produce H_2O_2 [32]. Similarly, NOX4 is induced in endothelial cells in response to a subset of ER stressors, leading to presumably locally restricted H_2O_2 signaling [33]. In both cases, proper activation of UPR pathways requires NOX4-derived H_2O_2. Of note, NOX4-dependent, ER-associated oxidative signaling through the RAS-ERK pathway in endothelial cells promotes prosurvival autophagy rather than cell death [33]. A related link operates in smooth muscle cells where NOX4-derived H_2O_2 stimulates autophagy by inhibiting autophagy-related gene 4B activity, which antagonizes ER stress and cell death [64].

Little is known about signaling roles of H_2O_2 sources other than NOX4 in the ER. Nevertheless, the available data on NOX4 strongly suggest that—in analogy to the situation in other compartments—H_2O_2 operates in the ER as a spatially restricted second messenger rather than a bulk metabolite.

3. Peroxiredoxin IV

PrxIV is the only ER-resident representative of the Prx family. Its predominant isoform harbors a classical signal peptide, which is cleaved upon cotranslational entry into the ER, but no ER retrieval motif to ensure its retention in the early secretory pathway (ESP) [65, 66]. Instead, similar to the ER retention mechanism of Ero1α, physical interactions with the ESP oxidoreductases ERp44 and PDI inhibit PrxIV secretion from cells [67]. Therefore, cell-specific differences and/or saturation of the retrieval machinery, for example, following exogenous overexpression, might explain the ambiguity in the literature on the intracellular or secreted nature of PrxIV [68–72]. This review will focus on the role of the ER-resident fraction of PrxIV.

PrxIV belongs to the subclass of typical 2-Cys Prxs and predominantly exists in decameric configuration. The toroid shaped pentamer of antiparallel dimers (Figure 2) is stabilized by hydrophobic interactions at dimer-dimer interfaces. In contrast to other family members [73], PrxIV does not show significant transition from the decameric to the dimeric state upon disulfide-bond formation between C_P and C_R, even though this process is associated with local unfolding [74]. Furthermore, PrxIV harbors a unique N-terminal extension. As judged from the positions of the truncated N-termini in the crystal structure, these flexible extensions protrude into the center of the decameric assembly of full length PrxIV protomers (Figure 2). In addition to hydrophobic interactions, neighboring antiparallel dimers are linked by Cys^{51}–Cys^{51} interchain disulfide bonds between N-terminal regions (Figure 2), but mutagenesis to serine

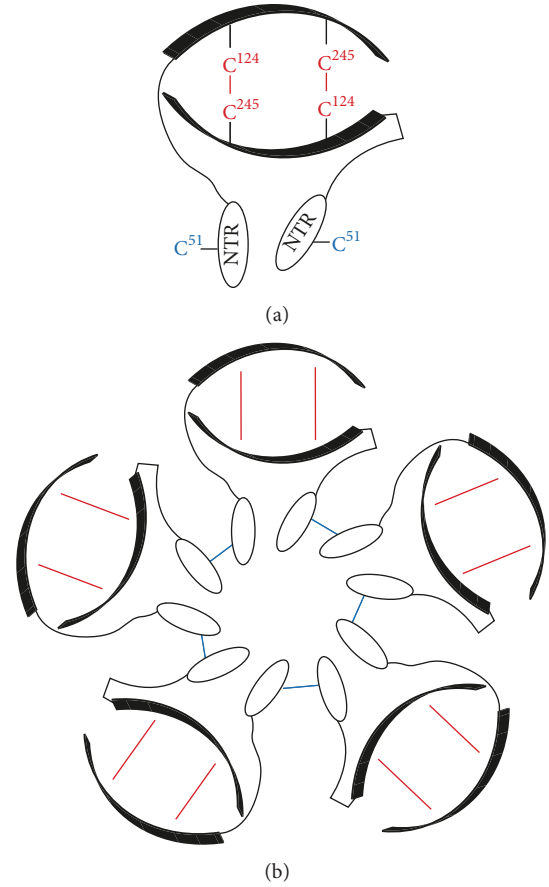

Figure 2: Oligomeric structure of PrxIV. (a) Upon peroxide-mediated oxidation, antiparallel PrxIV dimers are transiently linked by disulfide bonds between C_P (C^{124}) on one subunit and C_R (C^{245}) on the other subunit (depicted in red), which is the characteristic feature of typical 2-Cys Prxs. However, dimer formation relies on hydrophobic interactions and is redox state-independent. The flexible N-terminal region (NTR) of PrxIV is oriented towards the center of the toroid-shaped, decameric complex (b). The role of the disulfide bonds linking adjacent dimers via Cys^{51} in the NTR (depicted in blue) is currently unclear.

or alanine neither affected decamerization nor the catalytic parameters of PrxIV [74–76]. The impact of the N-terminal extensions for correct quaternary structure is still unclear. In an N-terminal truncation mutant, Wang et al. observed a significant transition from the decameric to the dimeric state upon oxidation. In contrast to this, Ikeda et al. reported a shift from decameric to higher oligomeric forms [76, 77].

Like other Prxs, PrxIV exhibits an exceptionally fast reactivity towards H_2O_2 ($2.2 \times 10^7\ M^{-1}\ s^{-1}$) [76]. As data on PrxIV reacting with peroxide substrates other than H_2O_2 is scarce, PrxIV may exclusively react with H_2O_2 *in vivo* (Table 1). PrxIV knockout cells stained with H_2O_2-reactive dye showed a bright signal, which was blunted upon reconstitution of PrxIV (Figure S(10) in [62]). Where does this H_2O_2 come from? A popular model implicates Ero1α-derived H_2O_2, a regular byproduct of oxidative protein folding [78], as oxidizing substrate of PrxIV [79]. This model is based

on the finding that activation of Ero1α in cells by dithiothreitol (DTT)-mediated reduction of its regulatory disulfide bonds increased the hyperoxidized fraction of PrxIV [80]. In further support, DTT-triggered hyperoxidation of PrxIV was inhibited by knockdown of Ero1α (Neil Bulleid, personal communication), and Ero1α-dependent accumulation of H_2O_2 in response to DTT treatment was increased by PrxIV knockdown and decreased by PrxIV overexpression (our unpublished observations). However, in contrast to GPx8 (see below), this crosstalk between Ero1α-derived H_2O_2 and PrxIV was only observed in the presence of DTT (our unpublished observations), which likely does not reflect normal physiology. Experiments with murine or fungal loss-of-function models of Ero1 strongly suggested that PrxIV can be coupled to (an) Ero1-independent source(s) of H_2O_2: ectopic expression of PrxIV rescues the thermosensitive *ero1-1* yeast strain by Ero1-independent oxidative protein folding [81] (see below) and PrxIV is required to protect Ero1-deficient mice against H_2O_2-mediated ascorbate depletion [82]. The H_2O_2 source(s) targeted by PrxIV remain(s) to be identified [12].

Following disulfide-bond formation between C_P and C_R, PrxIV acts as PDI peroxidase by using several different PDIs as electron donors [75, 83] (Table 1). As discussed further below, these PDIs can subsequently shuttle the disulfide onto various substrate proteins, implicating PrxIV as an important element of oxidative protein folding.

It is intriguing that despite the fact that the ER is devoid of sulfiredoxin activity, PrxIV has retained specific structural features to support H_2O_2-mediated hyperoxidation [74, 76]. Accordingly, sulfinylation of C_P in PrxIV could potentially serve a specific function. It has been speculated that hyperoxidized PrxIV could operate as a molecular chaperone or as a secreted damage associated molecular pattern [65].

4. GPx7 and GPx8

GPx7 and 8 are closely related ER-luminal members of the GPx family. Whereas GPx7 possesses a cleavable N-terminal signal sequence, GPx8 is a transmembrane protein with a short N-terminal cytoplasmic tail. Retention in the ESP is mediated by exposed, C-terminal motifs, -Arg-Glu-Asp-Leu and-Lys-Glu-Asp-Leu in GPx7 and 8, respectively, which are recognized in the Golgi by KDEL retrieval receptors [84]. This ESP-retention mechanism is noteworthy for GPx8, since ER membrane proteins are usually retrieved to the ER via cytosolic interactions with retrograde coat proteins [85]. The physiological implications of this peculiarity are currently unclear.

Whereas no other peroxide substrate besides H_2O_2 has been documented for GPx8 yet, GPx7 (also known as nonselenocysteine containing phospholipid hydroperoxide glutathione peroxidase, NPGPx) can efficiently react with phospholipid hydroperoxides *in vitro* ($k > 10^3\ M^{-1}\ s^{-1}$, Table 1) [86]. Although speculative at present, we consider it possible that also in its native context, GPx7 can reduce lipid peroxidation products in the luminal leaflet of the ER membrane. As to GPx8, which largely shares the active site architecture with GPx7 (Figure 3), the short linker between the transmembrane anchor and the catalytic domain might not confer enough flexibility for the active site to interact with the lipid bilayer. Accordingly, both GPxs (together with PrxIV) could protect ER-oriented lipids against peroxidation by scavenging ER-luminal H_2O_2, but only soluble GPx7, in analogy to GPx4 [87], would be able to directly reverse lipid peroxidation by enzymatic reduction.

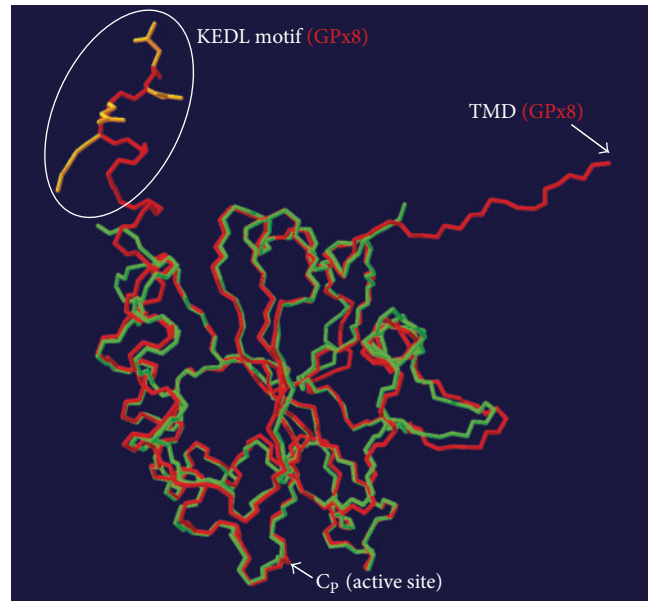

Figure 3: Superimposition of GPx7 and GPx8. Overlay of the carbon-nitrogen backbones of GPx7 (green; PDB ID 2KIJ) and GPx8 (red; PDB ID 2P31) was done using the Swiss PDB viewer software (available at http://www.expasy.org/). The close resemblance of the two three-dimensional structures is particularly appreciable in the peptide loops surrounding the active site Cys (C_P). The ESP retention signal (KEDL motif) and the location of the transmembrane domain (TMD) of GPx8 (not part of the crystal structure) are indicated.

Another prevailing model implicates Ero1 activity to provide H_2O_2 as oxidizing substrate for GPx7 and 8 [21, 88]. Using a split YFP complementation approach, Ero1α and GPx7 or 8 were found to associate within the ER, and addition of GPx7 increased the oxidase activity of Ero1α *in vitro* [88]. While the mechanistic basis for the latter finding remains to be elucidated, these data point to a functional interaction between GPxs and Ero1α. In line with this, knockdown of GPx8 but not PrxIV aggravated the accumulation of H_2O_2 induced by a deregulated Ero1α mutant (our unpublished observations). Therefore, despite their lower reactivity towards peroxide, the physical interaction with Ero1α likely places the GPxs in a privileged position relative to PrxIV to detoxify Ero1α-derived H_2O_2.

Irrespective of the peroxide source, the catalytic mechanism for the reductive regeneration of GPx7/8 remains controversial. Despite the absence of a canonical C_R, GPx7 and

Table 1: Published peroxide and reducing substrates of ER-resident peroxidases.

	Peroxide substrates	Reducing substrates
PrxIV	H_2O_2 [76]	PDIs (ERp46, P5, PDI) [75, 83]
GPx7	H_2O_2 [88] phospholipid hydroperoxide [86]	PDIs (PDI, ERp46, ERp57, ERp72, P5) [86, 88, 89], GRP78/BiP [90], GSH [86], XRN2 [93]
GPx8	H_2O_2 [88]	PDIs (PDI, ERp46, ERp57, ERp72, P5) [88]

8 harbor an additional cysteine in a conserved Pro-$Cys^{86/108}$-Asn-Gln-Phe motif [86]. Studies with GPx7 have highlighted two possible mechanisms of peroxidase reduction [86, 89, 90] (Figure 4(a)). Of note, one of the possibilities features Cys^{86} as a noncanonical C_R. However, since C_P and Cys^{86} are ~11 Å apart in the crystal structure (Figure 4(b)), this implies a major conformational change. Indeed upon H_2O_2 addition, the intrinsic fluorescence of Trp^{142}, which, in reduced GPx7, is particularly solvent-exposed and in close proximity to C_P (Figure 4(b)), readily resumes in the time scale of 2-3 sec after initial decline [88, 89]. This likely indicates the translocation of Trp^{142} away from the fluorescence-quenching C_P sulfenic acid. In this connection, we note the adjacent aromatic side chain of Phe^{89}, which is part of the conserved motif surrounding Cys^{86} (see above), and speculate that stacking of Phe^{89} and Trp^{142} upon C_P oxidation could promote formation of the C_P–Cys^{86} disulfide (Figure 4(b)). Interestingly, in addition to the Pro-Cys-Asn-Gln-Phe motif, the exposed Trp residue is conserved throughout the GPx family [86].

If GPx7 (and likely GPx8) can oxidize reducing substrates in the absence of $Cys^{86/108}$, what could be the reason for its conservation? We suggest that the function of C_R-dependent intramolecular disulfide-bond formation is to prevent the accumulation of sulfenylated GPxs, which may display reactivity towards nonnative thiol substrates. Rapid reaction with Cys^{86} largely prevents the accumulation of the C_P-sulfenylated form of purified GPx7 in presence of H_2O_2 [89]. It will be interesting to assay the oxidation state of GPx7 and 8 in living cells. At all events, evidence for a possible toxic gain-of-function of sulfenylated GPxs came from experiments with an engineered H_2O_2-sensing fluorescent protein [91]. This protein is a fusion of redox-sensitive GFP (roGFP2) and Orp1, which is yeast GPx3. Mutation of C_R in Orp1 accelerated disulfide-bond formation in roGFP2 in response to H_2O_2 *in vitro*. In living cells, however, the C_R-mutant sensor failed to respond to H_2O_2 addition, which was due to competing reactions with reducing substrates other than roGFP2 including glutathione [91].

5. Reducing Substrates of ER-Resident GPxs

In analogy to PrxIV, oxidized GPx7 and 8 were demonstrated to act as PDI peroxidases by using several different PDIs as electron donors [88] (Table 1). The utility of disulfide transfer onto PDIs shall be discussed in the next section. Here, we will touch upon alternative reducing substrates, which have been found to interact with GPx7 (Table 1). For instance, although glutathione reduces sulfenylated GPx7 at a far lower rate compared to PDI, it has been calculated to potentially represent a competing substrate taking into account its millimolar concentration *in vivo* [86]. However, since the reaction of glutathione with oxidized PDI is very fast [92], the physiological relevance of direct glutathione-mediated reduction of GPx7 is questionable.

In contrast, disulfide transfer from GPx7 to the abundant ER chaperone and UPR target GRP78/BiP—as evidenced by cysteine-dependent coimmunoprecipitation from H_2O_2-treated cells—appears to have critical influence on ER physiology [90]. GRP78/BiP carrying the resulting Cys^{41}–Cys^{420} disulfide exhibits increased chaperone activity towards misfolded clients, arguing for a role of GPx7 as oxidative stress sensor and positive regulator of GRP78/BiP [90]. Consistently, cells lacking active GPx7 were more susceptible to H_2O_2 and ER-stress-induced toxicity than wild-type control cells [90]. Very much like PrxIV knockout cells (see above), they also displayed increased staining with a H_2O_2-reactive dye compared to wild-type [90].

Nontargeting siRNA-transfected GPx7 knockout cells displayed harmfully elevated levels of siRNA compared to transfected wild-type cells, indicating a potential link between ER-resident GPx7 and the degradation machinery of nontargeting cytoplasmic siRNA [93]. This link was proposed to involve thiol-disulfide transfer between GPx7 and the nuclear exoribonuclease XRN2, although this reaction appears topologically prohibited [93]. Irrespective of this paradox but consistent with a role of GPx7 in the processing of small RNAs, nontargeting siRNA selectively induced GPx7 expression in wild-type fibroblasts [93], a process mediated by the nuclear protein nucleolin and its activity as transactivator of the GPx7 promotor [94]. It is interesting to note that the cytosolic membrane leaflet of the rough ER is emerging as a central nucleation site of miRNA/siRNA processing in plants and animals [95, 96], and the interplay between the RNA silencing machinery and GPx7 (and possibly other ER-resident peroxidases) deserves further attention.

Compared to GPx7, the enzymatic characterization of GPx8 including the identification of its reducing substrates is far less developed. However, since the structures of their active sites are nearly superimposable (Figure 3), GPx7 and 8 are likely to share many of their catalytic properties.

6. The Two-Disulfides-out-of-One-O_2 Concept

Oxidative protein folding relies on *de novo* disulfide generating enzymes and on oxidants, which accept the electrons derived from thiol oxidation. While several such electron transfer cascades exist in the mammalian ER, resulting in a certain degree of redundancy, Ero1 oxidases (using O_2 as

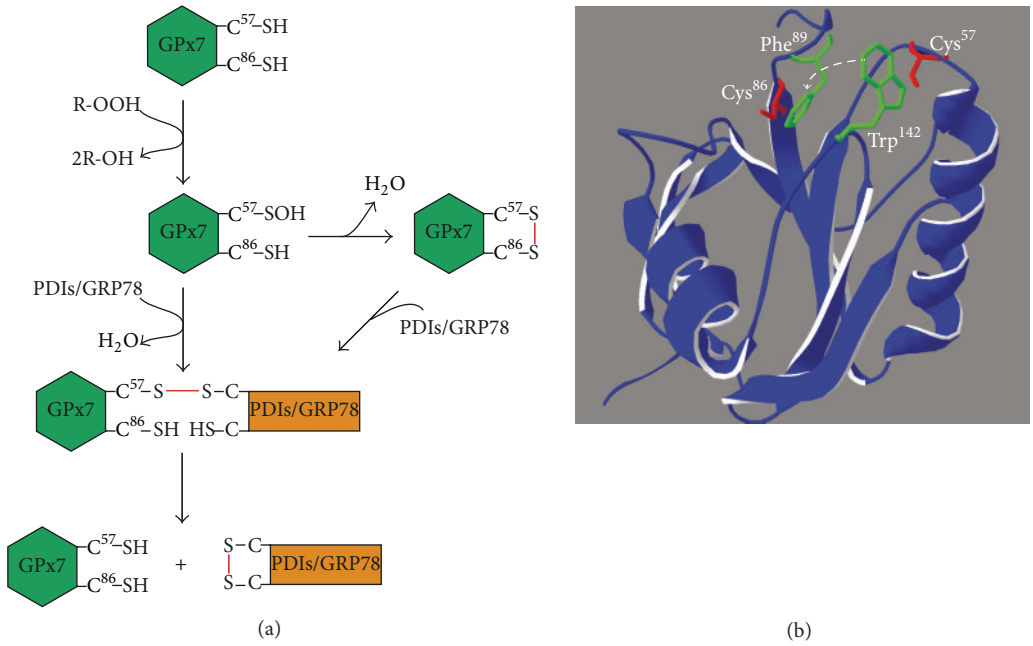

FIGURE 4: Suggested reaction mechanisms of GPx7. (a) Following peroxide-mediated oxidation of the active site Cys (C^{57}), sulfenylated C^{57} is either directly subjected to nucleophilic attack by a (deprotonated) Cys in the reducing substrate (PDIs/GRP78) or attacked by (deprotonated) Cys^{86}, which results in formation of an intramolecular disulfide bond. In a second step, this intramolecular disulfide is attacked by a Cys in the reducing substrate. Both pathways converge in the formation of an intermolecular disulfide-bonded intermediate between GPx7 and the reducing substrate prior to the completion of the reaction cycle, which gives rise to regenerated, reduced GPx7 and oxidized PDIs/GRP78. (b) Hypothesized conformational change prior to formation of a Cys^{57}–Cys^{86} disulfide bond in GPx7 is depicted on the structure of reduced GPx7 (PDB ID 2KIJ). Active site rearrangement upon oxidation of Cys^{57} might involve a stacking interaction between the conserved aromatic side chains of Phe^{89} and Trp^{142} (green), which would move away Trp^{142} from Cys^{57} (dashed white arrow).

oxidant) and PrxIV (using H_2O_2 as oxidant) are evidently the dominant disulfide sources [29, 36, 81]. The fact that both enzymes can oxidize PDIs [75, 78, 81, 83, 97, 98] has led to the intriguing concept that the four oxidizing equivalents in O_2 can be exploited by the consecutive activity of Ero1 and PrxIV to generate two disulfides for oxidative protein folding [79, 99] (Figure 5). Along the same lines, the PDI peroxidase activity of GPx7 constitutes a pathway for the productive use of Ero1α-derived H_2O_2 in the biosynthesis of disulfides [88, 89].

Evidence for a contribution of ER-resident peroxidases to oxidative protein folding is manifold. Mixed disulfide reaction intermediates between peroxidase and PDI were isolated from cells [75, 81, 89], and in the case of PrxIV, interactions with the PDI family members ERp46 and P5 were also reported [75, 83]. Interestingly, of the two Cys-X-X-Cys active sites in PDI, PrxIV preferentially oxidizes the **a′** domain active site and GPx7 the **a** domain active site [75, 89]. Since the mixed-disulfide complexes were stabilized by a Cys-X-X-Ala active site configuration in PDI [75], they must have resulted from the reaction of reduced PDI with oxidized peroxidase [100]. Accordingly, consumed peroxidase molecules can be activated/recycled by PDIs. It is possible that the availability of reduced PDIs actively adjusts the activation state of ER peroxidases. Thus, peroxidases could be kept in an inactive state unless new disulfides are needed, as indicated by the accumulation of reduced PDIs. In a very related manner, the intramolecular disulfides, which shut off Ero1α, are feedback-regulated by the availability of reduced PDI [101]. In contrast to Ero1α, however, the redox state of PrxIV appears to be predominantly reduced in cells at steady state [83].

Peroxidase/PDI-catalyzed oxidative protein folding can be reconstituted. Refolding of reduced RNase A, a process requiring introduction of four disulfides, occurs in the presence of PDI together with PrxIV or GPx7 [81, 89]. It is important to note though that PrxIV-driven refolding appears to depend on the addition of H_2O_2, whereas GPx7-driven refolding readily works in presence of Ero1α, which generates H_2O_2 by reducing ambient O_2 [81, 89]. This difference parallels the evidence discussed above for a preference of GPx7 or 8 over PrxIV to detoxify Ero1α-derived H_2O_2.

The role of PrxIV as a source of disulfide bonds is also strongly supported by genetics. Ero1-deficient mouse embryonic fibroblasts are hypersensitive to the loss of PrxIV, which causes hypooxidation of an ER-targeted thiol-disulfide sensor, ER dilation, and decreased cell viability [81]. Somewhat counterintuitively, compound loss of Ero1α/β and PrxIV also leads to oxidative phenotypes such as glutathione depletion and cell senescence [82]. These phenotypes are attributed to the failure to reduce H_2O_2 from as yet unidentified origin, which causes shortage of intracellular ascorbate (vitamin C) associated with defects in collagen synthesis and scurvy [82]. Last but not least, codepletion of PrxIV in hepatocytes exacerbates the cytotoxic phenotype of Ero1α/β depletion

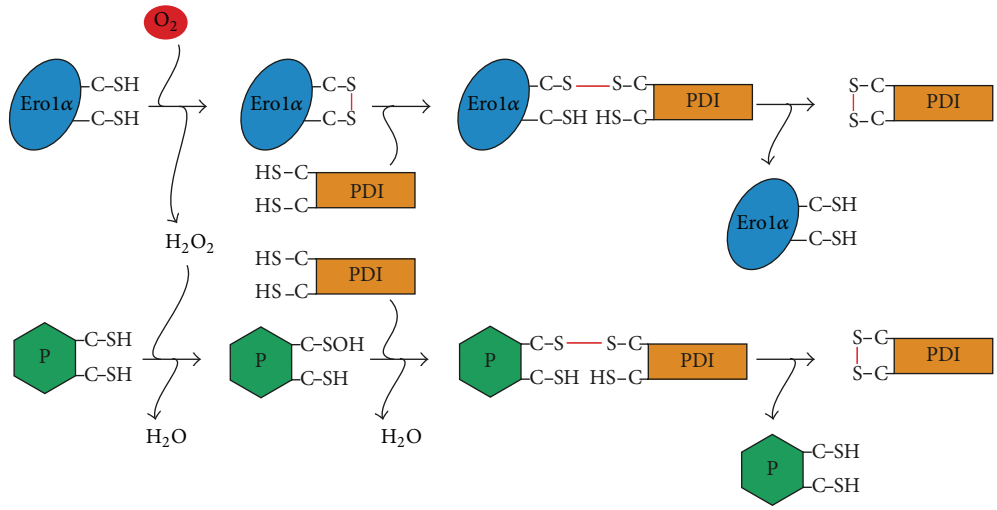

FIGURE 5: The two-disulfides-out-of-one-O_2 concept. O_2 (red)-mediated oxidation of Ero1α results in the generation of one disulfide bond (red), which is transferred to a reduced PDI, and of one molecule of H_2O_2. ER-resident peroxidases (P)—probably exclusively of the GPx family (see main text for details)—can couple the reduction of Ero1α-derived H_2O_2 to H_2O with the introduction of a second disulfide bond (red) into a PDI family member, thereby exploiting the oxidizing capacity of H_2O_2.

and further slows ER reoxidation after reductive challenge [36].

Taken together, a role in oxidative protein folding is particularly well documented for PrxIV but is also shared by the ER-resident GPxs. Still, although appealing, we consider it likely that the concept of peroxidase-dependent exploitation of Ero1α-derived H_2O_2 (Figure 5) only applies to GPxs (see above).

7. Organismal Roles of ER Peroxidases

For PrxIV and GPx7, *in vivo* studies have been performed in different model organisms. One striking conclusion of these studies is that whole-body loss-of-function of GPx7 in mice shows a stronger organismal phenotype compared to PrxIV deficiency. No *in vivo* characterization of the role of GPx8 has been published so far.

Male mice lacking a functional X-chromosomal *PRDX4* gene ($PrxIV^{-/y}$) display a mild phenotype, which manifests predominantly by testicular atrophy accompanied by increased DNA fragmentation and peroxidation of lipids and proteins [69]. The number of sperms is markedly decreased in the epididymis of $PrxIV^{-/y}$ mice, which, however, does not affect their fertility [69]. These phenotypes are likely attributed to loss of the testis-specific transmembrane isoform of PrxIV [65].

Similarly, in fruit flies a decrease in PrxIV expression to 10–20% of wild-type levels is associated with increased $[H_2O_2]$ and lipid peroxidation in membrane preparations from whole animals [102]. However, negative impact on longevity was only observed under oxidative stress conditions induced by H_2O_2 or paraquat treatment. Strikingly, 6–10 fold, global overexpression of PrxIV in flies, which shifted its subcellular distribution from predominantly ER-resident to cytosolic and secreted, resulted in dramatically shortened lifespan under nonstress conditions and increased apoptosis in thoracic muscle and fat body tissue [102]. Since this proapoptotic phenotype upon PrxIV overexpression was not reproducible in cultured fly cells, noncell autonomous and/or fly-specific *in vivo* effects of secreted PrxIV need further consideration.

In contrast to this, overexpression of PrxIV in mice has beneficial effects in the context of metabolic diseases. For instance, elevated levels of PrxIV in apolipoprotein E negative mice, which were fed a high cholesterol diet, have antiatherogenic effects with less oxidative stress, a decrease in apoptosis, and suppressed T-lymphocyte infiltration [103]. In addition, cytoprotective effects of overexpressed PrxIV were evident in nongenetic mouse models of both type 1 and type 2 diabetes mellitus (T1DM and T2DM) [104, 105]. Specifically, autoimmune-induced apoptosis of pancreatic β-cells (in T1DM) and fatty liver phenotypes and peripheral insulin resistance (in T2DM) were diminished upon PrxIV overexpression. It is possible that more efficient clearance of inflammatory ROS is the underlying reason for the ameliorated phenotypes of these mice [104, 105]. However, one has to bear in mind that overexpression of PrxIV above a certain threshold exceeds ERp44-mediated ESP retrieval [67] and therefore may result in abnormally high levels of secreted peroxidase. Overexpression studies therefore need careful evaluation, before implications on normal physiology can be conclusively deduced.

Interestingly, endogenous PrxIV is dramatically upregulated during terminal B-cell differentiation [106], a process accompanied by increased ROS levels but not by discernible hyperoxidation of the ER lumen [107, 108]. PrxIV knockout splenocytes, however, develop normally and do not show a defect in antibody secretion, arguing for redundancy among different oxidant control mechanisms [106].

In contrast to the relatively mild PrxIV knockout phenotype [69], quite dramatic changes including a shortened lifespan were documented for GPx7$^{-/-}$ compared to control mice [90]. Besides induction of UPR hallmarks in different organs, these mice exhibited oxidative DNA damage and apoptosis predominantly in the kidney. Furthermore, multiple organ dysfunctions including glomerulonephritis, spleno- and cardiomegaly, fatty liver, and multiple malignant neoplasms were diagnosed [90]. Carcinogenesis and premature death were concluded to reflect systemic oxidative stress [90].

Along this line, Peng and coworkers proposed a tumor-suppressive role for GPx7 in oesophageal epithelial cells [109]. Progression from healthy tissue to premalignant Barrett's oesophagus (BO) and further to malignant oesophageal adenocarcinoma (OAC) is associated with gastro-oesophageal reflux, leading to ROS accumulation and increased oxidative DNA damage. BO/OAC neoplastic transformation is accompanied by decreased expression of GPx7 [110]. The diminished levels of GPx7 in BO and OAC tissues are due to DNA-hypermethylation within the respective promoter region. Bile acid-mediated intracellular and extracellular ROS accumulation in oesophageal epithelial cell culture was also responsive to overexpression or downregulation of GPx7 [111]. Furthermore, reconstitution of GPx7 expression suppressed growth and promoted cellular senescence in both *in vitro* and *in vivo* OAC models [109]. Therefore, inactivation of GPx7 is a crucial step in BO/OAC formation. Despite these conclusive links between oxidative injury and GPx7 expression *in vivo*, it is important to emphasize that the actual source of peroxide that causes ROS accumulation in absence of GPx7 remains to be identified. A possible involvement of Ero1α [112] remains to be experimentally verified.

8. Conclusions and Perspectives

The reaction cycle of a peroxidase is split into an oxidizing part, which uses a source of hydroperoxide, and a reductive part, which uses a dithiol substrate. As such, available data highlight a twofold function of ER-resident peroxidases; on one hand, they can reduce and spatially restrict local H_2O_2 or lipid hydroperoxides and on the other hand, they are net producers of disulfide bonds.

The model, which has probably generated the highest resonance, holds that ER peroxidases eliminate the obligatory and potentially harmful side product of Ero1-catalyzed disulfide-bond formation, H_2O_2, by exploiting its oxidizing power to generate a second disulfide in PDI for oxidative protein folding (Figure 5). The fact that all ER peroxidases—PrxIV, GPx7, and GPx8—can catalyze steps of this pathway *in vitro* [75, 81, 88, 89] has led to the understanding that they basically perform the same function [65]. But do ER peroxidases really all do the same? Are their functions redundant? We believe that this is clearly not the case. For instance, the prominent phenotype of the GPx7$^{-/-}$ mouse strongly suggests that neither PrxIV nor GPx8 can broadly substitute for the loss of GPx7 [90]. This could be due to the fact that GPx7 uses unique reducing substrates (other than PDI family members) or metabolizes phospholipid hydroperoxides in the ER-facing membrane leaflet *in vivo*. Alternatively, tissue-specific expression levels might prohibit functional compensation between ER peroxidases. These questions are exciting subjects for future research. Clearly, it will also be interesting to learn about the phenotypes of GPx8$^{-/-}$ and GPx7/8 double knockout animals. Whether or not other human GPx isoforms like for example, the ubiquitously secreted GPx3 [21] have an additional intracellular function in the ER is another open question.

Differences between ER peroxidases also manifest in terms of the source of hydroperoxide. There is clear proof for PrxIV reacting with Ero1-independent H_2O_2 [81, 82], and unpublished data from our laboratory has demonstrated that this peroxidase does not react with Ero1α-derived H_2O_2 in cells under steady-state conditions. In this respect, one of the most urgent questions is which is the H_2O_2 source that drives PrxIV-dependent oxidative protein folding [36, 81, 82]. Identification of this source will likely provide major new insights into the diffusion pathways of this metabolite.

Another area for future investigation concerns potential signaling roles of H_2O_2 in the ER lumen and beyond. For instance, the interplay of ER-resident NOX family members and peroxidases is largely unexplored. Likewise, it is currently unclear whether or not the known proapoptotic role of Ero1α during ER stress [113–115] is mediated by diffusion of Ero1α-derived H_2O_2 into the cytoplasm, as is suggested [7]. It is foreseeable that aquaporins will be found to play a central function in these processes at the ER membrane [8]. As every discovery arouses further interest and curiosity, we are expecting new insights and again new questions to come.

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Review Article

Oxidative Protein-Folding Systems in Plant Cells

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Plants are unique among eukaryotes in having evolved organelles: the protein storage vacuole, protein body, and chloroplast. Disulfide transfer pathways that function in the endoplasmic reticulum (ER) and chloroplasts of plants play critical roles in the development of protein storage organelles and the biogenesis of chloroplasts, respectively. Disulfide bond formation requires the cooperative function of disulfide-generating enzymes (e.g., ER oxidoreductase 1), which generate disulfide bonds de novo, and disulfide carrier proteins (e.g., protein disulfide isomerase), which transfer disulfides to substrates by means of thiol-disulfide exchange reactions. Selective molecular communication between disulfide-generating enzymes and disulfide carrier proteins, which reflects the molecular and structural diversity of disulfide carrier proteins, is key to the efficient transfer of disulfides to specific sets of substrates. This review focuses on recent advances in our understanding of the mechanisms and functions of the various disulfide transfer pathways involved in oxidative protein folding in the ER, chloroplasts, and mitochondria of plants.

1. Introduction

The endoplasmic reticulum (ER) is the first organelle in the secretory pathway, and this dynamic and highly specialized organelle contains enzymes and chaperones that mediate the folding and assembly of newly synthesized proteins [1]. A key step in oxidative protein folding is the formation of disulfide bonds, which covalently link the side chains of pairs of Cys residues, impart thermodynamic and mechanical stability to proteins, and control protein folding and activity [2]. The introduction of disulfide bonds into polypeptides requires the cooperative function of a pair of enzymes, a de novo disulfide-generating enzyme (e.g., ER oxidoreductase 1 [ERO1]) and a disulfide carrier protein (e.g., protein disulfide isomerase [PDI]) [3]. PDIs, which are ubiquitous thiol-disulfide oxidoreductases in all eukaryotic cells, directly donate disulfides to substrate proteins by means of thiol-disulfide exchange reactions. The oxidized form of PDI is regenerated by ERO1, which relays the oxidizing power from molecular oxygen to the reduced form of PDI (Figure 1). The genomes of higher plants, including *Arabidopsis thaliana*, *Glycine max* (soybean), *Oryza sativa* (rice), and *Zea mays* (maize), encode approximately 10 to 20 members of the PDI family, which show wide variation in the organization of their thioredoxin (TRX)-fold domains [4, 5]. Although the network of enzymes involved in disulfide bond formation has not yet been fully elucidated, emerging evidence suggests that the molecular and structural diversity of the PDIs and the specific combinations of disulfide-generating enzymes and PDIs are key to determining the functions of these enzymes and their substrate specificity.

Plant cells have two distinct types of vacuoles: the prototypical lytic vacuole (LV), which has high hydrolytic activity and shares functions with the yeast vacuole and mammalian lysosome, and the protein storage vacuole (PSV), a plant-specific organelle that is specialized in accumulating reserve proteins and is prevalent in seeds [6]. The developing endosperm cells of seeds actively synthesize large amounts of storage proteins that acquire disulfide bonds in the ER. Among the physicochemically and structurally diverse disulfide-rich seed storage proteins, soluble storage proteins are transported through the endomembrane system from the ER to PSVs, where they accumulate [7]. Insoluble proteins, however, form large oligomeric accretions within the ER and are deposited in ER-derived protein bodies (PBs), which bud and disconnect from the ER but remain surrounded by ER membranes [8]. The development of the PSVs and PBs enables massive, structurally stable accumulations of storage

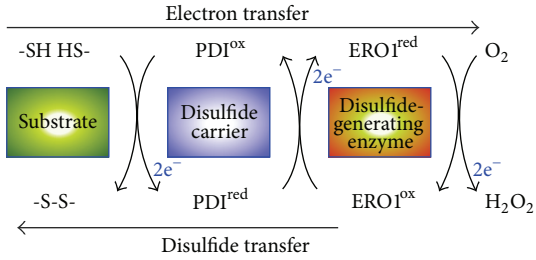

Figure 1: Disulfide relay from ERO1 to PDI in the ER. Disulfide bond formation involves electron transfer from the substrate to a disulfide carrier protein (e.g., PDI) and then to a de novo disulfide-generating enzyme (e.g., ERO1). PDI directly transfers a disulfide to two Cys residues of a substrate protein by means of a thiol-disulfide exchange reaction. The reduced form of PDI is reoxidized by ERO1, which relays the oxidizing power from molecular oxygen, via the FAD cofactor, to the reduced form of PDI.

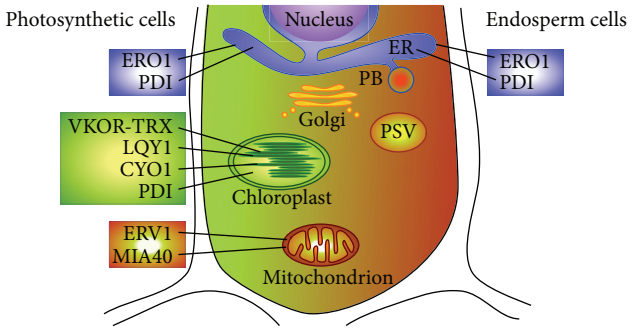

Figure 2: Overview of the sites of oxidative protein folding in photosynthetic and endosperm cells of plants. Disulfide-generating enzymes and disulfide carrier proteins characterized to date and their subcellular localizations are shown in photosynthetic (left) and endosperm (right) cells of plants. ER, endoplasmic reticulum; PB, protein body; PSV, protein storage vacuole.

proteins, which serve as nutritional reserves of nitrogen, sulfur, and carbon for the germinating seedlings (reviewed in [9, 10]). The redox state of storage proteins containing disulfide groups dramatically changes during seed development, maturation, and germination [11]; seed storage proteins are synthesized in reduced form on the rough ER and are converted to disulfide-bonded forms during maturation; then they are converted back to the reduced form to facilitate rapid mobilization during germination. The *h*-type TRX functions in the reduction of seed storage proteins in the endosperm, increasing their proteolytic susceptibility and making nitrogen and sulfur available during germination [12]. In contrast to the TRX-dependent system that acts mainly in the reduction of disulfides, the disulfide transfer systems involving ERO1 and PDIs function primarily to form the correct patterns of disulfide bonds, thereby facilitating the stable accumulation of seed storage proteins, preventing their denaturation, and decreasing their susceptibility to proteolysis.

In chloroplasts, the redox state of disulfide bonds in redox-regulated proteins is linked to the control of metabolic pathways and gene expression [11, 13, 14]. The most-studied redox regulatory system is the ferredoxin-TRX system that reversibly reduces and oxidizes thiol groups; reducing equivalents are transferred from photosystem I (PSI) to ferredoxin and then to TRX via ferredoxin-dependent TRX reductase [11, 13]. Inside chloroplasts, the thylakoid membranes, which enclose the lumen that originated as the bacterial periplasm, perform the primary events of oxygenic photosynthesis. Under strong illumination, however, photosystem II (PSII) in the thylakoid membranes is subjected to photodamage. To maintain photosynthetic activity, PSII requires the rapid reassembly of a thylakoid membrane protein complex composed of dozens of proteins [15], which is dependent on disulfide bond formation catalyzed by enzyme catalysts, including a zinc finger protein LQY1 and a vitamin K epoxide reductase (VKOR) homolog.

Emerging evidence has shown that disulfide transfer pathways that function in the ER and chloroplasts of plants play critical roles in the development of PSVs and PBs and the biogenesis of chloroplasts, respectively. This review focuses on recent advances in our understanding of the mechanisms and functions of the various disulfide transfer pathways involved in oxidative protein folding in the ER and chloroplasts of plants (Figure 2). The disulfide transfer pathways in plant mitochondria, which involve the disulfide-generating enzyme ERV1 and the disulfide carrier protein MIA40 (Figure 2), are also discussed in comparison to the respective pathways in yeast and mammalian cells.

2. Disulfide Bond Formation in the Plant ER

2.1. Seed Storage Proteins. Seed storage proteins are conventionally classified on the basis of their solubility in water (e.g., albumins), saline (e.g., α-globulin), alcohol (e.g., prolamins), and acidic or basic solutions (e.g., glutelins) [16]. The globulins, the most widely distributed group of storage proteins in both dicots and monocots, are divided into two subgroups on the basis of their sedimentation coefficients: the vicilin-like 7S globulins and the legumin-like 11S globulins. Rice glutelins (60% of total seed protein) [17] and soybean glycinins (40% of total seed protein) [18], both of which are 11S globulin homologs, are synthesized from their precursors, proglutelins and preproglycinins, respectively; the precursors are processed at a conserved Asp-Gly site by an asparaginyl endopeptidase [19, 20] to produce acidic and basic polypeptide subunits, which remain linked by disulfide bonds [21].

The prolamin family, which includes maize prolamins (referred to as zeins) and rice prolamins, is encoded by multiple genes and is composed of Cys-rich and Cys-poor members [16, 22]. Rice prolamins (20% of total seed protein) [17] are clustered into three subgroups: 13-kD prolamins containing 0–8 Cys residues, 10-kD prolamins containing 9–11 Cys residues, and 16-kD prolamin containing 13 Cys residues [22]. Maize prolamins include four structurally distinct types of proteins: α-, β-, γ-, and δ-zeins. The β-, γ-, and δ-zeins are rich in Cys residues, whereas the α-zeins are Cys poor [16]. Prolamins and 2S albumins contain conserved motifs

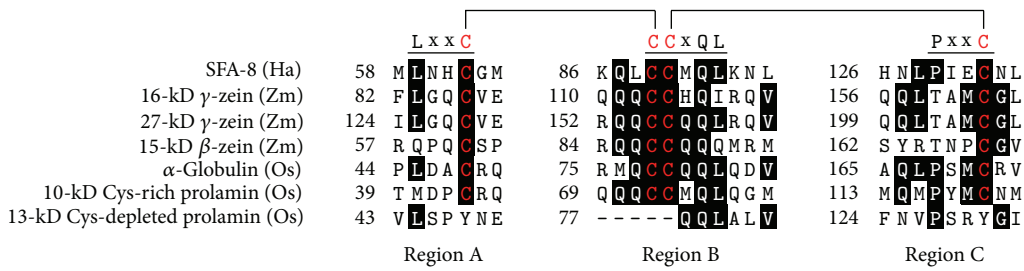

FIGURE 3: Conserved Cys residues in members of the prolamin superfamily. Amino acid sequences in regions A, B, and C of *Zea mays* (Zm) and *Oryza sativa* (Os) storage proteins are compared to *Helianthus annuus* (Ha) SFA-8. Amino acid sequences of SFA-8 (CAA40015), 16-kD γ-zein (NP_001105337), 27-kD γ-zein (NP_001105354), 15-kD β-zein (NP_001106004), α-globulin (BAA09308), and Cys-rich 10-kD prolamin (crP10; NP_001051380) were aligned by means of CLUSTALW. For comparison, the corresponding sequence of Cys-depleted 13-kD prolamin (cpP13; NP_001055213) is shown. Two disulfide bonds in SFA-8, Cys62–Cys89 and Cys90–Cys132, are indicated with solid lines. Red-on-black letters indicate Cys residues; white-on-black letters indicate amino acid residues conserved in at least four of the sequences analyzed.

in three separate regions [16]: LxxC in region A, CCxQL in region B, and PxxC in region C. Cys-rich prolamins of rice and the β- and γ-zeins of maize, but not the δ-zeins, contain the conserved Cys residues found in regions A, B, and C (Figure 3) [16, 22]. Structural analysis shows that the sunflower (*Helianthus annuus*) 2S albumin SFA-8 forms a disulfide bond between regions A and B (between Cys62 of $LxxC^{62}$ and Cys89 of $C^{89}CxQL$) and one between regions B and C (between Cys90 of $CC^{90}xQL$ and Cys132 of $PxxC^{132}$) [23]. These conserved Cys residues are also found in other members of the prolamin family, including a 10-kD Cys-rich prolamin from rice and a 16-kD γ-zein from maize (Figure 3) [22].

2.2. Protein Storage Organelles. To enable the stable accumulation of massive amounts of seed storage proteins and prevent their degradation, plants have evolved specialized membrane-bound storage organelles: PSVs that contain matrix and crystalloid components and PBs derived from the ER [10]. The multisubunit 7S and 11S globulins are synthesized in precursor form and then transported from the lumen of the rough ER to PSVs, where these proteins undergo proteolytic cleavage and assemble to form dense accretions [24]. In contrast, prolamins generally form PBs directly within the lumen of the rough ER. PBs in maize and rice remain in the ER lumen, whereas those in wheat and barley are transported from the ER to PSVs by an autophagic process [25].

Disulfide bonds play a critical role in the accumulation of seed storage proteins in PSVs and PBs [26, 27]. In the endosperm cells of rice seeds, proglutelins acquire intramolecular disulfide bonds in the ER before they are targeted, via the Golgi, to a PSV (designated type-II PB [PB-II]; a crystalloid structure with a diameter of 2–4 μm). They are processed into acidic (37–39 kD) and basic (22–23 kD) subunits by a vacuolar processing enzyme (VPE) and accumulate as higher-order complexes held together by intermolecular disulfide bonds and hydrophobic interactions in the crystalloids of PSV [28–31]. The formation of intramolecular disulfide bonds is also required for the sorting of α-globulins to the matrix of PSV [27]. The 10-kD Cys-rich prolamins (Figure 3), however, directly accumulate within the ER of rice endosperm cells to form the center core region of an ER-derived PB (designated type-I PB [PB-I]; a spherical structure with a diameter of 1–2 μm) at early stages of PB development, before the 13-kD Cys-depleted prolamins (Figure 3) are localized to the outer layer of ER-derived PB [32, 33]. The assembly of prolamins into the ER-derived PB is stabilized by the formation of intermolecular disulfide bonds [34, 35]. In the endosperm cells of maize seeds, however, the β- and γ-zeins form spherical accretions (with a diameter of 0.2 μm) at early stages of PB development. Subsequently, the α-zeins penetrate the network of the β- and γ-zeins and fill the central region of the PB (which has a diameter of 1–2 μm) [36, 37].

2.3. Roles of PDIs in the Development of Protein Storage Organelles: Different Subcellular Localizations and Substrate Specificities. PDIs are multifunctional enzymes that catalyze a wide range of thiol-disulfide exchange reactions, including oxidation, reduction, and isomerization reactions, and also display chaperone activity [38]. PDIs are multidomain proteins, which have at least one redox-active TRX domain, designated domain a. The oxidized form of the redox-active CxxC motif in the a domain transfers a disulfide to a substrate and is converted to the reduced form. Members of the PDI family vary widely in the number and arrangement of their a domains and of another type of TRX domain, designated domain b, which has a similar TRX-like structure but lacks the redox-active motif. For example, Pdi1p, one of the five PDIs encoded by the *Saccharomyces cerevisiae* genome (and the only one that is essential for viability [39]), has been described as a U-shaped molecule composed of four TRX-fold domains (a-b-b′-a′) and an acidic C-terminal tail; two redox-active CGHC motifs are in the N-terminal and C-terminal TRX domains (a and a′, resp.), and face each other on either side of the U shape [40]. The subsequently determined crystal structure indicates that the shape the yeast Pdi1p adopts is more that of a boat, with the b and b′ domains at the bottom and the a and a′ domains at the bow and stern [41]. The flexible arms composed of the a and a′ domains are connected to the relatively rigid core composed of the

Table 1: Components of the system for disulfide bond formation in higher plants.

Species	Name	Domain	Active sites	Location
		Disulfide-generating enzymes		
Rice	ERO1		$C_{x4}C$, $C_{xx}C$	ER^m
Arabidopsis	ERV1	Erv1/ALR	$C_{xx}C$, $C_{x4}C$	M
Arabidopsis	LTO1	VKOR, TRX	$C_{x6}C$, $C_{xx}C$, $C_{xx}C$	T^m
Arabidopsis	QSOX1	TRX, Erv1/ALR	$C_{xx}C$, $C_{xx}C$, $C_{xx}C$	W
		Thiol-disulfide oxidoreductases		
PDI family proteins				
Rice	OsPDIL1;1	a-b-b′-a′	$C_{GH}C$, $C_{GH}C$	ER^L
Rice	OsPDIL2;3	a-a′-b	$C_{GH}C$, $C_{GH}C$	PB^{ER}
Arabidopsis	AtPDIL1;1	a-b-b′-a′	$C_{GH}C$, $C_{GH}C$	ER, G, LV, PSV
Arabidopsis	AtPDIL1;3	c-a-b-b′-a′	$C_{GA}C$, $C_{GH}C$	C, ER
Arabidopsis	AtPDIL1;4	c-a-b-b′-a′	$C_{GH}C$, $C_{GH}C$	ER, G, N, V, W
Arabidopsis	AtPDIL2;1	a-a′-D	$C_{GH}C$, $C_{GH}C$	ER^L
Soybean	GmPDIL-1	a-b-b′-a′	$C_{GH}C$, $C_{GH}C$	ER^L
Soybean	GmPDIL-2	c-a-b-b′-a′	$C_{GH}C$, $C_{GH}C$	ER^L
Soybean	GmPDIS-1	a-a′-D	$C_{GH}C$, $C_{GH}C$	ER^L
Soybean	GmPDIS-2	a-a′-D	$C_{GH}C$, $C_{GH}C$	ER^L
Soybean	GmPDIM	a-a′-b	$C_{GH}C$, $C_{GH}C$	ER^L
Zn finger proteins				
Arabidopsis	LQY1	DnaJ Zn finger	$(C_{xx}C_{xGxG})_4$	T^m
Arabidopsis	CYO1	DnaJ Zn finger	$(C_{xx}C_{xGxG})_2$	T^m
Disulfide carrier protein				
Arabidopsis	MIA40		C_pC	M

Disulfide-generating enzymes and disulfide carrier proteins from rice (*Oryza sativa*), Arabidopsis (*Arabidopsis thaliana*), and soybean (*Glycine max*) characterized to date. C, chloroplasts; ER, endoplasmic reticulum; ER^L, ER lumen; ER^m, ER membranes; G, Golgi; LV, lytic vacuole; N, nucleus; M, mitochondria; PB^{ER}, ER-derived protein body; PSV, protein storage vacuole; T^m, thylakoid membranes; V, vacuole; W, cell wall.

b and b′ domains, resulting in a highly flexible structure [41]. The primary substrate-binding sites of yeast Pdi1p, as well as *Homo sapiens* (human, Hs) HsPDI, have been mapped to the hydrophobic pocket in the b′ domain, whose substrate-binding ability seems likely to be influenced by the neighboring domains (b and a′) and by the x-linker region between b′ and a′ [40, 42–44]. The domain organization of PDIs likely contributes strongly to their substrate specificity.

Whole-genome sequencing has identified 13 genes encoding PDI proteins in Arabidopsis (At), 22 in soybean (Gm), and 12 each in rice (Os) and maize (Zm) [4, 5]. Arabidopsis AtPDIL1;1 and AtPDIL1;2; rice OsPDIL1;1, OsPDIL1;2, and OsPDIL1;3; maize ZmPDIL1;1 and ZmPDIL1;2; and soybean GmPDIL-1 show the a-b-b′-a′ domain structure (Table 1; Figure 4(a)) [4, 5, 32, 45], similar to human HsPDI and HsERp57 [46]. Arabidopsis AtPDIL1;3 and AtPDIL1;4, rice OsPDIL1;4, maize ZmPDIL1;3 and ZmPDIL1;4, and soybean GmPDIL-2 show a c-a-b-b′-a′ domain structure with an acidic N-terminal domain (c domain; Table 1; Figure 4(a)) [4, 5, 45]. In contrast, PDIL2;1–2;3 members show a three-domain structure, in which two redox-active TRX domains repeated tandemly in the N-terminal region are followed by a redox-inactive domain, either a TRX-like b domain (a-a′-b domain structure; Arabidopsis AtPDIL2;2 and AtPDIL2;3, rice OsPDIL2;3, maize ZmPDIL2;3, and soybean GmPDIM [4, 5, 32, 47], similar to human HsP5 [46]) or an α-helical D domain, as found in human HsERp29 (a-a′-D domain structure; Arabidopsis AtPDIL2;1, rice OsPDIL2;1 and OsPDIL2;2, maize ZmPDIL2;1 and ZmPDIL2;2, and soybean GmPDIS-1 and GmPDIS-2 [4, 5, 48]) (Table 1; Figure 4(a)).

Specific members of the PDI family have been suggested to be involved in oxidative folding of storage proteins. For example, soybean GmPDIS-1 and GmPDIM (orthologs of AtPDIL2;1 and OsPDIL2;3, resp.) are localized in the ER and associate with proglycinin in the cotyledon cells [47, 48]; GmPDIL-1 and GmPDIL-2 (orthologs of AtPDIL1;1 and AtPDIL1;3, resp.), which show higher activity for refolding of reduced, denatured RNase than do GmPDIS-1, GmPDIS-2, and GmPDIM [45, 47, 48], are localized in the ER and associate with proglycinin and β-conglycinin [45]. Quantitative comparison of the transcript levels of maize *ZmPDIL* genes by massively parallel signature sequencing showed that *ZmPDIL1;1* is highly expressed in all organs and tissues surveyed, except for pollen [4]. In the maize endosperm, *ZmPDIL1;1* is by far the most highly expressed PDI, followed by *ZmPDIL2;3* [4]. The level of ZmPDIL1;1 is upregulated in the seeds of *floury-2* mutant, which accumulates an abnormally processed α-zein [49]. In *OsERO1*-knockdown mutant seeds of rice (*ero1*), which exhibit the abnormal accumulation of storage proteins and unfolded protein response-related induction of the protein-folding chaperone BiP, OsPDIL1;1

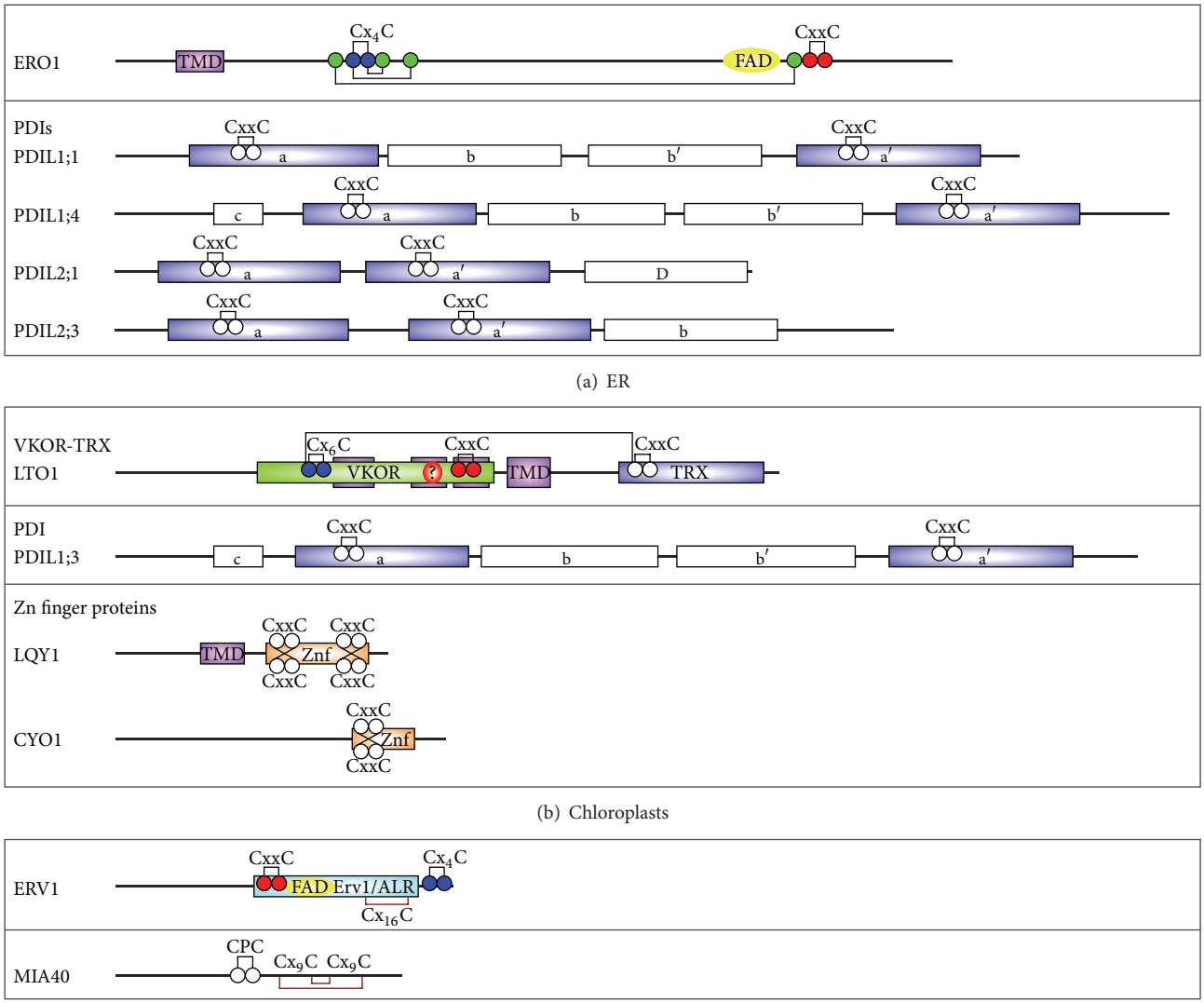

FIGURE 4: Main characterized components of the disulfide bond formation pathway in the ER, chloroplasts, and mitochondria of plant cells. (a) Schematic illustration of ERO1 and PDIs in the plant ER. (b) Schematic illustration of a VKOR homolog (LTO1) and thiol-disulfide oxidoreductases (PDIL1;3, LQY1, and CYO1) in chloroplasts. (c) Schematic illustration of ERV1 and MIA40 in mitochondria. In disulfide-generating enzymes (ERO1, LTO1, and ERV1), the predicted active-site Cys pairs, shuttle Cys pairs, and regulatory Cys pairs are indicated with red, blue, and green circles, respectively. The FAD cofactor is shown in yellow. No cofactor of LTO1 has been identified yet (question mark circled in orange). In disulfide carrier proteins and TRX domain of LTO1, the predicted redox-active Cys pairs (CxxC in TRX domains and Zn finger domains, CPC in MIA40) are indicated with white circles. The predicted structural Cys pairs in MIA40 (Cx_9C) and ERV1 ($Cx_{16}C$) are indicated with brown lines. Redox-active TRX (a and a′ domains; blue boxes), redox-inactive TRX (b and b′; white boxes), α-helical D (white box), VKOR (green box), DnaJ Zn finger (Znf; orange boxes), and Erv1/ALR (cyan box) domains are predicted by NCBI Conserved Domain searches (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). Transmembrane helices (TMD; purple boxes) are predicted by TMHMM v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). Acidic N-terminal c domains are also indicated (white boxes). Amino acid sequence data can be found in the GenBank/EMBL databases under the following accession numbers: OsERO1, OSJNEc05N03; OsPDIL1;1, NP_001067436; AtPDIL1;4, NP_001190581; AtPDIL2;1, NP_973708; OsPDIL2;3, NP_001063331; AtPDIL1;3, NP_191056; AtLTO1, NP_567988; AtLQY1, NP_177698; AtCYO1, NP_566627; AtERV1, NP_564557; AtMIA40, NP_680211.

(a ZmPDIL1;1 ortholog; a-b-b′-a′; Figure 4(a)) accumulates to lower levels than in the wild type, whereas OsPDIL2;3 (a ZmPDIL2;3 ortholog; a-a′-b; Figure 4(a)) accumulates to higher levels than in the wild type [50].

In a recent study, we demonstrated that rice OsPDIL1;1 and OsPDIL2;3, when expressed in the same cell, show distinct subcellular localizations, substrate specificities, and functions [32]. OsPDIL1;1, whose redox activity for oxidative protein folding depends on the redox state of the catalytic active sites (Cys69–Cys72 and Cys414–Cys417), is distributed in the ER lumen of endosperm cells [32, 50] and plays an important role in the formation of disulfide bonds in the

PSV-targeted storage proteins proglutelins and α-globulin [32, 50, 51]. OsPDIL2;3, which does not complement the function of OsPDIL1;1 in the formation of disulfide bonds in vivo, exhibits lower activity for oxidative folding of reduced, denatured α-globulin and RNase than does OsPDIL1;1, whereas OsPDIL2;3 exhibits higher activity for the formation of nonnative intermolecular disulfide bonds between α-globulin Cys79Phe mutant proteins [32]. Interestingly, the redox-active form of OsPDIL2;3 is predominantly targeted to the surface of the ER-derived PB, and this localization of OsPDIL2;3 is highly regulated by the redox state of the redox-active sites (Cys59–Cys62 and Cys195–Cys198) [32]. The knockdown of *OsPDIL2;3* inhibits the accumulation of a Cys-rich 10-kD prolamin (Figure 3) in the core of the ER-derived PB, indicating that OsPDIL2;3 has a specific function in the intermolecular disulfide bond-assisted assembly of polypeptides into the ER-derived PB [32, 52]. Note that BiP is localized on the surface of the ER-derived PB in an ATP-sensitive manner [53]. In cultured human cells, HsP5 forms a noncovalent complex with BiP and BiP client proteins [54, 55]. The high sequence similarity between human HsP5 and rice OsPDIL2;3 suggests that OsPDIL2;3 may also interact with BiP. If so, OsPDIL2;3 and BiP may be involved in recruiting a specific group of proteins to the ER-derived PB. In addition, because the knockout of *OsPDIL1;1* causes aggregation of proglutelins through nonnative intermolecular disulfide bonds and inhibits the development of the ER-derived PB and PSV [50, 51], the distinct but coordinated function of OsPDIL1;1 and OsPDIL2;3 likely supports the development of the ER-derived PB and PSV in rice endosperm.

2.4. Role of PDI in the Regulation of Programmed Cell Death. In vivo and in vitro evidence strongly suggests that members of the PDI family play different physiological roles in different types of plant cells. For example, Arabidopsis AtPDIL1;4 (c-a-b-b′-a′; Figure 4(a)), which restores the wild-type phenotype in the *E. coli* protein-folding mutant *dsbA*, is highly expressed in root tips and developing seeds and interacts with BiP in the ER, thereby establishing that it is involved in oxidative protein folding [56], whereas the ER-localized AtPDIL2;1 (a-a′-D; Figure 4(a)), which can restore the wild-type phenotype in a yeast *PDI1* null mutant, is highly expressed in the micropylar region of the ovule and plays a role in embryo sac development [57].

In contrast, Arabidopsis AtPDIL1;1 (PDI5; a-b-b′-a′; Figure 4(a)) may play a role in the regulation of programmed cell death (PCD), a ubiquitous physiological process that occurs in both prokaryotes and eukaryotes during development and under various stresses [58]. The PCD pathways in plants and animals share some components, including specialized Cys proteases called caspases (Cys-containing Asn-specific proteases) that function as integration points for life-or-death decisions by cells [59, 60]. Vacuoles have been proposed to play a central role in various plant PCD pathways, including developmentally controlled and pathogen-induced PCD pathways; the collapse of vacuoles releases enzymes into the cytosol that attack organelles and DNA, leading to cell death [61, 62]. Vacuole-localized VPE, which was discovered originally as a novel Cys protease responsible for the maturation of seed storage proteins [63], shares enzymatic properties with caspase-1, and both enzymes undergo self-catalytic conversion/activation from their inactive precursors (reviewed in [64]). VPE cleaves caspase-specific substrates and regulates the death of cells during the hypersensitive response and the death of limited cell layers during early embryogenesis [64, 65]. Andeme-Ondzighi et al. have shown that Arabidopsis AtPDIL1;1 acts as a redox-sensitive regulator of the activity of the noncaspase-type, PCD-related Cys proteases RD21 and CP42, which contain a redox-sensitive active site ($_{Gx}C_{GS}C_{W}$) composed of two Cys residues. AtPDIL1;1, which travels from the ER via the Golgi to vacuoles (LVs and PSVs; Table 1), specifically interacts with RD21 and CP42 to prevent their premature activation during embryogenesis and to control the timing of the onset of PCD by protease activation in endothelial cells [66].

2.5. The ERO1 System in the Plant ER. The introduction of disulfides into substrate proteins leads to concomitant reduction of PDIs, the reduced form of which needs to be reoxidized for the next round of the reaction cycle (Figure 1). A key question is how the plant ER establishes a redox environment that favors PDI regeneration and promotes oxidative folding of nascent polypeptides. As described in the introductory section, disulfide bond formation involves the transfer of electrons from a substrate protein to a disulfide carrier protein (e.g., PDI) and then to a de novo disulfide-generating enzyme (Figure 1) [3]. An example of disulfide-generating enzyme is the flavoenzyme Ero1p, which was first identified in yeast by mutation studies of the temperature-sensitive conditional mutant *ero1-1* [67, 68]. The ERO1 family members are highly conserved in eukaryotic organisms, including yeast, humans, and plants. The *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Drosophila melanogaster* genomes each encode only a single-copy *ERO1* gene. Genome duplication is observed in humans: *HsERO1α* and *HsERO1β* [69–71]. In most higher and lower plants, including Arabidopsis [72], maize, and moss (*Physcomitrella patens*), there are two paralogues of *ERO1* [73]. Rice contains a single ortholog of *ERO1* [50], as do *Chlamydomonas reinhardtii* and *Volvox carteri* [73].

ERO1 introduces disulfides in PDI by relaying oxidizing power from molecular oxygen to the reduced form of PDI via the ERO1 flavin cofactor [74–76]. ERO1 proteins possess two conserved catalytic Cys pairs, a shuttle Cys pair and an active-site Cys pair (Cx_4C and CxxC, resp.; Figure 4(a)). The shuttle Cys pair of Ero1p (Cys100–Cys105; yeast Ero1p numbering) directly oxidizes the active site of Pdi1p by means of a thiol-disulfide exchange reaction, and is subsequently reoxidized by the active-site Cys pair (Cys352–Cys355; yeast Ero1p numbering) [77–79]. The active-site Cys pair of Ero1p is reoxidized by transferring their electrons to the flavin cofactor and then to molecular oxygen [74–76]. Plant ERO1 proteins share greater homology with human HsERO1α than with yeast Ero1p [50, 73]. Modeling analysis of Arabidopsis AtERO1 shows that the locations of the conserved Cys

residues in AtERO1 are similar to the locations in HsERO1α (PDB ID code 3AHQ; [80]) [73]. Rice OsERO1 possesses two pairs of Cys residues, Cys134–Cys139 and Cys391–Cys394, which correspond to Cys94-Cys99 (shuttle Cys pair) and Cys394–Cys397 (active-site Cys pair) of human HsERO1α, respectively (Figure 4(a)) [50].

Yeast Ero1p is tightly associated with the luminal face of the ER membrane via the 127-aa C-terminal tail [67, 81]. Human HsERO1α, which lacks the C-terminal domain found in yeast Ero1p, contains a 23-aa signal peptide and is retained in the ER by covalent interactions with HsPDI and HsERp44 [70, 81, 82]. Although the subcellular localization of Arabidopsis AtERO1 proteins has not been characterized, AtERO1 proteins have been shown to be glycosylated [72]. Unlike ERO1 proteins from yeast and human, rice OsERO1 is localized to the ER membrane, and this localization depends on the N-terminal region (Met1-Ser55), which contains an Ala/Pro-rich sequence (Met1-Trp36) and a putative transmembrane domain (TMD; Ala37-Ser55; Figure 4(a)) [50].

We studied the functions of rice OsERO1 by means of RNAi-induced knockdown of *OsERO1* under the control of an endosperm-specific promoter. The *OsERO1*-knockdown seeds, which produce OsERO1 at dramatically reduced levels, fail to develop the typical PSV and ER-derived PB, and abnormal aggregates form instead [50]. Knockdown of *OsERO1* inhibits the formation of native disulfide bonds in proglutelins and promotes the formation of proglutelin-containing aggregates through nonnative intermolecular disulfide bonds in the ER, as does knockout of *OsPDIL1;1* [50]. These results indicate that the ERO1-dependent pathway plays an important role in the formation of native disulfide bonds in the ER of rice endosperm cells.

ERO1 proteins possess a feedback system that regulates ERO1 activity in response to fluctuations in the ER redox environment, allowing the cell to maintain the redox conditions in the ER favorable for native disulfide bond formation [83, 84]. Yeast Ero1p contains two noncatalytic, regulatory Cys pairs (Cys90–Cys349 and Cys150–Cys295); reduction of the disulfide bonds between these Cys pairs increases Ero1p activity [83]. Human HsERO1α possesses a different mode of regulation; HsERO1α activity is regulated by an internal disulfide rearrangement in which Cys94 forms a catalytic disulfide bond with Cys99 (shuttle Cys pair Cys94–Cys99) or a regulatory disulfide bond with Cys131 [80, 84]. A similar regulatory system may also exist in plant ERO1 proteins, including Arabidopsis AtERO1 and rice OsERO1, in which the Cys residues corresponding to Cys150–Cys295 of yeast Ero1p are missing but those corresponding to Cys94, Cys99, and Cys131 of human HsERO1α are present, as indicated by sequence similarity analysis [50].

Additionally, the ERO1-mediated disulfide transfer system produces hydrogen peroxide, a reactive oxygen species (ROS), as a by-product (Figure 5(a)) [76]. Overexpression of ERO1 has been shown to cause a significant increase in ROS, whereas a partial reduction of ERO1 activity suppresses ROS accumulation and increases resistance to the lethal effects of ER stress [85, 86]. Although hydrogen peroxide and other ROS are cytotoxic, hydrogen peroxide sometimes plays a key role as a signal transduction messenger in eukaryotic cells [87]. High concentrations of hydrogen peroxide may cause peroxidation of membrane lipids and gradually destroy the membrane integrity in plant cells, leading to the leakage of small molecules, including water [88]. The production of hydrogen peroxide in the ER of rice endosperm cells correlates with the oxidation of sulfhydryl groups in seed storage proteins [50]. The homozygous mutant seeds of EM49, which have fewer sulfhydryl groups and generate less hydrogen peroxide than wild-type seeds, contain a higher proportion of water than wild-type seeds during seed development and show delayed seed desiccation and maturation [50]. Unlike the adjacent 2n embryo, the 3n endosperm cells, which synthesize vast amounts of disulfide-rich proteins during early seed development, are destined for PCD during seed desiccation and maturation [89]. The hydrogen peroxide generated during the formation of disulfide bonds in the ER of endosperm cells may serve as a signal for inducing PCD and subsequent seed desiccation and maturation.

Another key question is how ERO1 recognizes and oxidizes specific PDI proteins in the plant ER. Yeast Ero1p preferentially oxidizes the CxxC motif in the N-terminal TRX domain of Pdi1p [90]. In mammalian cells, specific combinations of ERO1 and PDI members drive the oxidative folding pathways. For example, human HsERO1α shows different affinities for human HsPDI and HsERp57 [82, 91, 92]. Crystallographic and biochemical analyses have revealed that electrostatic and hydrophobic interactions between HsERO1α and the PDI b$'$ domain allow for effective oxidation of specific PDI proteins by HsERO1α [80]. Although human HsERp57 shares the a-b-b$'$-a$'$ domain organization of human HsPDI, HsERp57 exhibits a different electrostatic potential on the b-b$'$ domain and lacks the hydrophobic pocket that is essential for substrate binding in the b$'$ domain [80, 93]. It remains unclear which members of the rice PDI family are specific partners of OsERO1 in the ER (Figure 5(a)). However, given that OsPDIL1;1 and OsPDIL2;3 differ in domain organization (a-b-b$'$-a$'$ and a-a$'$-b, resp.) and that sequence similarity between OsPDIL1;1 and OsPDIL1;4 is higher in domains a and a$'$ than in domains b and b$'$, it is plausible that OsERO1 may show different affinities for these PDI proteins.

2.6. Alternative Pathways for Disulfide Bond Formation in the Plant ER. The yeast *ERO1* gene is essential for viability [67, 68]. In human cells, HsERO1α is expressed in most cell types, whereas HsERO1β is expressed only in selected tissues [69–71]. Interestingly, disruption of HsERO1α and HsERO1β, which have selective and nonredundant functions in oxidative protein folding, causes only a modest delay in IgM production and is not lethal, suggesting that there are ERO1-independent pathways for disulfide bond formation in mammalian cells [94]. In fact, a candidate enzyme for a parallel ERO1-independent pathway has recently been uncovered; ER-localized peroxiredoxin (PRX) IV uses hydrogen peroxide to convert thiols into disulfides in the ER and transfers these disulfides to PDI, which in turn introduces disulfide bonds into nascent polypeptides [95]. In addition, the human glutathione peroxidase family members GPx7 and

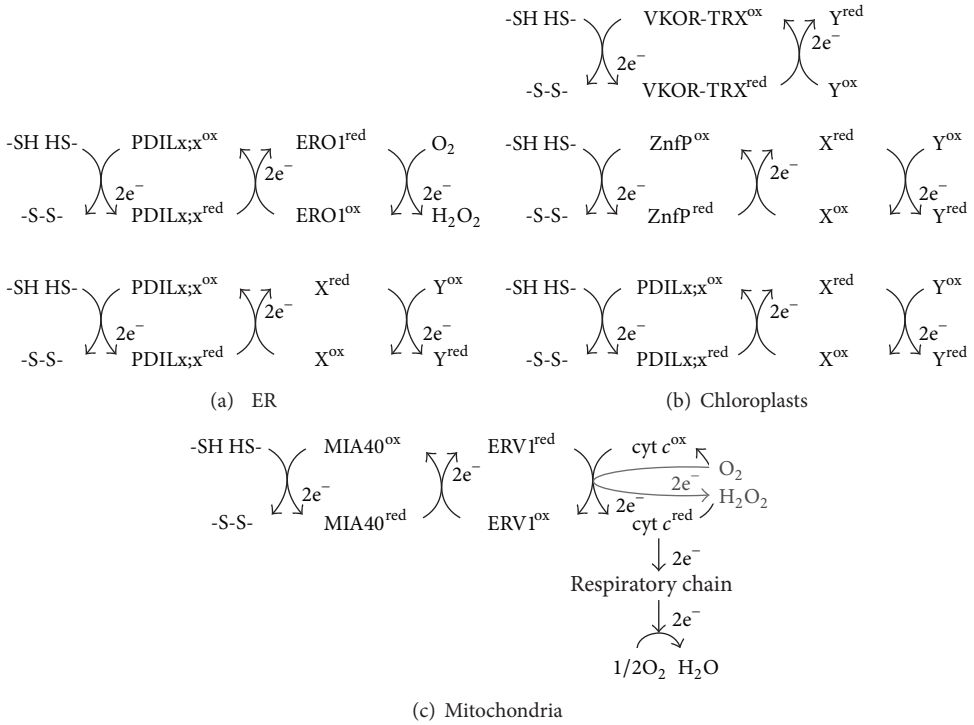

Figure 5: Multiple electron transfer pathways for oxidative protein folding in the ER, chloroplasts, and mitochondria of plant cells. (a) ERO1-dependent and PDI-dependent pathways in the ER. (b) VKOR-TRX-dependent, Zn finger protein-dependent, and PDI-dependent pathways in chloroplasts. LTO1 is indicated as VKOR-TRX. LQY1 and CYO1 are indicated as ZnfP. (c) ERV1-MIA40-meditaed pathway in mitochondria. One-electron carrier cytochrome *c* is indicated as cyt *c*. X^{ox} and Y^{ox} represent unidentified de novo disulfide-generating enzymes and electron acceptors, respectively. Whether de novo disulfide-generating enzymes (X^{ox}) are involved in the LQY1-dependent and CYO1-dependent disulfide transfer pathways is not clear.

GPx8 are involved in the pathway that couples hydrogen peroxide production to disulfide bond formation; GPx7 and GPx8 are localized in the ER and preferentially oxidize PDI in the presence of hydrogen peroxide, enabling disulfide bond formation in a reduced unfolded protein [96].

Native and nonnative disulfide bonds form in *OsERO1*-knockdown mutant seeds of rice, suggesting that unidentified de novo disulfide-generating enzymes are also involved in the oxidative protein folding in the ER of endosperm cells (Figure 5(a)) [50]. In plants, PRXs play central roles in the antioxidant defense system, including thiol-based reduction of hydrogen peroxide, and in the dithiol-disulfide redox regulatory network [97]. The members of the plant PRX family are localized in chloroplasts, mitochondria, the cytosol, and the nucleus, but no ER-localized PRX has yet been identified in plants [97–99]. Among the putative TRX-dependent peroxidases of plants, glutathione-dependent peroxidase Gpx5 has been predicted to show dual localization to the ER and cytosol [98].

3. Disulfide Bond Formation in Chloroplasts

3.1. Role of PDI in Chloroplasts. Because it is derived from a cyanobacterial ancestor, the chloroplast combines prokaryotic and eukaryotic features of gene expression. Thousands of chloroplast proteins, including photosynthesis-related proteins, are encoded by nuclear genes and are synthesized in the cytoplasm. Cytoplasmically synthesized chloroplastic precursors must be unfolded for translocation across the chloroplastic double envelope membranes [100]. Most of the unfolding takes place within the membranes themselves, and proteins are then refolded before localizing to their target destinations. In addition to the nuclear-encoded chloroplast proteins, there are also approximately 100 chloroplast proteins encoded by the organelle's own genome [101]. The transcription of chloroplast genes is driven by plastid transcription systems, nuclear-encoded and plastid-encoded plastid RNA polymerases [102].

Oxygen-evolving photosynthetic organisms are subject to irreversible light-induced damage. Photodamage occurs primarily in PSII in the thylakoid membranes and leads to inactivation of photosynthetic electron transport and subsequent oxidative damage of the reaction center proteins [103]. To maintain PSII activity, the damaged D1 and D2 proteins in the PSII reaction center are degraded and replaced, and expression of plastid-encoded *psbA* and *psbD* (which encode D1 and D2, resp.) is rapidly activated at the level of transcription in response to light (reviewed in [102, 104]). In chloroplasts, reducing equivalents are generated from light by PSII and PSI and are transferred to the ferredoxin-TRX system, which in turn reduces regulatory redox-active sites of target proteins, including key proteins involved in carbon

dioxide assimilation and ATP synthesis [13, 105]. It has been suggested that a reductive signal transduced by TRX activates a 5′ protein complex with high affinity for the 5′ UTR of *psbA* mRNA [14]. The genomes of the green algae *Chlamydomonas reinhardtii* and *Volvox carteri* contain 8 and 7 genes encoding PDI proteins, respectively [5]. *Chlamydomonas reinhardtii* RB60, which contains two CGHC motifs and the C-terminal KDEL signal, functions as a redox-responsive translational regulator in chloroplasts [106, 107]. The reduction-oxidation of RB60, located in a complex bound to the 5′ UTR of *psbA* mRNA, plays a key role in determining the rate of *psbA* translation [106, 108].

Arabidopsis AtPDIL1;3 (Figure 4(b)), which shows high sequence similarity to RB60 and contains the KDEL signal but not a cleavable chloroplast-targeting signal, is localized to the stromal-starch interface in chloroplasts, in addition to the ER [109–111]. The expression of *AtPDIL1;3* is upregulated by light during chloroplast development, and this upregulation is correlated with starch synthesis [109, 112]. AtPDIL1;3 likely plays an important role in protein folding or in enzyme regulation of proteins associated with starch metabolism in chloroplasts [109].

3.2. VKOR Homolog-Dependent Disulfide Transfer Pathway in Chloroplasts: Roles in Assembly of the Photosystem Complexes. Although plants are subject to irreversible photodamage, as described above, they have evolved a highly specialized repair mechanism that restores the functional status of PSII. PSII repair requires disassembly and reassembly of a thylakoid membrane protein complex composed of dozens of proteins (including the reaction center proteins and oxygen-evolving proteins of PSII) and hundreds of cofactors and the breakage and reformation of disulfide bonds among PSII proteins [15, 113, 114]. Recently, a disulfide-generating VKOR homolog has been demonstrated to play important roles in assembly of the photosystem complexes in chloroplasts.

In mammals, VKOR catalyzes a step in the vitamin K cycle, which occurs in the ER membrane and is required for sustaining blood coagulation. Human HsVKOR, an integral membrane protein of the ER, consists of four TMDs with the same membrane topology as the core of a bacterial homolog of VKOR from *Synechococcus* sp. [115, 116]. The physiological redox partner of HsVKOR is a membrane-anchored member of the PDI family, with which HsERO1α interacts only poorly [116]. VKOR is a member of a large family of homologs that are present in various organisms, including vertebrates, plants, bacteria, and archaea [117]. All homologs contain an active-site CxxC motif and an additional pair of loop Cys residues [115, 117]. In some homologs from plants and bacteria, including Arabidopsis, cyanobacteria *Synechococcus* sp., and *Synechocystis* 6803, the domain homologous to HsVKOR is fused with the TRX-like domain typical of the PDI family (Figure 4(b)) [115, 117–119]. A crystal structure of the *Synechococcus* VKOR has revealed that electrons are transferred from a substrate protein to the reduced Cys pair (Cys209–Cys212) of the soluble C-terminal TRX-like domain and then to the two conserved loop Cys residues (Cys50 and Cys56) of the VKOR domain. Subsequently, electrons are transferred from the loop Cys pair (Cys50–Cys56) to the active-site CxxC pair (Cys130–Cys133) [115]. Like *E. coli* DsbB, VKOR accepts the oxidizing power from quinone. The C-terminal Cys residue (Cys133) in the active-site Cys pair is located on the side of the ring of quinone [115].

Arabidopsis VKOR homolog (designated LTO1) is a thylakoid membrane-localized protein that is composed of one VKOR domain, one TRX domain, and four TMDs (Figure 4(b)), as found in the *Synechococcus* VKOR, and catalyzes the formation of disulfide bonds (Figure 5(b)) [119–121]. LTO1 is required for the assembly of PSII by means of the formation of a disulfide bond in PsbO, a luminal subunit of the PSII oxygen-evolving complex [120]. Additionally, LTO1 may regulate the redox state of Cys-containing proteins residing in and facing the thylakoid lumen. In vitro enzymatic assays indicate that LTO1 is active in reducing phylloquinone, a structural cofactor tightly bound to PSI [122], to phylloquinon, but does not reduce either phylloquinone epoxide or plastoquinone [119]. Whether phylloquinone is involved in the LTO1-mediated thiol-oxidation pathway in chloroplasts is not clear.

3.3. Zinc Finger Protein-Dependent Disulfide Transfer Pathways in Chloroplasts: Roles in Photosystem Repair and Chloroplast Biogenesis. Other chloroplast proteins for which disulfide bond-forming activity has been reported are zinc finger-like proteins LQY1 [123] and CYO1 [124] (Figures 4(b) and 5(b)). The LQY1 protein, which is localized in the thylakoid membranes and binds to the PSII core monomer, is suggested to be involved in maintaining PSII activity and regulating the repair and reassembly of PSII under high-light conditions [123]. Arabidopsis mutants that lack the LQY1 protein show elevated accumulation of ROS and reduced levels of PSII-light-harvesting complex II supercomplex under high-light conditions [123]. Proteins homologous to Arabidopsis CYO1 are widely found in plants, including rice, maize, and soybean, but not in moss and algae [124]. The levels of the *CYO1* transcript and the CYO1 protein increase in response to light, and the CYO1 protein is localized to the thylakoid membranes in Arabidopsis [124]. The *cyo1* mutation does not affect the biogenesis of etioplasts under dark conditions but severely impairs the development of thylakoid membranes under light conditions, indicating that CYO1 plays an important role in chloroplast biogenesis [124]. Taken together with the evidence that LQY1 and CYO1 each contain a zinc finger motif (Figure 4(b)) similar to that in *E. coli* DnaJ, bind Zn^{2+}, and exhibit PDI-like activity [123–125], the above data suggest that the PDI-like activity (including disulfide isomerization activity and chaperone activity) of LQY1 and CYO1 may be required for the repair of PSII and thylakoid biogenesis, respectively.

4. Disulfide Bond Formation in Plant Mitochondria

In mitochondria, which are also surrounded by a double membrane, the disulfide transfer pathway for oxidative protein folding is linked to the redox-regulated import

of precursor proteins. Mitochondria play critical roles in numerous cellular processes, including energy metabolism and PCD. Mitochondria are derived from bacterial endosymbionts, and their genomes possess limited coding capacity (approximately 40–50 genes) [126]; thus, they largely depend on the energy-dependent import of nuclear-encoded proteins that are synthesized in the cytosol. The translocase of the outer membrane, designated the TOM complex, is the main entry gate for importing mitochondrial preproteins. In yeast, N-terminal presequence-carrying and hydrophobic precursor proteins are initially recognized by Tom20 and Tom70, respectively; subsequently transferred to the central receptor Tom22; and then inserted into the channel of the central component Tom40 [127]. Among the four subsequent sorting pathways leading to intramitochondrial destinations, the mitochondrial intermembrane space (IMS) import and assembly machinery (MIA) directs small, Cys-rich precursors into the IMS [127]. The MIA pathway is a redox-driven import pathway that requires the cooperative function of the essential IMS proteins. Yeast Mia40 binds to the precursors of IMS proteins with either twin Cx_3C or Cx_9C motifs, including members of the inner mitochondrial membrane chaperone complex (TIM) Tim8–Tim13. In yeast, a ternary complex composed of Mia40 (disulfide carrier protein), the FAD-bound sulfhydryl oxidase Erv1 (disulfide-generating enzyme), and the substrate facilitates the relay of disulfides from Erv1 to the precursor protein via Mia40; and the reduced form of Mia40 is reoxidized by Erv1, which transfers electrons via cytochrome *c* (one-electron carrier) to the respiratory chain and then to molecular oxygen or can use molecular oxygen directly, resulting in the generation of hydrogen peroxide (Figure 5(c)) [127–130].

Plant orthologs of yeast Mia40 and Erv1 have been found: Arabidopsis AtMIA40 and AtERV1 each contain a highly conserved redox-active Cys pair (CPC motif in AtMIA40, CxxC in AtERV1; Figure 4(c)) and structural Cys pairs (two Cx_9C in AtMIA40, $Cx_{16}C$ in AtERV1; Figure 4(c)) and localize to mitochondria [131, 132]. Arabidopsis AtERV1 also contains noncovalently bound FAD (Figure 4(c)) and shows sulfhydryl oxidase activity [132]. Conservation of Mia40 and Erv1 orthologs and the presence of MIA import pathway substrates in yeast, animals, and plants suggest the common use of the MIA pathway [133]. Both Mia40 and Erv1 are essential in *Saccharomyces cerevisiae* [134, 135], and deletion of Arabidopsis AtERV1 causes embryonic lethality [131]. However, deletion of AtMIA40 in Arabidopsis causes no clear phenotype [131]. Additionally, unlike yeast Mia40, Arabidopsis AtMIA40, which is localized to both mitochondria and peroxisomes, does not seem to play an essential role in the import and/or assembly of small Tim proteins in mitochondria. Instead, the loss of AtMIA40 leads to the absence from mitochondria of two IMS-localized proteins, copper/zinc superoxide dismutase (CSD1; which has a dissimilar Cys pair from the Cys motif found in Mia40 substrates) and the chaperone that inserts copper into CSD1, although both are still successfully imported into mitochondria [131]. Loss of AtMIA40 also results in a decrease in the capacity of complex I and the efficiency of the assembly of subunits into complex I [131]. Arabidopsis AtERV1, which is unable to substitute for the yeast enzyme in vivo, contains both the common features of the Erv1/ALR protein family and unique features. AtERV1 lacks CxxC (shuttle Cys pair) in the N-terminal domain found in yeast Erv1 and human ALR, but contains a C-terminal Cx_4C motif conserved among plant ERV1 homologs, which is likely to function in the interdisulfide relay [132]. MIA40 and ERV1 may thus play both conserved and distinct roles in plants.

5. Disulfide Bond Formation Outside the Cell

Quiescin sulfhydryl oxidase (QSOX) is an atypical disulfide catalyst, localized at various subcellular locations or outside the cell. Mammalian QSOX proteins are localized to the Golgi in the post-ER secretory pathways or secreted from cells [136–138], and Arabidopsis AtQSOX1, when expressed in the leaf epidermis, is found in the cell wall rather than the plasma membrane (Table 1) [139]. QSOX, in which the Erv domain has been linked with a redox-active TRX domain during evolution, catalyzes both de novo disulfide generation and disulfide transfer. Generation and transfer of disulfide bonds by QSOX are mediated by two redox-active Cys pairs, one in an Erv domain and the other in a TRX-like domain. Intramolecular disulfides are catalytically generated at the noncovalently bound FAD-proximal Cys pair in the Erv domain of QSOX and are in turn transferred to the Cys pair in the TRX domain by means of interdomain electron transfer, which is followed by intermolecular thiol-disulfide exchange between QSOX and a substrate [140–142]. AtQSOX1 contains a CxxC motif in the TRX domain, and this motif exhibits sulfhydryl oxidase activity on the small-molecule substrate dithiothreitol but not electron transfer activity from the TRX domain to the Erv domain [143]. The expression of *AtQSOX1* in leaves is upregulated by K^+ starvation, and AtQSOX1 plays a role in regulating cation homeostasis by activating root systems that load K^+ into the xylem [139]. This K^+ efflux system may be regulated by AtQSOX1-mediated oxidation of sulfhydryl groups of the transporter at the external side of the plasma membrane [139].

In conclusion, plant cells have evolved multiple and distinct oxidative protein-folding systems in the ER, chloroplasts, and mitochondria. These systems, which require the cooperative functions of disulfide-generating enzymes and disulfide carrier proteins, support cell function and development and protect against environmental fluctuations. Individual disulfide carrier proteins (e.g., PDIs) recognize specific sets of substrate proteins. Current interest lies in uncovering the broad and specific network for oxidative protein folding in plant cells. Further study is needed to define the functions of known disulfide-generating enzymes that supply oxidizing power to specific sets of disulfide carrier proteins (e.g., PDIs), to discover additional such enzymes, and to identify the final electron acceptors in the oxidative protein-folding systems in plant cells.

Abbreviations

ER: Endoplasmic reticulum
ERO1: ER oxidoreductase 1
PB: Protein body

PCD: Programmed cell death
PDI: Protein disulfide isomerase
PRX: Peroxiredoxin
PSV: Protein storage vacuole
QSOX: Quiescin sulfhydryl oxidase
ROS: Reactive oxygen species
TMD: Transmembrane domain
TRX: Thioredoxin
VKOR: Vitamin K epoxide reductase
VPE: Vacuolar processing enzyme.

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Research Article

Quantifying Changes in the Cellular Thiol-Disulfide Status during Differentiation of B Cells into Antibody-Secreting Plasma Cells

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Plasma cells produce and secrete massive amounts of disulfide-containing antibodies. To accommodate this load on the secretory machinery, the differentiation of resting B cells into antibody-secreting plasma cells is accompanied by a preferential expansion of the secretory compartments of the cells and by an up-regulation of enzymes involved in redox regulation and protein folding. We have quantified the absolute levels of protein thiols, protein disulfides, and glutathionylated proteins in whole cells. The results show that while the global thiol-disulfide state is affected to some extent by the differentiation, steady-state levels of glutathionylated protein thiols are less than 0.3% of the total protein cysteines, even in fully differentiated cells, and the overall protein redox state is not affected until late in differentiation, when large-scale IgM production is ongoing. A general expansion of the ER does not affect global protein redox status until an extensive production of cargo proteins has started.

1. Introduction

The cellular thiol-disulfide redox environment is defined by protein thiols (PSH) and disulfides (PS_{ox}) as well as low molecular weight thiols and disulfides. In mammalian cells, by far the most abundant low molecular weight sulfhydryl molecule is glutathione (GSH). Together with its disulfide (GSSG), this pair is often referred to as the cellular thiol-disulfide redox buffer.

In the cytosol of eukaryotic cells, glutathione is highly reducing with a ratio of GSH to GSSG of at least 3,000 [1, 2], and consequently the majority of protein cysteines are found as PSH. The high concentrations of PSH and GSH in this compartment are important in the cellular defense against thiol oxidants [3], during thiol-disulfide stress, formation of mixed disulfides between protein and glutathione (PSSG) serves as a mechanism for protecting PSH and GSH from irreversible oxidation. In contrast to cytosolic proteins, secretory proteins often contain disulfide bonds, and the glutathione redox pool in the secretory compartments of the cell is found to be considerably more oxidizing than the cytosolic pool [4]. Disulfide bond formation is an essential step for the correct folding of many secretory proteins [5], and in eukaryotic cells their folding and assembly takes place in the endoplasmic reticulum (ER). In this compartment, molecular chaperones and enzymes for disulfide bond formation and glycosylation support protein folding. The maintenance of a proper ER redox environment is crucial for the folding of secretory proteins. If the redox environment becomes too reducing, the formation of disulfide bonds is hampered [5]. If too oxidizing, folding intermediates with nonnative disulfide bonds can accumulate [6]. A number of oxidoreductases, which may have different functions and/or substrate or tissue specificities in the assistance of folding secretory proteins, are found in the ER of mammalian cells [7]. The best characterized oxidoreductase is protein disulfide isomerase (PDI), which introduces, reduces, and reorganizes disulfide bonds in a broad variety of substrate proteins [8]. The oxidative pathway

remains unresolved, but PDI may be reoxidized by a number of enzymes including PDI peroxidases, GPx7 and GPx8 [9], peroxiredoxin 4, and the flavoprotein Ero1 (endoplasmic reticulum oxidoreductin 1), for review see [10, 11].

Professional secretory cells are specialized in producing secretory proteins and are characterized by their abundant ER. One example is the terminally differentiated B cell, also referred to as plasma cell, which secretes enormous amounts of antibodies, that is, immunoglobulins (Ig). While resting B cells do not secrete antibody, they do express a membrane-bound Ig on their cell surface as a subunit of the B cell receptor, which upon binding of antigen activates a signaling cascade that can lead to differentiation into antibody-secreting plasma cells. The differentiation is accompanied by many morphological changes to accommodate production of large amounts of secreted antibody. This includes a general increase in cell volume with a preferential expansion of the ER [12]. In addition, the differentiation is accompanied by dramatic changes in the proteome of the cell [13, 14]; as expected, the ER proteins are significantly up-regulated.

IgM is the first antibody produced in the adaptive immune response. IgM is typically secreted as disulfide-linked pentamers or hexamers of a subassembly consisting of two identical heavy chains (μ) and two light chains (λ). The pentameric holoprotein in addition contains a J-chain, which the hexamer does not. As each subassembly contains 16 disulfide bonds and the J-chain contributes 4 disulfide bonds, almost 100 disulfide bonds need to be formed for each secreted IgM [15]. This oxidative folding may generate reactive oxygen species (ROS). ROS production is increased during B cell differentiation and counterbalanced by a strong antioxidant response [16].

We set out to investigate how this enormous load on the secretory machinery affects the global thiol-disulfide environment of the B cell. We have applied a previously developed method for quantitative determination of the absolute levels of PSH, PS_{ox}, and PSSG on all cellular proteins (including membrane proteins) in cultured mammalian cells, and combined these data with quantifications of GSH and GSSG in the same cells. In this way, we have obtained a picture of the global changes in cellular thiol-disulfide redox status during differentiation of the resting B cell into an antibody-secreting plasma cell.

2. Results

2.1. Strategy for Global Quantification of the Thiol-Disulfide Environment. Quantitative studies of the cellular redox status involve a variety of technical challenges due to the reactive nature of the SH group. Great care must be taken to avoid artificial air oxidation and to eliminate cross-reactivity between the thiol and disulfide specific reagents, which can otherwise lead to deceptive conclusions [17]. By applying a previously developed technology that carefully considers these technical pitfalls [3], we can quantitatively determine the cellular levels of total sulfhydryl equivalents in low molecular mass thiols and in protein. The key features of the experimental approach are illustrated in Figure 1. To avoid perturbation of the cellular thiol-disulfide status during cell lysis and sample preparation, cells were acidified by the addition of TCA to a final concentration of 10%, resulting in immediate protein denaturation and precipitation. This combination of rapid trapping and deprotonation simultaneously unfolds redox enzymes, some of which have low thiol pK_a and are fairly acid-stable, and quenches generic thiols by protonation. To fully exploit the strength of our approach, we did not FACS-sort cells before analysis, nor did we homogenize and fractionate cells.

The TCA pellets were solubilized by sonicating in appropriate buffers with high concentrations of SDS or urea to quantify the different sulfhydryl species in all cellular proteins including membrane proteins. PSH and PS_{ox} levels were determined with a highly sensitive HPLC assay based on the thiol quantification agent 4-DPS [18]. The total value of protein cysteines (Total PS) was calculated by the addition of PSH and PS_{ox} and to verify the method, this value was also determined experimentally. For experiments performed on resting B cells, there is an excellent agreement between the experimentally determined value and the calculated sum of the experimentally determined PSH and PS_{ox} (data not shown). Finally, PSSG levels were selectively quantified by the use of the thiol derivatization agent SBD-F. The SBD-GS derivative is highly fluorescent and can be quantified specifically due to its unique retention time in an HPLC chromatogram [3]. In addition to protein sulfhydryls, the total protein content of each sample was determined and used as a common denominator to compare the individual samples. This is a crucial step as it eliminates any bias from a possible uneven division of the TCA pellet into fractions. Furthermore, the requirement for a common denominator in this study is particularly important, as it also eliminates any bias due to morphological differences between cell samples. The total protein content was quantified using a method based on complete hydrolysis in HCl followed by quantification of released amino acids with ninhydrin [3]. This constitutes a highly reproducible and sensitive method and, with a proper standard, it yields numbers that can be calibrated to "amino acids in protein." Furthermore, the ninhydrin assay is independent of protein solubility and hence includes both soluble and membrane proteins. Thus, all the following data will be shown as sulfhydryl per amino acid (SH/aa).

2.2. Quantifying the Thiol-Disulfide Environment of the Resting B Cell. As a model for B cell differentiation, we used a previously established system based on the murine B cell lymphoma 1.29μ^+ which can be induced by lipopolysaccharide (LPS) to secrete IgM [13, 19]. To obtain a well-defined reference point for the differentiation of B cells into plasma cells, we quantified the thiol-disulfide status in uninduced B cells to obtain a reference point for differentiation into plasma cells. Cells were seeded to a density of 0.2×10^6 cells/mL, and samples for redox quantification were taken each day during the next four days. Although cell density increased considerably during this period, the protein redox state remained constant throughout the experiment (Figures 2(a) and 2(b)). In addition, levels of PSSG, GSSG, and GSH remained constant (data not shown), and we concluded that

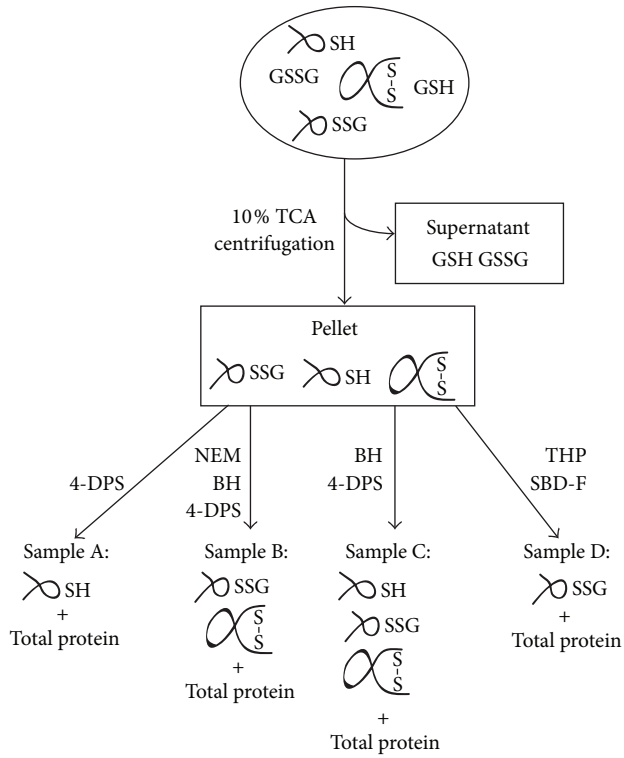

Figure 1: Flow chart of the experimental approach for global quantification of cellular redox species. To avoid contribution of protein disulfides from serum, cells were suspended and washed in phosphate buffered saline prior to addition of trichloroacetic acid (TCA) to 10%. After centrifugation, soluble GSH and GSSG were quantified from the supernatant while protein sulfhydryls were quantified from the pellet. The TCA pellet was divided into four samples (A-D). Sample A was directly incubated with 4,4′-dithiodipyridine (4-DPS) to quantify PSH. To quantify PS_{ox}, free thiols in sample B were first alkylated with N-ethylmaleimide (NEM). Disulfides then were reduced using sodium borohydride (BH) followed by thiol quantification with 4-DPS. The main advantage of this strategy is that thiol alkylation, disulfide reduction, and thiol quantification can be performed in the same test tube as excess NEM is inactivated by BH, whereas excess BH is easily removed by the addition of acid. As a control, Total PS was measured experimentally in sample C by directly reducing disulfides with BH followed by thiol quantification with 4-DPS. Finally, PSSG in sample D was quantified by reduction of disulfides with tris(hydroxypropyl)phosphine (THP) and fluorescent labeling of thiols with 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F). Selective quantification of GS-SBD derivative was performed using HPLC as described [3]. In addition to quantification of redox species, the total protein content in each pellet was quantified and used as a common denominator to compare the individual samples.

the global thiol-disulfide status is independent of cell density. Accordingly, the mean value of data obtained for each of the redox species during the four days was calculated, and the relative distributions of the different protein and glutathione sulfhydryl equivalents are given in Table 1. From these data, we concluded that the vast majority of cellular sulfhydryl equivalents exists in the reduced thiol form with only 5% and 9% of the PS and GS equivalents engaged in disulfide bond formation, respectively. An extremely small fraction of cellular sulfhydryl equivalents is found as PSSG. Together, these data describe the total thiol-disulfide environment of resting B cells, and we used them as reference point for studying the differentiation into antibody-secreting plasma cells. In the remaining part of this study, these data will be referred to as day 0 in the differentiation. Interestingly, the distribution of thiol and disulfide equivalents in resting B cells is very similar to that of HEK (human embryonic kidney) cells, where 6% of the PS equivalents were found as PS_{ox} and 8.5% of the GS equivalents were found as GSSG. In addition, our observation that cellular PSSG levels are extremely low is supported by results in HEK and HeLa cells [3]. It should be mentioned that although cells are grown in the presence of β-mercaptoethanol, which was detected in the TCA supernatant and also in very small amounts in the TCA pellet, it did not interfere with the glutathione or PSSG measurements because fluorescent β-mercaptoethanol derivatives were separated efficiently from GS derivatives by HPLC (data not shown).

2.3. Changes in the Cellular Thiol-Disulfide Status Induced by Differentiation. Resting $1.29\mu^+$ cells were treated with LPS to induce differentiation into antibody-secreting plasma cells. Samples were prepared for global thiol quantification after 1, 2, 3, and 4 days of LPS treatment. From the SH/aa of total PS equivalents (Figure 3(a)), we find that the frequency of cysteine residues in proteins is fairly constant (~2%) throughout the differentiation. This is in excellent agreement with the experimentally determined values for other mammalian cell lines as well as calculated values for eukaryotes in general Hansen [3, 20]. The SH/aa for total PS was expected

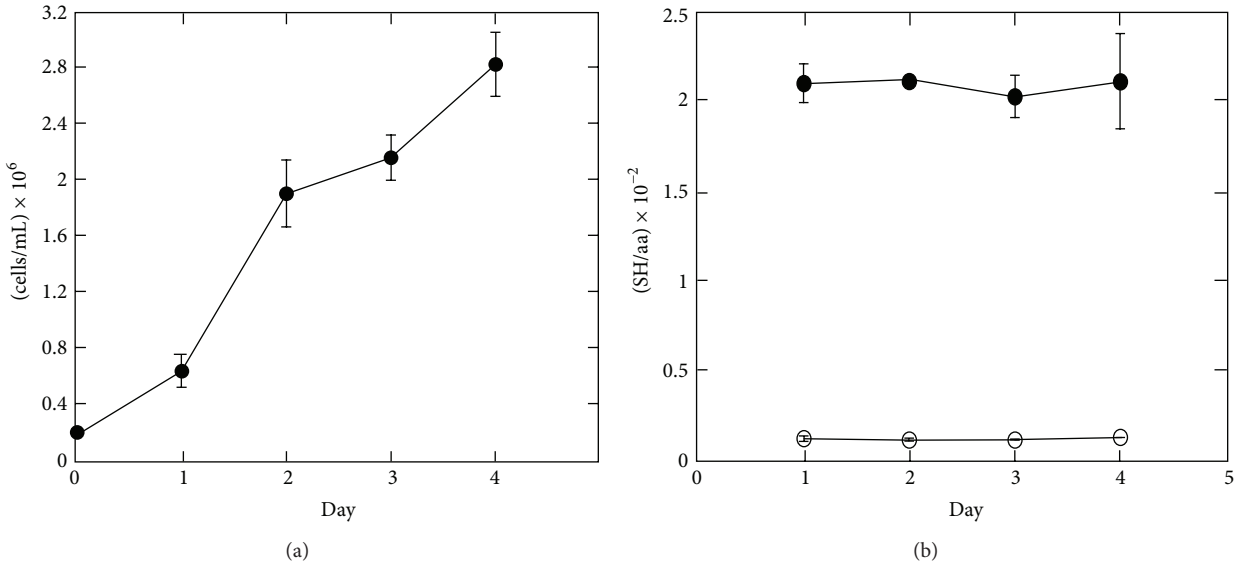

Figure 2: Cell density does not affect protein thiol-disulfide status in resting B cells. Experiments were performed on two independent cultures. (a) Growth curve of 1.29μ^+ lymphoma cells cultured in suspension as described in [13]. Each day a fraction of the culture was harvested for global quantification of the thiol-disulfide status, as illustrated in Figure 1. The graph shows cell densities at the times of sample preparation. (b) Distribution of protein sulfhydryl equivalents in samples harvested on days 1–4. SH/aa values for PSH (•) and PS_{ox} (○) are shown.

Table 1: Relative distribution of protein and glutathione sulfhydryl equivalents in resting B cells.

	Protein[a]	Glutathione[b]
% Thiols	95 ± 4	91 ± 5
% Disulfides[c]	5.1 ± 0.2	8.8 ± 0.5
% PSSG[d]	0.11 ± 0.02	0.15 ± 0.02

Values are given as the means ± SDM.
[a]Percentage of measured SH/aa relative to total protein SH/aa.
[b]Percentage of measured SH/aa relative to total glutathione SH/aa. Total glutathione equivalents are calculated by addition of the SH/aa values of [Total soluble GS], quantified from the TCA supernatant, and [GS in PSSG], quantified from the TCA pellet, according to Figure 1. GSH is calculated by subtracting [GS in GSSG] from [Total soluble GS].
[c]Values are calculated as SH/aa equivalents engaged in disulfide bond formation.
[d]Values are calculated as SH/aa equivalents engaged in PSSG formation.

to be largely unaffected during differentiation as the IgM monomer has a cysteine frequency of 2.5% (IgM has 1,540 amino acids, of which 38 are cysteines [21]). Although the absolute value of total PS equivalents remained unchanged throughout the experiment, the distribution of protein thiols and disulfides was influenced by the LPS-induced cell differentiation. The percentage of protein thiols engaged in disulfide bond formation remained largely unaffected for two days after LPS induction, but at day three the PS_{ox} values had doubled, and a total increase by a factor of 3.3 was found at day four (Figure 3(b)). Likewise, after a lag time of two days, on day three PSSG had increased by a factor of 2.2, but in contrast to PS_{ox}, the PSSG value did not increase further on day four (Figure 3(c)). Interestingly, the ratio of PSSG to PS_{ox} remained essentially unchanged throughout the experiment (Figure 3(d)). Quantification of soluble glutathione equivalents revealed that the absolute concentrations of GSSG remained largely unaffected (Figure 4(a)), but the fraction of oxidized GS equivalents (GS in GSSG) relative to total GS equivalents (total GS) increased from 8.8% to 14.5% (Figure 4(b)). This was caused by a gradual decrease in GSH (Figure 4(a)) resulting in an overall decrease of 56% in (total GS) at day 4 compared to day 0. The GS equivalents were not recovered as PSSG, which throughout the study remained a minute fraction of total GS equivalents (Figure 4(c)). To rule out that the decrease in intracellular GS was caused by an increase in dying cells, the level of apoptotic and necrotic cells was measured using flow cytometry and staining with the cell-impermeable dye propidium iodide (PI). Although the fraction of viable (PI-negative) cells decreased during the differentiation by a factor of 1.5 (Supplementary Material Figure 1 available online at http://dx.doi.org/10.1155/2013/898563), it could not account for our observed decrease in intracellular glutathione (see Supplementary Material text). As the decrease in intracellular GS was not explained by an increase in cell death, we measured whether GS equivalents were secreted by the cells. No glutathione was detectable in a blank medium sample (data not shown); media from uninduced cells, however, contained measurable amounts of glutathione, and the values increased significantly on day three and four after induction (Figure 4(d)). It should be noted that these numbers are only rough estimates and that the values are most likely underestimated due to extracellular GS degradation catalyzed by γ-glutamyl transferase. From the data shown in Figures 4(a) and 4(d), the increase in extracellular GS/aa equivalents at day 4 relative to day 0 was calculated to $(0.23 \pm 0.1) \times 10^{-2}$ while the decrease in intracellular GS/aa was calculated to $(0.46 \pm 0.05) \times 10^{-2}$. As the two numbers are in the same

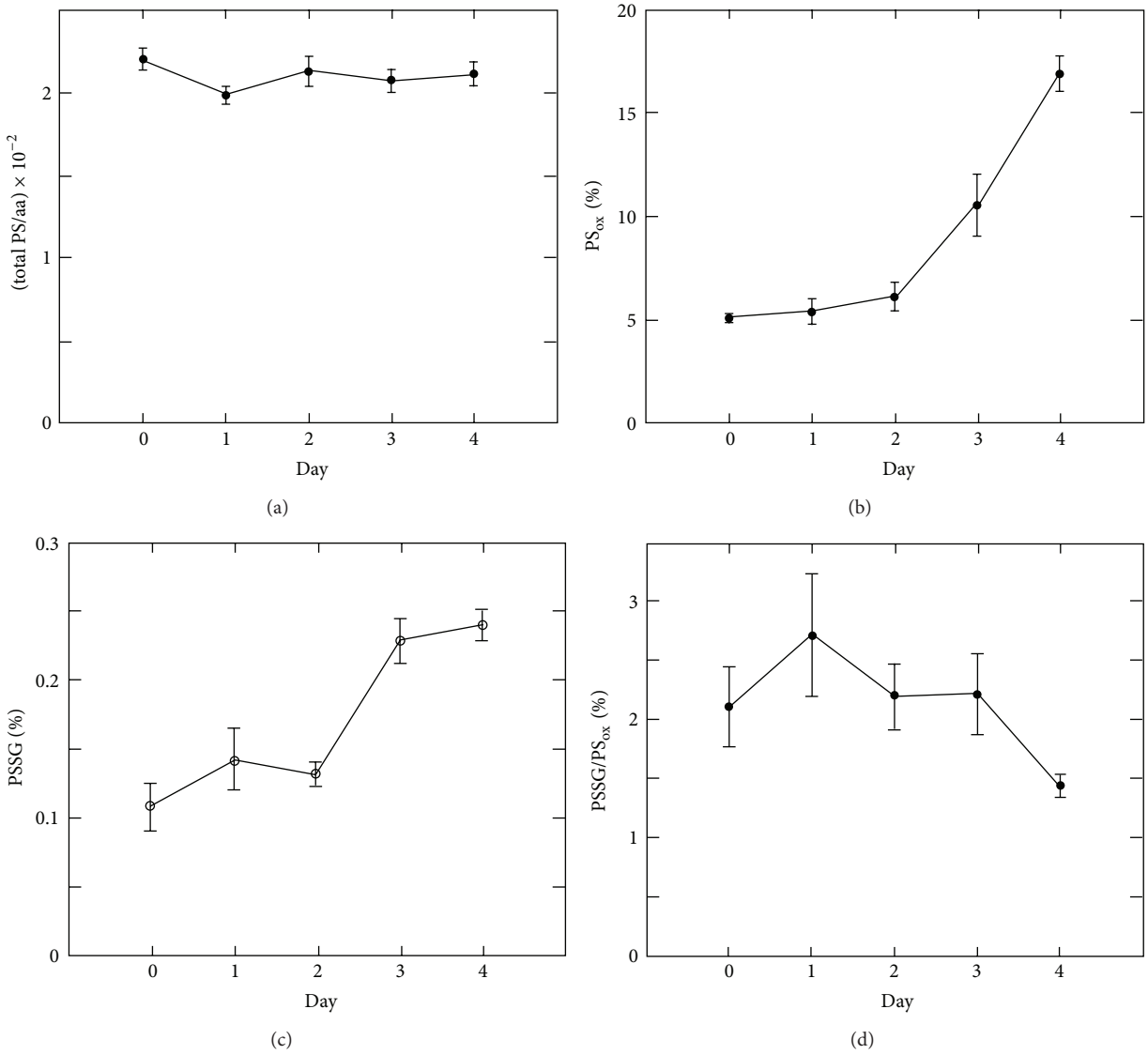

Figure 3: Quantitative changes in protein thiol-disulfide redox state during B cell differentiation. Protein thiol-disulfide status was measured each day during B cell differentiation in three independent cultures induced with LPS, as illustrated in Figure 1. Values are given as mean ± SEM. The values for resting B cells are represented as "Day 0." (a) The total fraction of protein cysteines per amino acid (total PS cal). (b) Percent of PS_{ox} relative to total PS cal. (c) Percent of PSSG relative to total PS cal. (d) PSSG as a percent of PS_{ox}.

size range, it is possible that the differentiating cells secrete glutathione (details are given in Supplementary Material text).

3. Discussion

The transformation of resting B cells into antibody-secreting plasma cells involves an extensive expansion of the ER [12] and both ER resident proteins and proteins involved in redox balance are up-regulated linearly during differentiation [13]. How cells cope with this sudden increase in secretory activity has been the subject of numerous studies [22, 23]. This study, for the first time, provides a quantitative overview of the cellular thiol-disulfide status during differentiation of resting B cells into antibody-secreting plasma cells. We applied a previously developed method to quantify soluble GSH and GSSG as well as protein thiols and disulfides in cells [3]. Importantly, the method includes both soluble and membrane proteins, which precludes bias due to morphological changes during B cell differentiation. We find that the differentiation process affects the global protein thiol-disulfide status with an increase factor of 3.3 in the fraction of oxidized protein thiols at day 4 of differentiation, compared to day 0. The effects on the glutathione redox status are less significant with an increase factor of 1.6 in the fraction of oxidized GS equivalents. The changes in glutathione redox state were caused by a general depletion of GS equivalents from differentiating B cells.

The differentiation of B cells into plasma cells has been studied in great detail at the proteomic level [13, 14]. Full

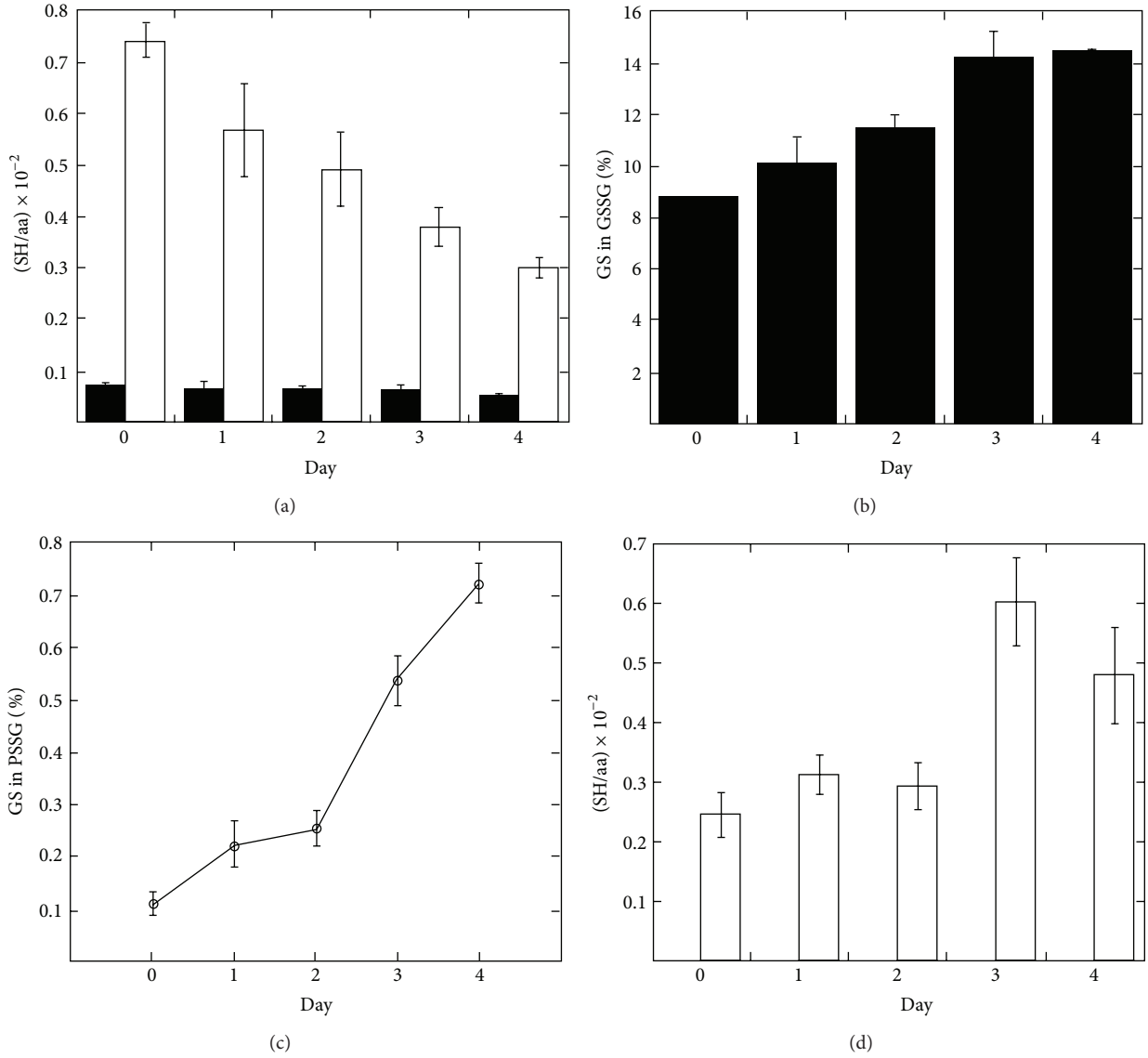

Figure 4: Quantitative changes in glutathione thiol-disulfide redox state during B cell differentiation. Values from experiments performed on three independent cultures are given as mean ± SEM. The values for resting B cells are represented as "Day 0." All data are normalized to the amino acid content of the TCA precipitated cells and data processing is performed as described in the legend of Table 1. (a) SH/aa values of intracellular GSH (white bars) and (GS in GSSG) (black bars). (b) Percent (GS in GSSG) relative to (Total GS). (c) Percent (GS in PSSG) relative to (Total GS). (d) SH/aa values of GS equivalents quantified from the media.

IgM production is not initiated until two days after activation. Until then, the cell prepares by ensuring that metabolic capacity and secretory machinery can cope with the mass production of antibody molecules. We did not find any change in protein thiol-disulfide status until the third day of activation, when the fraction of PS_{ox} increased by a factor of 2.2. The kinetics for the change in protein redox state were identical to the kinetics for IgM production. Our results suggest that a general expansion of the ER does not affect protein redox status until an extensive production of cargo proteins is initiated. The increase of IgM production at day three is possibly preempted by a process known as "proactive" unfolded protein response (proactive UPR) [24]. The UPR is a stress signaling process that is initiated when unfolded polypeptides accumulate in the ER [25]. This process leads to an up-regulation of ER chaperones and folding enzymes which prevents ER stress. While unfolded protein stress (or ER stress) is not involved in the initial expansion of the ER in professional secretory cells [13, 14], it is essential for B cell differentiation [26, 27]. UPR-induced oxidases such as Ero1β may facilitate the change in protein redox state on the third day of differentiation to initiate disulfide-dependent IgM polymerization and its subsequent secretion. The role of glutathione in the ER has been a subject of intense debate. Initially GSSG was thought to provide oxidizing equivalents for disulfide bond formation, but after identification of

the Ero1 proteins this hypothesis was discarded. Instead, GSH now is considered to be involved in the isomerization of nonnative disulfide bonds [28–30] to consume excess oxidizing equivalents produced by the Ero1 proteins [31] and to activate Ero1 by reducing its regulatory disulfides [32]. Consequently, the abundance of GSSG in the ER is altogether assumed to be at least partially caused by Ero1 activity. The mechanisms by which the ER maintains its GSH/GSSG redox balance are unknown. Excess GSSG could be reduced by an ER resident glutathione reductase, it could be transported to the cytosol for reduction or it could be secreted from the cells [28]. During B cell differentiation Ero1α is up-regulated by factors of 3.0 and 2.4 at day 3 and 4, respectively [14], and consequently we expected GSSG levels to increase. Surprisingly, GSSG levels remained constant throughout differentiation, but the overall cellular glutathione redox status did become more oxidizing gradually (Figure 4(a)). This was the result of a depletion of total GS equivalents at the expense of GSH (Figure 4(b)). There are three possible explanations for the decrease in total intracellular GS, (1) differentiation of B cells leads to secretion of GS equivalents, (2) GSH is irreversibly oxidized to sulfinic or sulfonic acids, which is not detected by the quantification method, and (3) GS equivalents are released by the fraction of PI positive cells. We found an increase in extracellular GS on days 3 and 4 of the differentiation that was of about the same magnitude as the intracellular decrease (Figure 4(d)). This result supports model (1) and suggests that GSH converted to GSSG by the up-regulation of Ero1 proteins and is exported to the media. This export could serve as a mechanism for relieving cells from any oxidative load caused by up-regulation of Ero1 proteins. We can, however, not make any certain conclusions regarding the cause of intracellular GS depletion, as the levels of extracellular GS could in principle be explained by the fraction of PI-positive cells releasing their intracellular GS content to the media (see Supplementary Material text).

Due to the extremely reducing glutathione redox potential of the cytosol, the vast majority of PSSG is expected to be found in the oxidizing compartments of the cell [3]. In a study of liver microsomes, 50% of the GS equivalents were found as PSSG [33], suggesting that a major fraction of the glutathione in the ER is associated with protein. However, this fraction was subsequently estimated to be significantly lower (i.e., less than 2% PSSG) based on whole-cell quantification with the assumption that no PSSG is found in the cytosol [3]. Under the same assumption; that is, that all PSSG equivalents are found in the ER and that the concentrations of GS equivalents are the same in all cellular compartments, we can estimate that the maximal fraction of PSSG is relative to total GS equivalents in the ER, in fully differentiated B cells. The ER volume is reported to constitute at least 10% of total cell volume in antibody-secreting B cells [12] and, accordingly, maximally 7% (0.7%/10%) of the GS equivalents in ER are found as PSSG. Thus, even in highly active secretory cells, PSSG only constitutes a minor fraction of total GS equivalents of the ER. These results support the notion that the ER glutathione redox environment is more reducing than previously assumed [34]. It is generally assumed that the level of GSSG is critical for the amounts of PSSG formed [35]. Interestingly, we found that the ratio of PSSG to PS_{ox} was independent of differentiation (Figure 3(d)), suggesting that the oxidizing compartments of the cell maintain a constant level of PSSG, and consequently, that the ER glutathione redox status is tightly regulated throughout differentiation. This may be explained by the activation of oxidative stress during the early stage of B cell differentiation and followed by a strong antioxidant response [16, 36]. Maintenance of a proper ER glutathione redox environment can be a crucial factor in securing the correct folding of IgM. In this study, we have for the first time characterized the changes in thiol-disulfide state during differentiation of B cells. In general, the differentiation does not cause massive thiol-disulfide stress to the cells. The steady state levels of PSSG are maintained at very low levels, even in fully differentiated cells, and the overall protein redox state is not affected until late in differentiation, when large-scale IgM production has started and the ER stress response has been activated.

4. Materials and Methods

4.1. Cell Culture and Activation of B Cells. $1.29\mu^+$ cells were maintained in suspension as described in [13] and after 2 days, cell culture medium was replaced by fresh medium. For differentiation, cells were cultured in the presence of 20 μg/mL LPS (Sigma). Three independent cultures were induced with LPS and samples were taken after 1, 2, 3, or 4 days of differentiation. As a control, samples from 3 independent cultures grown without LPS were collected as well. Each day after LPS activation, samples were collected for flow cytometric analysis. Cells were stained with PI (Sigma) and flow cytometry data were obtained with FACScalibur (BD Biosciences) and analyzed using Cellquest software (BD Biosciences).

4.2. Quantifying Intracellular Redox Species. Cells were harvested by centrifugation followed by a wash step with 10 mL Dulbecco's 1x PBS (PAA laboratories) to eliminate traces of protein from the media. Cells were resuspended in 1 mL ice-cold 10% (w/v) TCA and incubated on ice for 30 minutes followed by centrifugation. The supernatant, used for quantification of GSH and GSSG, was immediately frozen in N_2 and kept at −80°C until later use. The TCA pellet, used for protein thiol quantification, was washed in 10% TCA by four cycles of sonication and centrifugation. Before the final centrifugation step, the suspension was divided in four to quantify PSH, PS_{ox}, PSSG, and total PS. The PSH, PSSG, and total PS samples were immediately frozen in N_2 and kept at −80°C until later use. The PS_{ox} sample was directly solubilized and alkylated by sonicating the pellet in 500 μL of 5% SDS, 1 mM EDTA, 20 mM NEM (Sigma) in 0.5 M Tris-Cl pH 8.3. The alkylation was allowed to proceed for at least 20 min before the sample was transferred to −80°C. Thiol and disulfide species in TCA supernatant and TCA pellet fractions were quantified as described in [3]. Briefly, PSH samples were solubilized in 5% SDS, 1 mM EDTA in 0.4 M sodium citrate, pH 4.5 and protein thiols were quantified by the addition of 4-DPS (Sigma) to a final concentration

of 0.5 mM followed by HPLC analysis. For quantification of PS_{ox}, samples alkylated with NEM were reduced by the addition of BH (Sigma) to a final concentration of 3.3% (w/v). Prior to quantification with 4-DPS, BH was destroyed by the addition of HCl, as described in [18]. Total PS was quantified by solubilizing the pellet in 5% SDS, 1 mM EDTA, and 0.5 M Tris-Cl pH 8.3, reducing all thiols with BH, followed by thiol quantification with 4-DPS. The PSSG sample was solubilized in 6 M urea, 8 mM EDTA, and 200 mM bicine pH 9.2. Disulfides were reduced with 2.1 mM THP (Calbiochem) and thiols were derivatized by the addition of 6.4 mM SBD-F (Fluka) for 1 hour at 60°C. To compare samples, total protein content was determined by using a ninhydrin based assay [3].

4.3. Quantifying Intracellular and Secreted Glutathione. An HPLC assay based on thiol derivatization with N-(1-pyrenyl)maleimides (Fluka) was used to quantify GSSG and total GS (GSH + GSSG) from the supernatant as described in [1]. GSH was quantified by subtracting oxidized glutathione from total glutathione. For quantification of secreted glutathione, proteins in media from harvested cells were precipitated by the addition of TCA to 10% (v/w) and incubated on ice for 30 minutes followed by centrifugation. Total glutathione was quantified from the TCA supernatant as described above.

Abbreviations

GSH: Glutathione
GSSG: Glutathione disulfide
PSH: Protein thiols
PS_{ox}: Protein disulfides
PSSG: Mixed disulfides between protein and glutathione
ER: Endoplasmic reticulum
PDI: Protein disulfide isomerase
Ero1: Endoplasmic reticulum oxidoreductin 1
Ig: Immunoglobulin
TCA: Trichloroacetic acid
SBD-F: 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate
THP: Tris(hydroxypropyl)phosphine
NEM: N-ethylmaleimide
BH: Sodium borohydride
4-DPS: 4,4′-dithiodipyridine
LPS: Lipopolysaccharide
ROS: Reactive oxygen species.

Conflict of Interests

The authors declare that they have no conflicting financial interests.

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