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Tuning of Peroxiredoxin Catalysis for Various Physiological Roles

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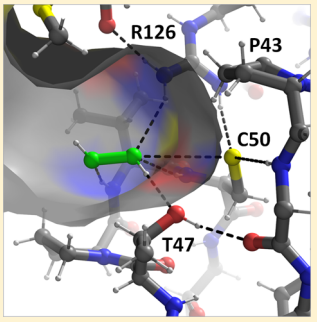
Supporting Information

ABSTRACT: Peroxiredoxins (Prxs) make up an ancient family of enzymes that are the predominant peroxidases for nearly all organisms and play essential roles in reducing hydrogen peroxide, organic hydroperoxides, and peroxynitrite. Even between distantly related organisms, the core protein fold and key catalytic residues related to its cysteine-based catalytic mechanism have been retained. Given that these enzymes appeared early in biology, Prxs have experienced more than 1 billion years of optimization for specific ecological niches. Although their basic enzymatic function remains the same, Prxs have diversified and are involved in roles such as protecting DNA against mutation, defending pathogens against host immune responses, suppressing tumor formation, and—for eukaryotes—helping regulate peroxide signaling via hyperoxidation of their catalytic Cys residues. Here, we review the current understanding of the physiological roles of Prxs by analyzing knockout and knockdown studies from ∼25 different species. We also review what is known about the structural basis for the sensitivity of some eukaryotic Prxs to inactivation by hyperoxidation. In considering the physiological relevance of hyperoxidation, we explore the distribution across species of sulfiredoxin (Srx), the enzyme responsible for rescuing hyperoxidized Prxs. We unexpectedly find that among eukaryotes appearing to have a "sensitive" Prx isoform, some do not contain Srx. Also, as Prxs are suggested to be promising targets for drug design, we discuss the rationale behind recently proposed strategies for their selective inhibition.



INTRODUCTION TO PEROXIREDOXINS AND SCOPE OF THIS REVIEW

Peroxiredoxins (Prxs) are nature's dominant peroxidases. From archaea to humans, they are widely expressed and possess the same catalytic components.[3] Prxs serve to protect cells from oxidative stress and prevent damage to DNA, lipids, and other proteins by reducing hydroperoxides and peroxynitrite.[4] With catalytic rates of $\sim 10^7$ M^{-1} s^{-1} and an abundance that implies that they account for the reduction of more than 90% of cytosolic peroxide, they are crucial for regulating intracellular peroxide levels in most organisms.[5] Cells encounter peroxides in a variety of ways—as a byproduct of cellular processes, as a consequence of environmental conditions, or even as a result of deliberate attacks by other cells[6]—and Prxs have been finely tuned to address the needs of their respective organisms. Given the ubiquity of Prxs, it is presumed that they make up an ancient enzyme family that arose at the time of the great Oxidation Event, some 2.4 billion years ago, to aid cells in coping with increased oxygen levels and to facilitate aerobic metabolism.[7] Because of their retention over the millennia, with no major alterations in the protein fold or catalytic mechanism, Prxs can be seen as being integral to the existence of life on Earth.

We have come to understand that Prxs serve a function much more complex than simply purging cells of a toxic molecule. This is in part due to the discovery that peroxide not only creates oxidative stress and participates in stress-related signaling, such as activating the bacterial transcription regulator OxyR,[8] but also in eukaryotes is an integral part of normal, "non-oxidative-stress-related"[a] cell regulation events.[1] Such non-stress-related peroxide signaling is now known to be an important factor involved in cell proliferation, angiogenesis, senescence, and apoptosis.[6,9,10] Non-oxidative-stress-related peroxide signaling occurs, for instance, as a part of insulin-stimulated activation of NADPH-oxidases (NOXs),[11] or adrenocorticotropic hormone (ACTH)-stimulated activation of a cytochrome P450 that contributes to peroxide buildup (Figure 1). The peroxide bolus produced by such enzymes becomes a chemical signal that leads to changes in protein activities through the reversible oxidation of protein residues, like an active site cysteine of protein tyrosine phosphatases.[4] Other phospho-regulatory enzymes, such as kinases CAMKII, PKA, and PKG, are oxidant-sensing and also can be regulated by hydrogen peroxide (recently reviewed by Burgoyne et al.[11]). Thus, a complex interplay exists between Prxs and transcription factors, phosphatases, kinases, and any cellular molecule capable of being modified by peroxide (Figure 1). The important influence of Prxs in cell homeostasis is supported by the observations that Prxs are overexpressed in some human breast,[12] lung,[13] and thymic[14] cancers, and that when the most abundant Prx isoform is knocked out in mice the animals develop malignant tumors and hemolytic anemia and die prematurely.[15]

There have been a number of recent reviews of Prxs that have highlighted structure−function relations,[1,5] enzymology,[16,17] and their roles in signaling.[18] Here, we seek to

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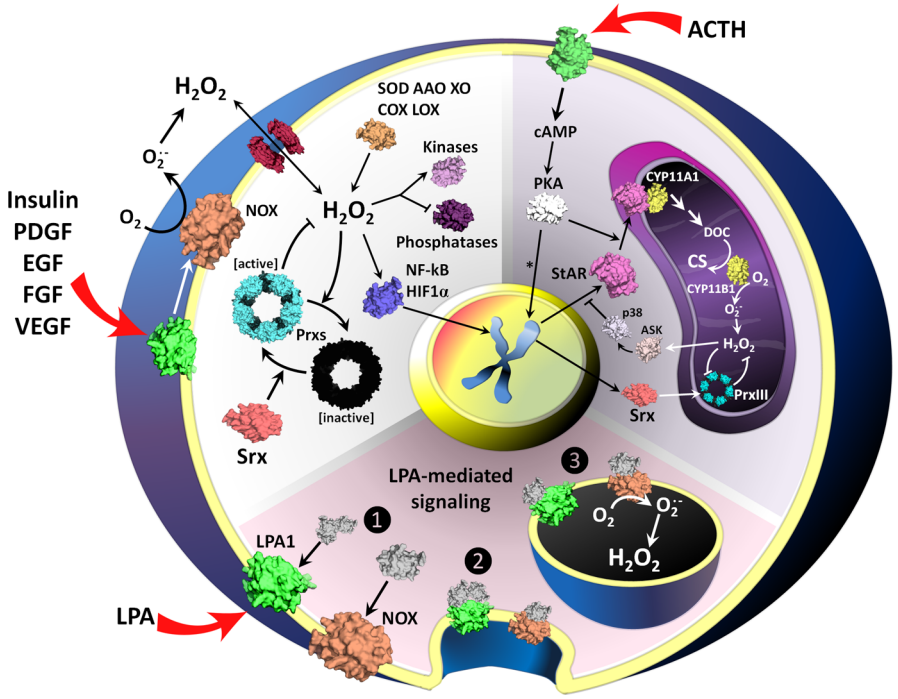

Figure 1. Examples of non-stress-related peroxide signaling. The white panel (left) shows a general scheme of growth factor-triggered peroxide signaling.[6] Binding of growth factor to receptors (green) leads to the activation of oxidases (orange) and the production of superoxide that is subsequently converted to peroxide. Certain aquaporins (dark red) facilitate the entry of peroxide into the cell[137] where kinases (light purple), phosphatases (dark purple), and transcription factors[6,100] (dark blue) can be oxidatively activated or deactivated.[137] Active Prxs (cyan toroid) degrade peroxides but also can be inactivated by hyperoxidation (dark toroid); Srx (light red) reactivates hyperoxidized Prxs. The magenta and purple panels convey other examples of peroxide signaling highlighted in the text. In LPA-mediated signaling[104] (magenta, bottom), binding of LPA to its receptor (green) activates NADPH oxidase (NOX, orange), and through endocytosis, a "redoxosome" is formed. Superoxide/peroxide accumulates in the redoxosome, and it serves as a hub for modifying regulatory factors. In murine adrenal corticosteroid production[108] (purple, top right), binding of ACTH to its receptor (green) leads to the activation of the cAMP-PKA pathway (the transcription factor cAMP response element-binding protein is denoted with an asterisk) and then phosphorylation and activation of steroidogenic acute regulatory protein (StAR); StAR makes cholesterol available for CYP11A1- and CYP11B1-catalyzed conversion via 11-deoxycorticosterone (DOC) to corticosterone (CS) and also produces superoxide from which superoxide dismutase (SOD) produces peroxide. The peroxide increasingly inactivates PrxIII and after further buildup initiates a negative feedback loop by activating p38, which in turn suppresses the synthesis of StAR.

complement these reviews by organizing current knowledge of the physiological roles of Prxs, exploring how evolution has optimized Prx dynamics and thermodynamics to modulate their sensitivity to hyperoxidation, assessing the distribution of its partner enzyme sulfiredoxin (Srx),[19] and describing how drug design might take advantage of the conformational changes that Prxs undergo.[20−22] At present, there are ∼120 Prx structures in the Protein Data Bank (PDB) and more than 15000 annotated Prx genes,[23] so a wealth of data is available.

■ PEROXIDASE FUNCTION OF PRXS

Catalytic Cycle. Prxs have been classified into subgroups on the basis of functional site sequence similarity.[3] These are Prx1, Prx5, Prx6, Tpx, AhpE, and PrxQ [proposed recently[24] to replace the uninformative name of BCP (bacterioferritin comigratory protein) that has been used for some members of this group]. These subgroups have variations in their oligomerization, conformation, and some secondary structure elements, and most organisms possess multiple isoforms[5] (for example, humans contain four Prx1 subtypes, one Prx5 subtype, and one Prx6 subtype, whereas *Escherichia coli* has one Prx1, one Tpx, and one PrxQ). For all Prxs, however, catalysis is facilitated by a peroxidatic Cys (C_P) contained within a universally conserved Pxxx(T/S)xxC active site motif[3] (Figure 2). The active site lowers the C_P side chain pK_a from ∼8.4 to ∼6 or even lower so that it is kept predominantly in a nucleophilic, thiolate state.[25−27]

The conformation of the enzyme that possesses a substrate-ready active site pocket (Figure 2A) is termed "fully folded" (FF). In the catalytic cycle (Figure 2B), the peroxide substrate binds to the FF active site where it is attacked by the nucleophilic C_P in an S_N2-type reaction to form Cys-sulfenic acid (C_P-SOH) and water or alcohol. Subsequently, the active site locally unfolds, an event that for some Prxs involves the rearrangement of as many as ∼35 residues[20] (Figure 2B, center). As discussed later in more detail, a second peroxide can react with C_P-SOH to hyperoxidize the enzyme to a dead-end C_P-SO_2^-. Some organisms, mainly eukaryotes, contain Srx, which converts the hyperoxidized form back to C_P-SOH in an ATP-dependent reaction.[28] For a minority of Prxs, termed "1-Cys" Prxs, the C_P-SOH form is reduced directly by an intracellular reductant such as glutathione or ascorbate.[29] The majority of Prxs, called "2-Cys" Prxs, have a second resolving Cys (C_R) that forms a disulfide bond with C_P.[5] Depending on the Prx, the C_R may be contained within the same chain or, for some oligomeric Prxs, in the chain of another subunit. The formation of the C_P−C_R disulfide requires the active site to locally unfold, i.e., adopting a "locally unfolded" (LU) conformation, that often involves substantial rearrangements of both the C_P and C_R regions (Figure 2B).[5]

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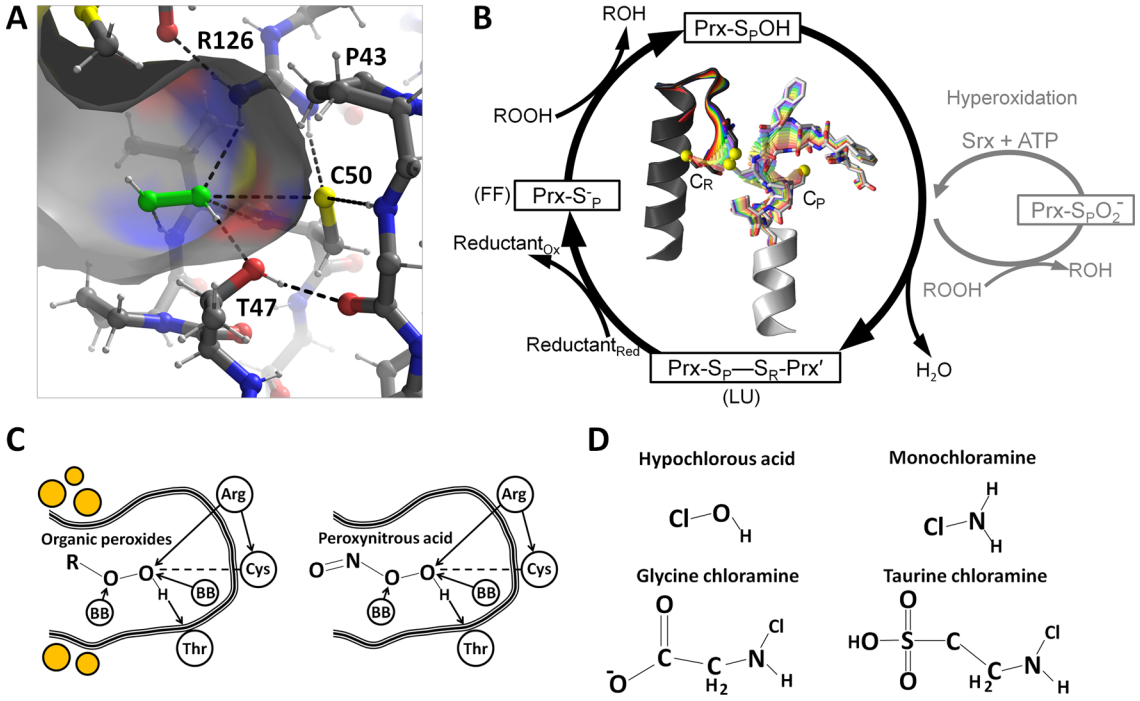

Figure 2. Catalysis by peroxiredoxins. (A) Michaelis complex of peroxide (green) bound to the FF active site of *Ap*Tpx (PDB entry 3a2v) with atom coloring (gray carbons, white hydrogens, yellow sulfurs, red oxygens, and blue nitrogens) showing key hydrogen bonds (dashed lines). (B) The normal Prx catalytic cycle (black) is shown along with the hyperoxidation shunt (gray). To illustrate the change in conformation necessary for Prx catalysis, the center shows a morph between FF and LU conformations for the Prx1 subfamily member *St*AhpC; the C_P- and C_R-containing chains are colored white and dark gray, respectively, and the C-terminal region beyond C_R is not shown. (C) An organic peroxide and peroxynitrous acid are shown bound to the active site in ways that mimic the interactions made by peroxide in panel A. "BB" refers to a backbone NH hydrogen bond donor. The placement of the hydrophobic collar seen in some organic peroxide selective Prxs is noted by orange circles. (D) Chemical structures of some other molecules recently reported to react with Prxs (see the text).

To complete the catalytic cycle, the disulfide is commonly reduced by thioredoxin (Trx), or a thioredoxin-like protein,[30] and the Prx is returned to the FF conformation. Recently, the first structure of a Prx−Trx complex was obtained, showing one Trx on each side of a Prx dimer trapped in a mixed disulfide with C_R.[31] However, given that this particular yeast Prx possesses an unconventional N-terminal C_R, it is unclear how representative the details of this interaction may be for Prxs in general.

Reactivity toward Various Substrates. Structural work has greatly elucidated the features important for substrate interactions, with a peroxide-bound complex of *Aeropyrum pernix* thiol peroxidase[32] providing a view of a true Prx Michaelis complex (Figure 2A). Other ligands bound at the active sites of Prx crystal structures include molecules such as oxidized dithiothreitol (DTT),[24,33] benzoate,[34] acetate,[32] formate,[35] and glycerol,[32] with the oxygens of these molecules mimicking those of a peroxide. Analysis of these complexes led to a proposal that the roughly 10^5-fold rate increase of the enzyme over that of free cysteine is largely due to an extensive set of hydrogen bonds that stabilize the transition state of the reaction,[33] and this was supported by recently determined experimental thermodynamic activation energies as well as quantum mechanics/molecular mechanics simulations.[36,37]

Interestingly, though Prxs share a universal catalytic cycle and active site, some are observed to have relatively broad substrate specificity, while others are more selective.[38] For instance, *Salmonella typhimurium* alkyl hydroperoxide reductase C (*St*AhpC) is ∼100-fold more reactive with hydrogen peroxide than with organic peroxides primarily because of differences in K_m.[39] In contrast, human PrxV[26] and *E. coli* thiol peroxidase (*Ec*Tpx)[38] are ∼100- and ∼200-fold, respectively, more reactive with organic peroxides. The preference of some Prxs for organic peroxides has been attributed to a "hydrophobic collar" of apolar side chains around their active site that can make favorable hydrophobic interactions with the hydrocarbon part of the substrate (Figure 2C). Such a conserved hydrophobic collar was first observed in the Tpx subfamily,[38] but other Prxs that efficiently reduce organic peroxides, such as human PrxV,[33] also possess analogous collars. One commonality among various hydrophobic collars is that a dimer partner is frequently seen to contribute a bulky hydrophobic side chain to the collar across the dimer interface.[24,31,33,38] The significance of this interaction is not fully understood but may be related to a positive cooperativity seen for one Prx when consuming organic peroxides.[31] Another possible contributor to substrate specificity proposed for a PrxQ from *Xanthomonas campestris* is for an extended β-strand to fold down and cap the active site after binding an organic peroxide.[35]

The ability of Prxs to reduce peroxynitrite is also well-established.[40] AhpCs from the genera *Salmonella, Mycobacterium*, and *Helicobacter* were shown to efficiently reduce peroxynitrite,[41] as were Prxs from other organisms such as *Trypanosoma cruzi* tryparedoxin peroxidase[42] and human PrxV.[26,43] Experiments indicate that the C_P thiolate reacts with peroxynitrous acid[44] (i.e., the protonated form that is readily formed at physiological pHs[45]), and this is consistent with the protonated form being better able to mimic peroxide binding in the Prx active site (Figure 2C). Additionally, lowering the pH from 7.8 to 7.4 (and increasing the fraction of

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peroxynitrous acid present) increased the rate of peroxynitrite reduction by human PrxV[26] from ~10^7 to ~10^8 M^{-1} s^{-1}.

Hypochlorous acid (HOCl) is among the reactive oxygen species released extracellularly by neutrophils to overwhelm pathogen redox systems,[46] and HOCl can also lead to generation of chloramines via spontaneous HOCl-amino reactions[47,48] (Figure 2D). HOCl and chloramines readily oxidize thiol groups, and recent studies indicate Prxs are targets of these chemical species.[47,48] Human PrxIII did become oxidized when cells were treated with micromolar levels (thought to be representative of *in vivo* concentrations) of NH_2Cl and HOCl, but reported rates are similar to that of free thiols, suggesting the reaction is not substantially facilitated by the enzyme.[47,48] Given the prevalence of glutathione and other cellular thiols, Prxs are not thought to be major sinks for HOCl or chloramines.[47] Nevertheless, it can be seen that a major evolutionary advantage conferred by Prxs is the ability to eliminate many forms of peroxide and apparently even some other reactive species.

KNOCKDOWN AND KNOCKOUT STUDIES AS PROBES OF THE PHYSIOLOGICAL ROLES OF PRXS

Prxs influence a variety of cellular processes, and one approach to discern their various physiological roles is to observe the phenotypes that arise when cells or whole organisms are made deficient in terms of these enzymes. Summarized here are the results of extensive knockdown studies in cells from humans and in other organisms (Table S1 of the Supporting Information) and of knockout studies for vertebrates (Table 1), other eukaryotes (Table S2 of the Supporting Information), and prokaryotes (Table S3 of the Supporting Information).

Prx Deficiency in Eukaryotes. Humans have six Prx isoforms, which are localized in discrete parts of the cell. PrxI, PrxII, and PrxVI are primarily cytosolic. PrxIII is mitochondrial. PrxIV is in the endoplasmic reticulum and PrxV is in the cytosol as well as the mitochondria and peroxisomes.[4] The effects of Prx knockdowns have been characterized in at least one cell line for each isoform (Table S1 of the Supporting Information). One commonality of these studies is an increase in the level of oxidative damage to cellular components such as increases in levels of protein carbonylation[49] and DNA oxidation.[50] These effects are typically accompanied by reduced rates of growth and survival and an increased rate of apoptotic cell death, especially under conditions of oxidative stress.[49,51−56] It is perhaps not surprising, therefore, that Prx deficiency also contributes to cellular degeneration and decreases the viability of cancer cells. For example, PrxI was designated as a tumor suppressor upon the discovery that a histone deacetylase exerted its antitumor properties by increasing the level of PrxI expression in cancerous esophageal cells.[57] Additionally, knockdowns of PrxII[51] and PrxVI[55] in breast cancer cells were found to inhibit metastases.

Further elucidating the protective role of Prxs in mammals are knockout analyses conducted on the homologous mouse enzymes (Table 1). As was seen in the human cell knockdowns, Prx knockout mice show increased levels of oxidative damage to proteins, lipids, and DNA that detrimentally affect a host of cellular processes and often result in abnormal cellular regulation and growth.[58−64] Mouse PrxI knockouts exhibit the most severe phenotype in which c-Myc levels increase,[58] Akt kinase levels are elevated in fibroblasts and mammary epithelial cells,[65] and death occurs by 9 months because of the development of malignant tumors.[66] PrxII-knockout animals showed an increased level of atherosclerosis,[67] an increased number of splenocytes, bone marrow differentiation, and peripheral blood mononuclear cells,[68] an enlarged thymus, an increased rate of T-cell proliferation,[69,70] and elevation of p21 and p53 levels and increased cell senescence.[71] PrxIII-null mice exhibited alterations in fat metabolism, with increased fat mass, downregulation of adiponectin, impaired glucose tolerance and insulin resistance,[62] and a reduced litter size and general sensitivity to oxidative stress as observed in placenta,[72] macrophage,[73] and lung cells.[61] PrxIV was also found to influence reproductive success, as PrxIV-knockout mice displayed testicular atrophy and reduced sperm viability under conditions of oxidative stress.[74]

Prxs are further seen to be important for the viability of less complex eukaryotes (Tables 1 and 2 of the Supporting Information). *Caenorhabditis elegans* Prx knockdowns show a 70% reduction in brood size, and individual growth is retarded.[75,76] Also, studies of Prx-deficient disease-causing eukaryotes have implicated Prxs as pathogenicity factors for a number of organisms, with *Schistosoma* showing decreased rates of survival and larval size,[77−79] *Trypanosoma brucei* exhibiting a 16-fold increase in sensitivity to peroxide-induced death,[80] and *Leishmania infantum* having decreased infectivity in mice.[81] In addition, Tpx1 knockouts of *Plasmodia* have increased sensitivity to paraquat and nitroprusside,[82] produce 60% fewer gametes, exhibit delayed gaetocytemia,[83] grow fewer sporozoites in mosquitoes, and are less effective at infecting

Table 1. Summary of Prx Knockout Studies in Vertebrates

organism/enzyme	ref	brief phenotypic observations
Mus musculus-PrxI	66	malignant cancers, hemolytic anemia, premature death
M. musculus-PrxI	58	increased DNA oxidation, increased c-Myc activation in embryonic fibroblasts
M. musculus-PrxI	65	increased susceptibility to Ras-induced breast cancer
M. musculus-PrxII	59	increased protein oxidation in red blood cells, hemolytic anemia
M. musculus-PrxII	67	increased plaque formation, predisposition to develop atherosclerosis
M. musculus-PrxII	69	enlarged thymus, increased T cell proliferation
M. musculus-PrxII	68	increased splenocytes, bone marrow differentiation
M. musculus-PrxII	71	increased p21 and p53 levels, increased cellular senescence
M. musculus-PrxII	60	increased protein cysteine oxidation in red blood cell fractions
M. musculus-PrxIII	61	increased lung damage from inflammation, increased DNA damage
M. musculus-PrxIII	62	increased fat mass, increased protein carbonylation in adipose tissue
M. musculus-PrxIII	72	reduced litter size, increased oxidative stress in placenta tissue
M. musculus-PrxIII	73	increased macrophage apoptosis by lipopolysaccharide treatment
M. musculus-PrxIV	74	testicular atrophy, reduced sperm viability in oxidative stress
M. musculus-PrxVI	134	increased lung damage, decreased animal survival due to hyperoxia
M. musculus-PrxVI	131	increased ischemic reperfusion injury, increased cardiomyocyte apoptosis
M. musculus-PrxVI	135	decreased lung surfactant degradation
M. musculus-PrxVI	64	increased LDL oxidation by macrophages, increased plasma lipid H_2O_2 levels
M. musculus-PrxVI	136	increased UPR, increased apoptosis in lens epithelial and aging cells

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mice.[84] Thus, these results are consistent with Prxs being crucial components of pathogen redox defenses.

Several studies have utilized fungal model organisms to analyze the effects of Prx knockouts (Table S2 of the Supporting Information). In *Saccharomyces cerevisiae*, which has multiple Prx and glutathione peroxidase (Gpx) isoforms, the knockout of individual Prxs resulted in increased sensitivity to reactive oxygen and nitrogen species as well as an increased number of DNA mutations.[85] Not surprisingly, these effects were magnified when all Prx isoforms were knocked out,[85] and dual Prx/Gpx-null strains exhibited a ∼50% shorter life span.[86] The less extensively studied *Neurospora crassa* showed altered circadian periods and phases when a Prx was knocked out and peroxide-dependent transcriptional responses were lost.[87] Alterations to circadian rhythms were also seen for *Arabidopsis thaliana*, the only plant for which a Prx deficiency has been well-characterized.[87] Interestingly, Prx knockdowns in this model plant impacted several plant-specific processes, such as an increased level of foliar ascorbate oxidation,[88] altered gene expression in the chloroplast, and reduction of photosystem II and cytochrome b_6 content[89] (Table S1 of the Supporting Information).

These studies demonstrate that Prxs in eukaryotes are essential to normal function, as their absence results in damage to cell components and promotes deterioration of cell cycle regulation; the latter especially emphasizes that a vital role is played by Prxs in non-oxidative-stress-related peroxide signaling. An interesting observation that arises from the different effects seen in the knockout or knockdown of single Prxs is that, despite their high level of sequence similarity and shared peroxidase functionality, Prx isoforms do not have fully overlapping functions. This is illustrated especially well for humans and mice, for which the deficiency in each isoform resulted in distinct, deleterious phenotypes (Tables 1 and Table S1 of the Supporting Information). One obvious contributor to this lack of compensation is the discrete tissue expression profiles and cellular locations of eukaryotic Prx isoforms.[53] Besides the restrictions imposed by localization, the cytosol, nucleus, ER, and mitochondria all have distinct redox environments[90,91] (for a recent review, see ref 92), and therefore, Prx isoforms have been specifically tuned for optimal function in only certain cellular compartments.

Prx Deficiency in Bacteria. Unlike their eukaryotic counterparts, bacteria are not known to utilize non-oxidative-stress-related peroxide signaling. Thus, the lack of an evolutionary pressure to allow for the localized buildup of peroxide constitutes a major difference in the functional optimization of bacterial Prxs. As a consequence, many bacterial Prxs have evolved to be highly "robust" against inactivation by hyperoxidation, even at millimolar peroxide concentrations.[93] The advantage of this robustness is especially apparent for pathogenic bacteria as Prxs are utilized to defend against the reactive oxygen species employed by attacking macrophages.[94] Investigations into the role of bacterial Prxs, therefore, have been largely focused on disease-causing species (Table S3 of the Supporting Information).

The most extreme dependence on Prxs so far observed for a bacterial species is that of *Helicobacter*, for which knockouts displayed no growth under microaerobic conditions,[95] were more susceptible to killing by macrophages, and nearly lost their ability to colonize mouse stomachs.[96] Likewise, for *Staphylococcus aureus*[97] and *Mycobacterium bovis*,[98] Prx-deficient strains were shown to have reduced infectivity. In general, minimal effects of some Prx knockouts may be due to compensation by other redox defense enzymes. Some support for this is found in that more adverse phenotypes are observed for *Vibrio parahemolyticus*[99] and *Brucella abortus*[94] when two enzymes are knocked out at once. As discussed above, substrate specificity may influence the essentiality of a certain isoform or set of isoforms, and for Prxs specific for organic peroxides, like *E. coli* Tpx, it is important to note that the impact of the loss of its activity may be underestimated by challenges with H_2O_2 alone.

PRX HYPEROXIDATION

Potential Physiological Value of Prx Hyperoxidation. As noted above, the C_P-SOH state of a Prx can react with a second peroxide and become hyperoxidized to a Cys-sulfinate (C_P-SO_2^-), which inactivates the enzyme's peroxidase function (Figure 2B). Prokaryotic Prxs typically are rather resistant to hyperoxidation, requiring millimolar concentrations of substrate, and have been termed robust isoforms.[100] In contrast, many eukaryotic Prxs are quite readily hyperoxidized even though this makes them worse peroxidases. For example, human PrxII is converted almost entirely to the hyperoxidized state in the presence of only 40 μM peroxide (with no reducing agent present), with a $k_{SOH} \rightarrow k_{SO_2}$ rate on the order of $\sim 1.0 \times 10^3$ M^{-1} s^{-1} or higher.[101,102] Such isoforms are termed "sensitive", because even at low peroxide levels they are sensitive to being inactivated through hyperoxidation.[100] To facilitate comparisons of sensitivity between Prxs, the quantity $C_{hyp1\%}$ was recently introduced as a normalized way to quantify this property;[93] $C_{hyp1\%}$ defines the peroxide concentration at which 1% of Prx molecules become hyperoxidized during each turnover. Using this terminology, it is apparent that human PrxI ($C_{hyp1\%} = 62$ μM), human PrxII ($C_{hyp1\%} \sim 1.5$ μM), and human PrxIII ($C_{hyp1\%} \sim 18$ μM) are much more sensitive than *St*AhpC ($C_{hyp1\%} = \sim 10000$ μM).[93,102]

When Prx hyperoxidation was first discovered, its physiological relevance was questioned, as *in vivo* peroxide concentrations in healthy cells are thought to rarely exceed 1−15 μM.[103] It has since been hypothesized that peroxide levels may locally reach concentrations at which hyperoxidation can occur,[4] such as in the vicinity of peroxide-producing enzymes such as NOXs (Figure 1). Recently, the growth factor lysophosphatidic acid (LPA) was shown to stimulate cellular internalization of NOX components into early endosomes, termed "redoxosomes", to serve as hubs for oxidative regulation[104] (Figure 1). Strong support for the existence of local peroxide buildup is an elegant study proving that protein tyrosine phosphatases, which are not highly reactive with peroxide, actually do become oxidized *in vivo*.[105] Further, Prx hyperoxidation is observed *in vivo* in a variety of organisms and has been discussed as a marker of ancient circadian rhythms,[87,106] though the meaning or relevance of this latter observation is not yet clear.

In terms of what evolutionary advantages could be conferred to the many eukaryotes that contain sensitive Prxs, there is as of yet no final consensus. One explanation, termed the "floodgate hypothesis", proposes that Prx hyperoxidation is important for enabling non-stress-related peroxide signaling in eukaryotes.[100] In this model, low peroxide concentrations are reduced efficiently, but when levels spike locally because of the purposeful production of H_2O_2 by enzymes such as NOX during signaling events,[6] Prxs are inactivated to allow the H_2O_2

to build up sufficiently in a local area to oxidize downstream target proteins (Figure 1). The dysregulation of this signaling pathway provides an explanation for how knockouts of sensitive isoforms in mammals (PrxI–IV) could result in the development of cancers,[66] increased cell senescence,[71] and malformed tissue and organs[72,74] (Table 1 and Table S1 of the Supporting Information). As noted above, the downstream targets that have been most extensively studied are the protein tyrosine phosphatases that become inactivated through the oxidation of a catalytic Cys residue (reviewed by Frijhoff et al.[107]). Nevertheless, the best documented example of such a floodgate-style function of a Prx is in fact the role of PrxIII in the negative feedback control of mammalian corticosteroid production (Figure 1). As this process occurs in adrenal gland mitochondria as a circadian cycle, an ACTH-activated cytochrome P450 produces H_2O_2 as a byproduct of making corticosteroids, and the inactivation of PrxIII allows peroxide to build up sufficiently to lead to p38 activation and a shutting down of the synthesis of the steroidogenic acute regulatory protein[108] (Figure 1).

Additional proposals that have been put forth for the possible benefits of Prx hyperoxidation include their serving as chaperones,[81,109,110] regulating senescence through protein–protein interactions with p38MAPKα,[111] and peroxide exposure dosimeters.[2] Also, most recently, Day et al.[112] showed that under extreme oxidative conditions the inactivation of Prxs can serve to preserve the Trx pool for use by more essential cellular systems.[2] In that study, the survival of *Schizosaccharomyces pombe* was greatly diminished when its single Prx was *not* inactivated by millimolar levels of peroxide.[112] The authors showed the Prx inactivation allowed the reduced Trx pool to be retained for use by Trx-dependent repair enzymes such as methionine sulfoxide reductase.[112] Though *S. pombe* in nature would not normally encounter such high peroxide levels, these results provide a valuable insight into the importance of maintaining a reduced Trx pool. In relation to this, it was proposed that the eukaryotic pathogen *Schistosoma* might possess both sensitive and robust isoforms because it allows for the switching between reduction sources; because the latter enzyme is preferentially reduced by the glutathione/glutathione reductase system,[113] the organism does not exclusively rely on Trx when enduring a peroxide burst from a macrophage. It is also noteworthy that although *Schistosoma* do not possess catalase, peroxide disproportionation by catalases, present in most cells, is in principle an alternative approach by which cells can prevent the depletion of their reduced Trx.[2]

Structural Features Influencing the Sensitivity of Prx to Hyperoxidation. So what are the structural features that give rise to sensitivity to hyperoxidation? It was discovered that many Prx1 subfamily sensitive Prxs contain two motifs that pack against the FF active site, a "GGLG" and a C-terminal extension with a "YF", which are not present in most robust isoforms[100] (Figure 3). By inhibiting the local unfolding of the active site, these motifs serve to rigidify and stabilize the FF active site and make the enzyme more susceptible to hyperoxidation.[100] This mode of action and the greater importance of the C-terminal YF motif to sensitivity were proven shortly thereafter by a study showing that C-terminal swapping between sensitive and robust isoforms from the eukaryotic parasite *Schistosoma* resulted in variants with reversed sensitivity.[113] Likewise, a truncation of the C-terminal YF motif in human PrxIV greatly diminished the enzyme's sensitivity.[114] On the basis of such results, it has sometimes

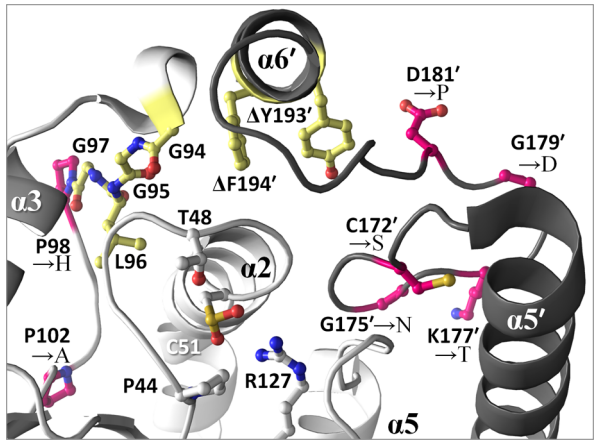

Figure 3. Studies probing the structural basis for Prx hyperoxidation. The active site and C-terminal region are shown for *Hs*PrxII (PDB entry 1qmv), with the GGLG and YF regions colored yellow. Sites where mutations have been introduced as a means to explore the impact on hyperoxidation for PrxI subfamily enzymes are colored pink.[101] Elimination of the YF motif by C-terminal truncation (indicated by Δ) has also been conducted.[114]

been generalized that only eukaryotes possess sensitive isoforms and that sensitive and robust Prxs can be reliably distinguished by the presence or absence of the GGLG and YF motifs, but these are both oversimplifications.

With regard to the first point, some prokaryotes do possess sensitive Prxs. A number of bacterial Prx isoforms have the GG(L/V/I)G and YF (or YL or FL) motifs, and some have been shown to be sensitive,[110,115,116] although they appear to be used for antioxidant defense rather than regulating peroxide signaling. Examples of this are two cyanobacterial species, *Anabaena* and *Synechocystis*, that both have sensitive Prxs.[115] *Anabaena* expresses its sensitive isoform abundantly and utilizes an Srx to rescue any hyperoxidized forms, while *Synechocystis* (which has no Srx) expresses its moderately sensitive Prx only at low levels to mop up endogenous peroxide and rapidly produces catalase to defend against higher peroxide levels.[115] Similarly, the bacterium *Vibrio vulnificus* was shown to possess both a sensitive and a robust Prx,[116] with trace amounts of peroxide inducing the expression of the sensitive isoform, whereas only high levels of peroxide induced the robust isoform, suggesting that the two Prxs are utilized for discrete levels of oxidative stress.

With regard to the second point, a recent study of human PrxII and PrxIII explored through mutagenesis the importance of secondary features associated with the two regions[101] (Figure 3). Both PrxII and PrxIII contain the GGLG and YF motifs, but nevertheless, PrxIII is ∼10-fold more robust. Swapping the identities of nearby residues between these two isoforms generated more robust PrxII variants and also more sensitive PrxIII variants, although again it was the presence of the C-terminal YF positions that was most critical to promoting sensitivity.[101] This proves that positions other than the GGLG and YF motifs can also contribute to sensitivity or robustness. This is especially exemplified by *E. coli* Tpx, which is a fairly sensitive Prx ($C_{hyp1\%}$ of 156 μM for cumene hydroperoxide[93]) even though it does not contain either motif and is actually in a different Prx subfamily. Also, Perkins et al.[20] showed that even conservative mutations such as C_R → Ser or Ala, commonly used to study the properties of Prxs, can actually perturb the C-terminal packing sufficiently to shift the FF ↔ LU equilibrium

toward LU and make the enzyme less sensitive. Such modulations of sensitivity have been recently shown to occur physiologically, as the C-terminal lysine acetylation of human PrxI[117] and N-terminal acetylation of human PrxII[118] led the enzymes to become robust. Further, nitration of human PrxII Tyr193 (in the YF motif), detected in Alzheimer patient brains, converted the enzyme to being robust and may play a role in the development of the disease.[119] Thus, a small alteration to even one residue can potentially reduce the fraction of the active FF population by orders of magnitude and thereby inhibit hyperoxidation.

These complexities reinforce the point that various Prxs have been optimized to suit diverse needs, and although trends do exist, caution must be employed when attempting to draw firm conclusions about Prx sensitivity solely from a sequence fingerprint. In general, enzymatic characterization is necessary to be certain, and there remains much to learn about the occurrence and roles of sensitive versus robust Prxs.

Distribution of Sulfiredoxin among Eukaryotes. Sulfiredoxin (Srx) catalyzes the ATP-driven rescue of C_P-SO_2^- back to C_P-SOH[19] and is present in many eukaryotes and a few cyanobacteria.[115] Upon its discovery,[120] Srx provided an explanation for how eukaryotes could allow sensitive Prxs to be hyperoxidized without being wastefully irreversibly inactivated. A crystal structure of a Prx−Srx complex[140] revealed that the two enzymes embrace with the locally unfolded Prx C-terminus wrapping around the backside of Srx, and the Prx C_P being placed into the Srx Gly-Cys-His-Arg (GCHR) active site pocket near the bound ATP (Figure 4).[28]

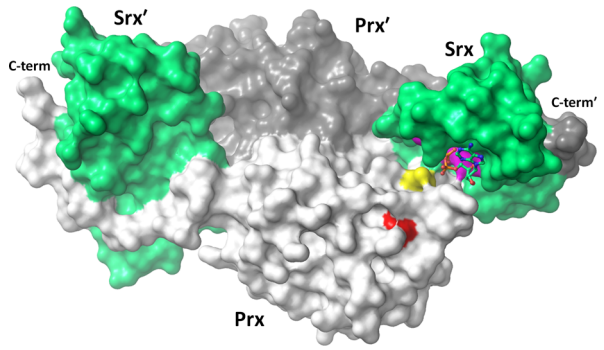

Figure 4. Prx−Srx embrace. Shown is a crystal structure of a human PrxI dimer (light and dark gray) in complex with two Srx chains (green, PDB entry 3hy2). Highlighted are the Prx C_P (yellow), the GGLG motif (red), the Srx active site (purple), and its bound ATP (sticks). The Prx C-terminal YF motif is disordered and not shown.

Srx appears to be remarkably important for organisms that express it. Knockouts of Srx cope poorly with oxidative stress,[121] with cells showing dramatically increased levels of Prx hyperoxidation, apoptosis, and mitochondrial membrane potential collapse.[122] Conversely, the overexpression of Srx has been observed to influence cell proliferation and pro-cancerous activity, including altering the states of p21, p23, and p53.[123] In yeast, the overexpression of Srx was shown to increase the replicative life span by 20%.[124] We expect that these phenotypes are largely due to altered Prx regulation, but Srx has also been reported to possess deglutathionylation activity.[125] Two recent reviews provide further details about the structure, function, and physiology of Srx.[125,126] Here, assuming that the presence of Srx in an organism would suggest a signaling-related physiological role for Prx hyperoxidation, we have investigated the distribution of Srx in nature to seek insight into the occurrence, and evolutionary roots, of peroxide signaling pathways.

To perform an updated analysis of the distribution of Srx, we used BLAST[127] to retrieve 335 Srx sequences from the nonredundant protein database. Only sequences containing the "GCHR" Srx active site fingerprint[121] were included as a way to filter out proteins such as the functionally unrelated bacterial chromosomal partition protein B (ParB), which is a known homologue.[128] An evolutionary tree (Figure 5) reveals that Srx is present and clusters distinctly in animals, fungi, plants, and some protists, and as reported in a 2005 Srx evolution study,[128] some cyanobacteria are the only prokaryotes to contain an *srx* gene. This apparent wide distribution of Srx among eukaryotes implies a relatively ancient existence of functional Prx hyperoxidation.

As a next step, we analyzed the 220 available sequenced eukaryotic genomes and surprisingly found that only 56% of them contained an *srx* gene: fungi and protists quite commonly lack Srx, and while most animals and plants contain Srx, a few animal exceptions seem to exist (Table 2). For example, *Xenopus* apparently does not have Srx, and subsequent searches for an amphibian *srx* gene did not yield any examples. Also, especially noteworthy is the fact that many organisms causing human disease, some of which had been mentioned in the 2005 study,[128] do not possess Srx (Table 2). These include *Entamoeba*, apicomplexans (such as *Plasmodia* species and *Toxoplasma gondii*), the Diplomonad *Giardia lamblia*, the parabasalid *Trichomonas vaginalis*, euglenozoa (*Trypanosoma* and *Leishmania* species), the nematodes *Loa loa* (eye worm) and *Brugia malayi* (causes elephantitis), and the flatworm *Schistosoma mansoni*.

The fact that Srx is present in a diverse range of eukaryotes yet is apparently absent from certain groups seems to be an important observation. For those eukaryotes lacking Srx, some possibilities for how they differ are that the Prx repair function is performed by a different enzyme, that hyperoxidized Prxs are not rescued, and/or that non-stress-related peroxide signaling is either not as important or not similarly regulated by Prx hyperoxidation. In *Sc. mansoni*, which does not possess Srx but does have a sensitive Prx isoform,[113] it has been shown that Prxs that become hyperoxidized are not repaired.[77] Whether they use peroxide in non-stress-related signaling is unknown. Like *Schistosoma*, many of the eukaryotes that do not contain Srx *do* have at least one Prx isoform that contains the GGLG and YF motifs (Table S4 of the Supporting Information). As discussed earlier, the presence of the GGLG and YF motifs does not necessarily prove that a Prx is sensitive, but as is seen for the *Schistosoma* enzyme, some may indeed be sensitive.

From these analyses, the additional question of why organisms that seem to lack the ability to rescue hyperoxidized Prxs would retain sensitive isoforms arises. Perhaps some of these organisms, such as was seen for *Vibrio*,[116] minimize waste by tightly regulating their sensitive Prxs to be expressed only at basal levels of peroxide. A further consideration is that because of cellular compartmentalization, even organisms that do contain Srx may not necessarily efficiently rescue all hyperoxidized Prxs. This is illustrated by a recent study showing that in human fibrosarcoma cells, when ER-localized human PrxIV hyperoxidation is induced through ER stress-generating agents, no rescue was observed, leading the authors to conclude that no ER-localized Srx exists.[129] We propose that the distribution pattern of Srx in eukaryotes holds important clues about the

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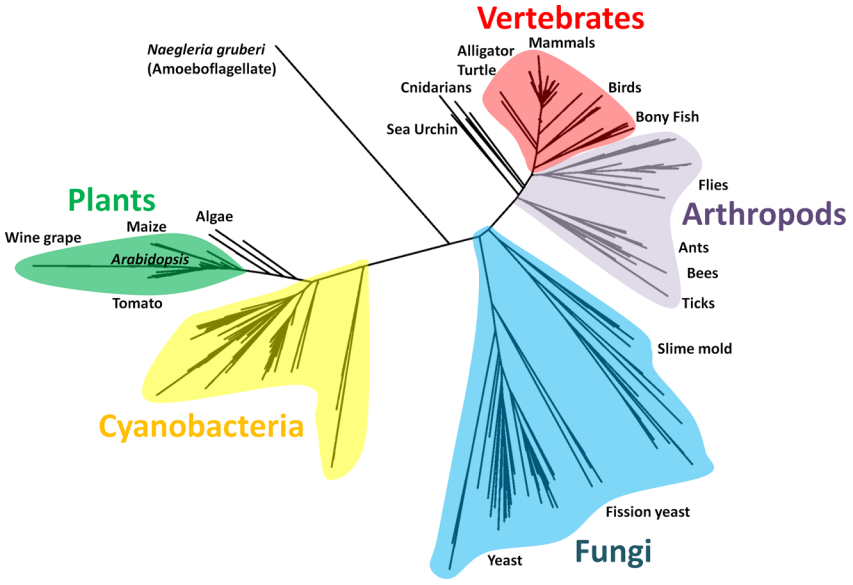

Figure 5. Relatedness tree for Srx sequences. An unrooted phylogenetic tree of 335 Srx sequences is shown. Select organisms or groups of organisms are noted. Sequences were retrieved from the nonredundant protein database by BLAST[127] on January 31, 2014, with an expect threshold of 100 using the human Srx1 sequence, and additional searches using distantly related Srx sequences did not identify further homologues. Sequences were aligned with MUSCLE,[138] and evolutionary distances were calculated using PhyML.[139]

Table 2. Presence of Sulfiredoxin in Eukaryotes[a]

animals	fungi	protists	plants
vertebrates	**ascomycetes**	choanoflagellates (0/1)	**eudicots (11/11)**
mammals (24/24)	**saccharomycetes (25/25)**	**amoebozoa**	**monocots (6/6)**
birds (9/9)	sordariomycetes (0/9)	**dictyostelium (3/3)**	**ferns (1/1)**
reptiles (1/2)[b]	leotiomycetes (0/2)	entamoeba (0/2)	**mosses (1/1)**
amphibians (0/2)[c]	eurotiomycetes (0/15)	**acanthamoeba (1/1)**	**green algae (6/8)**
fish (6/6)	dothideomycetes (0/3)	alveolates	red algae (0/3)
lancelets (1/1)	pezizomycetes (0/1)	apicomplexans (0/16)	
ascidians (1/1)	**schizosaccharomycetes (1/1)**	ciliates (0/2)	
echinoderms (1/1)	**basidiomycetes (3/11)**	stramenopiles	
arthropods	microsporidians (0/4)	diatoms (0/2)	
insects (19/20)[d]		oomycetes (0/1)	
mites/ticks (1/1)		eustigmatophytes (0/1)	
nematodes (1/5)		cryptomonads (0/1)	
flatworms (0/1)		haptophyta (0/1)	
cnidarians (1/2)		euglenozoa (0/7)	
placozoans (0/1)		**heterolobosea (1/1)**	
poriferans (0/1)		parabasalids (0/1)	
		diplomonads (0/1)	

[a]Across 220 organisms with sequenced genomes, the fractions of the total found to possess an Srx are given in parentheses. Groups containing any members with an Srx-encoding gene are highlighted in bold. [b]Searches of the *Anolis carolinensis* genome did not yield an Srx sequence, but that of *Ophiophagus hannah* (king cobra) did. [c]Frogs from the genus *Xenopus*. Additional searches yielded no amphibian Srx-possessing representatives. [d]The mosquito *Anopheles gambiae* had no Srx, but two other mosquitos, *Aedes aegypti* and *Culex quinquefasciatus*, possessed an Srx gene.

physiological roles of facile Prx hyperoxidation and that it is worthy of further study.

EFFICACY OF TARGETING PRXS FOR DRUG DESIGN

From the wealth of studies summarized above, we can conclude that Prxs play prominent roles in protecting DNA and other cellular components from oxidative damage, as well as influencing cell signaling, regulation, and proliferation in multicellular eukaryotes. So what rationale is there for the development of Prx-based therapeutics? A particularly interesting development for mammalian Prxs is the recent proposal that certain isoforms, especially PrxV and PrxVI, are danger signals associated with ischemic brain injury.[130,131] These enzymes are released poststroke by necrotic brain cells and are specifically detected by toll-like receptors of infiltrating macrophages, stimulating inflammatory cytokine production and promoting ischemic brain damage.[130] Antibodies against these Prxs were able to attenuate injury, providing evidence that implicates them as viable targets for future stroke therapeutics.[130] Also, given that some cancers overexpressing Prxs are resistant to radiation or other therapies,[12−14] it is tempting to envision that inhibiting human Prxs could have therapeutic value in some circumstances. For Prxs from pathogens,

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however, the case that they are drug targets seems very clear as Prx deficiencies in both prokaryotic and eukaryotic pathogens are linked to viability and infectivity.

The oft-noted challenge with regard to Prxs as drug targets is that the Prx active site is highly conserved, making it very challenging to make selective inhibitors targeting the active site. As an idea for designing inhibitors that would not target the active site, Perkins et al.[20] proposed that the delicately balanced FF ↔ LU equilibrium could be shifted by a small molecule to stabilize a single conformation (either the FF or the LU), thereby preventing the structural changes required for Prx catalysis. Surface regions of the protein that are involved in the FF ↔ LU transition are rather divergent in sequence and structure and can therefore be targeted. One such example is the C-terminal region of the Prx1 subfamily. If the LU form were stabilized, it would directly result in the loss of peroxidase activity. Alternatively, if the FF form were stabilized, and the C_P was blocked from resolving with the C_R, this would directly *enhance* activity but would indirectly lead to inhibition by promoting hyperoxidation.[20] Because most pathogens do not possess an Srx to rescue the hyperoxidized form (e.g., Table 2), these Prxs would be permanently inactivated. Further, the affinity of such an inhibitor could perhaps even be tuned so that it would dissociate and go on to inactivate other Prxs, thereby leading to an increased potency beyond a 1:1 ratio. Structures of many pathogenic Prxs are available (for detailed reviews, see refs 5 and 132)—including bacterial isoforms *St*AhpC, *Hp*AhpC, *Haemophilus influenza* Tpx, *Mt*AhpC, and *Mt*Tpx and eukaryotic isoforms[132] *Plasmodium yoelii* PrxI, *Plasmodium vivax* 2-Cys, and *Plasmodium falciparum* Trx-Px2—so rational drug design techniques such as virtual ligand screening[133] could be applied to identify leads. These approaches for Prx-targeted therapeutics warrant investigation, because two decades of Prx research can now be used for guidance, and if the effort is successful, it could provide novel antibiotics for some of the most virulent modern diseases.

■ ASSOCIATED CONTENT

Supporting Information

Summary of Prx knockdown studies (Table S1), summary of Prx knockout studies in various eukaryotes (Table S2), summary of Prx knockout studies in prokaryotes (Table S3), and a list of representative eukaryotes that lack Srx but have Prxs with GGLG/YF motifs (Table S4). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

Prx, peroxiredoxin; Srx, sulfiredoxin; NOX, NADPH oxidase; ACTH, adrenocorticotropic hormone; PDB, Protein Data Bank; C_P, peroxidatic cysteine; C_R, resolving cysteine; FF, fully folded; LU, locally unfolded; DTT, dithiothreitol; HOCl, hypochlorous acid; Gpx, glutathione peroxidase; LPA, lysophosphatidic acid; ParB, chromosomal partitioning protein B.

■ ADDITIONAL NOTE

[a]Although the term "peroxide signaling" can refer to both stress- and non-stress-related signaling,[1,2] here we exclusively use this term in reference to non-stress-related signaling.

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