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Thiol peroxidases mediate specific genome-wide regulation of gene expression in response to hydrogen peroxide

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Hydrogen peroxide is thought to regulate cellular processes by direct oxidation of numerous cellular proteins, whereas antioxidants, most notably thiol peroxidases, are thought to reduce peroxides and inhibit H2O2 response. However, thiol peroxidases have also been implicated in activation of transcription factors and signaling. It remains unclear if these enzymes stimulate or inhibit redox regulation and whether this regulation is widespread or limited to a few cellular components. Herein, we found that Saccharomyces cerevisiae cells lacking all eight thiol peroxidases were viable and withstood redox stresses. They transcriptionally responded to various redox treatments, but were unable to activate and repress gene expression in response to H2O2. Further studies involving redox transcription factors suggested that thiol peroxidases are major regulators of global gene expression in response to H2O2. The data suggest that thiol peroxidases sense and transfer oxidative signals to the signaling proteins and regulate transcription, whereas a direct interaction between H2O2 and other cellular proteins plays a secondary role.

Numerous cellular processes, including transcription and signaling, are redox regulated, but the molecular basis for this regulation is not clear. Hydrogen peroxide (H2O2) is a key molecule that is involved in redox regulation (1–3). It is both a toxic compound that can cause oxidative stress (4) and a second messenger that is required for cell proliferation (5). Its signaling function is thought to result from direct oxidation of various cell signaling and regulatory components, and its toxicity from stochastic oxidative damage to proteins, lipids, and nucleic acids (6, 7).

Several classes of enzymes, such as catalases and peroxidases, have evolved that specifically act on H2O2 or other hydroperoxides as substrates. Prominent among them are thiol-dependent peroxidases, which belong to peroxiredoxin (Prx) and glutathione peroxidase (Gpx) protein families. Thiol peroxidase genes are present in all previously characterized organisms, suggesting that these enzymes serve important functions conserved throughout evolution. Prx and Gpx have been implicated in cell signaling due to their ability to reduce intracellular levels of hydroperoxides and to serve as floodgates of H2O2 signaling (8–10). However, studies have also revealed that Saccharomyces cerevisiae Gpx3/Hyr1/Orp1 can serve as an H2O2 sensor and activate the transcription factor Yap1 by forming a disulfide in this protein (11), and a Schizosaccharomyces pombe thiol peroxidase Tsa1 was found to stimulate signaling through a MAP kinase pathway (12, 13). S. pombe thiol peroxidase Tpx1 similarly regulates transcription factor Pap1 (14). In addition, the ability to transfer oxidizing equivalents was demonstrated for a mammalian Gpx4 using a GFP-based redox sensor (15). It would be important to address the contribution of thiol peroxidases to stimulation and repression of redox regulation, particularly at a global, genome-wide scale.

In this work, we prepared a S. cerevisiae mutant lacking all eight thiol peroxidases. Surprisingly, this mutant was viable and could withstand significant oxidative stress. It responded to several redox stimuli by robust transcriptional reprogramming. However, it was unable to transcriptionally respond to hydrogen peroxide. The data suggested that thiol peroxidases transfer oxidative signals from peroxides to target proteins, thus activating various transcriptional programs. This study revealed a previously undescribed function of these proteins, in addition to their roles in removal of low levels of peroxides.

Results and Discussion

Yeast Cells Lacking All Thiol Peroxidases Are Viable and Can Withstand Redox Stresses. S. cerevisiae has five peroxiredoxins (Tsa1, Tsa2, Ahp1, nPrx, and mPrx) (16) and three glutathione peroxidases (Gpx1, Gpx2, and Gpx3) (17). We generated a series of mutants lacking multiple thiol peroxidases in different combinations (Fig. S1 and Table S1). These included several mutants that lacked seven (Δ7; three mutants with remaining Gpx2, Gpx3, or Tsa1) or all eight (Δ8) thiol peroxidase genes. The genome of the Δ8 strain was sequenced to 26.5× coverage on an Illumina platform, and the disruption of all eight thiol peroxidase genes was confirmed by DNA sequence analysis. All mutants lacking multiple thiol peroxidases, including Δ8, were viable, although their growth was affected compared to WT cells (Fig. L4 and Fig. S2). The mutant cells could withstand treatments with significant amounts of H2O2, diamide, DTT, and menadione, although some mutants were more sensitive than parental (WT) cells to these redox stresses (Fig. L4 and Fig. S2). Individual thiol peroxidases differentially contributed to this protection, with cells lacking multiple thiol peroxidases generally being more sensitive to stress. Removal of all eight thiol peroxidases also decreased cell growth in the absence of stressors (Fig. S2). We further compared viability of WT and multiple thiol peroxidase mutant cells following treatment with 1 or 2 mM H2O2, 3 mM diamide, or 25 mM DTT for 0.5–2 h and found smaller differences between WT and mutant strains (Fig. L1D). This observation suggests that the growth of Δ8 cells was inhibited by acute oxidative stress (Fig. L4 and Fig. S2), but cells were viable and could resume growth once stressors were removed (Fig. L1B). The unexpected oxidative stress resistance of Δ8A cells could be explained by the protective activities of catalase and cytochrome c peroxidase, which can remove hydrogen peroxide in thiol peroxidase mutant cells. Progressive


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removal of thiol peroxidases also resulted in lower life span (Fig. 1C). Taken together, these data suggested that the loss of thiol peroxidases decreased cell fitness and affected redox homeostasis. However, these enzymes were not essential, even when cells were treated with peroxide or other stressors.

Thiol Peroxidase Null Cells Are Unable to Activate and Repress Gene Expression in Response to H₂O₂. In S. cerevisiae, a large number of genes respond to H₂O₂ (18). Using cDNA microarrays with 6,692 gene features, we found that expression of 1,144 genes was induced and 574 genes repressed more than 2-fold upon incubation of WT cells with 0.5 mM H₂O₂ for 30 min (Fig. 2A). On average, gene expression was changed in WT cells 1.5-fold under these conditions (Fig. 2A and Fig. S3).

The main known function of thiol peroxidases is to scavenge hydroperoxides. Accordingly, these proteins may be expected to decrease the transcriptional response to H₂O₂ by lowering the cellular peroxide levels. Therefore, deletion of the peroxidase genes would be expected to stimulate gene expression in response to H₂O₂. However, in contrast to this prediction, the response to H₂O₂ was inhibited in cells lacking multiple thiol peroxidases. This effect was especially pronounced in the mutants that lacked six or more thiol peroxidase genes (Fig. 2A and Fig. S3) and was observed at both 0.1 and 0.5 mM H₂O₂ (Fig. S4). Moreover, cells lacking seven (i.e., all except Gpx2, all except Gpx3, and all except Tsa1) or all eight thiol peroxidases essentially lost the ability to regulate gene expression in response to H₂O₂. For example, 8Δ cells had a 37-fold reduced response to H₂O₂ treatment compared to WT cells. Importantly, both activation and repression of gene expression were inhibited (Fig. 2A and Fig. S3).

Thiol Peroxidase Null Cells Transcriptionally Respond to Stresses Other Than H₂O₂. To determine if the observed transcriptional effect was specific to H₂O₂ or if the mutant cells also lost the ability to respond to other stresses, we examined the response of WT and 7Δ cells in response to 0.5 mM H₂O₂, 2.5 mM DTT, 2.5 mM diamide, and 1 mM menadione treatments.

In S. cerevisiae, a large number of genes respond to H₂O₂ (18). Using cDNA microarrays with 6,692 gene features, we found that expression of 1,144 genes was induced and 574 genes repressed more than 2-fold upon incubation of WT cells with 0.5 mM H₂O₂ for 30 min (Fig. 2A). On average, gene expression was changed in WT cells 1.5-fold under these conditions (Fig. 2A and Fig. S3).
unfolded protein response (18), whereas menadione is a superoxide generator, and diamide is an oxidant that generates non-specific disulfide bonds. WT cells responded to all treatments. We also found that 7A cells responded to DTT and diamide similarly to WT cells; however, they did not respond to H$_2$O$_2$ treatment. Thus, deletion of multiple thiol peroxidase genes specifically disrupted the H$_2$O$_2$-induced transcriptional reprogramming without affecting the ability of cells to respond to other redox stresses. The response of mutant cells to menadione was twofold lower than that in WT cells (Fig. 2B), but this effect could be explained by the fact that this compound generates superoxide, which is further converted to H$_2$O$_2$ (to which these cells do not respond).

**Thiol Peroxidase Null Cells Do Not Respond to Varying H$_2$O$_2$ Concentrations and Treatment Times.** To examine the possibility that the response to H$_2$O$_2$ in the thiol peroxidase mutants was delayed or accelerated rather than the regulation was abolished, we analyzed gene expression in WT and 7A cells at 10, 30, 60, and 120 min after addition of H$_2$O$_2$ (Fig. 2B). In WT cells, gene expression (both activation and repression) changed by 30 min, peaked at 60 min, and diminished at 120 min. However, 7A cells did not respond to H$_2$O$_2$ at any time points. Likewise, we examined the regulation of gene expression in WT and 8A cells at different concentrations of H$_2$O$_2$ (0.05, 0.5, 1, 2, and 5 mM; 30-min treatment) (Fig. S5). In WT cells, activation and repression were most pronounced at 0.5 mM H$_2$O$_2$, but at concentrations above 1 mM the response diminished (Fig. 1B). However, the response of 8A cells was low at any H$_2$O$_2$ concentration. To test if other redox proteins functionally linked to thiol peroxidases were involved in the H$_2$O$_2$ response, we examined yeast cells deficient in cytochrome c peroxidase (Ccp1) or sulfiredoxin (Srx1). Both mutants responded to H$_2$O$_2$ similarly to WT cells (Fig. S6).

The data presented so far are consistent with a model wherein thiol peroxidases were required for the transfer of the H$_2$O$_2$ signal to other cellular components for transcriptional reprogramming. Moreover, this requirement was not limited to certain gene groups. Thus, thiol peroxidases appeared to function as global mediators (rather than inhibitors) of gene expression in response to H$_2$O$_2$.

**Thiol Peroxidase-Dependent Repression of Ribosomal Protein Gene Expression.** As a representative example, we analyzed expression of cytosolic ribosomal protein genes in WT and thiol peroxidase mutant cells. Ribosomal protein genes (i) were not down-regulated by H$_2$O$_2$ in mutant cells (Fig. 3A), (ii) exhibited normal expression levels in untreated mutant cells compared to WT cells (Fig. 3B), (iii) were down-regulated by H$_2$O$_2$, diamide, and DTT in WT cells, and (iv) were down-regulated by DTT and diamide (but not by H$_2$O$_2$) in mutant cells (Fig. 3C). These data argue for the specific thiol peroxidase-dependent regulation of ribosomal protein gene expression.

Expression of ribosomal protein genes is regulated by transcription factor Sfp1 (19). We tested the response of Sfp1 mutant cells to H$_2$O$_2$ (Fig. 3D) and found no repression of ribosomal protein genes, suggesting that Sfp1 is a H$_2$O$_2$- and thiol peroxidase-regulated transcription factor. An additional related cluster of genes that failed to respond to H$_2$O$_2$ treatment in 8A cells includes genes involved in rRNA modification and translation (Figs. S7 and S8). Similar to cytosolic ribosomal protein genes, RNA modification and translation genes were not activated or repressed in response to H$_2$O$_2$. However, their responses to DTT and diamide treatments in mutant cells were similar to those in WT cells. Interestingly, although cytosolic transcriptional machinery was transcriptionally repressed by H$_2$O$_2$ in a thiol peroxidase-dependent manner (Fig. 3 and Figs. S7 and S8), mitochondrial ribosomal protein genes were induced (Fig. S9). A similar effect was observed for genes coding for ubiquitin-dependent protein degradation components (Fig. S10). Further examination of responses in individual thiol peroxidase mutants suggested that thiol peroxidases could compensate for each other in mediating the repression of cytosolic ribosomal and translation-related proteins. Nevertheless, the contributions of individual thiol peroxidases to the peroxide-dependent regulation varied (Fig. 3D and Fig. S8).

**Thiol Peroxidase Null Cells Do Not Show Elevated Levels of Reactive Oxygen Species (ROS).** Thiol peroxidases have been suggested to serve as key enzymes in antioxidant defense. If so, a possibility had to be considered that the deletion of these enzymes led to oxidative stress (and therefore resulted in the H$_2$O$_2$ response even in the absence of stress) and that treatment with H$_2$O$_2$ did not further change or exacerbate the expression profile or response. Because the definition of oxidative stress is complex, we examined this possibility in a number of ways, as described in several sections below. First, we analyzed ROS levels in WT and mutant cells by monitoring 2,7'-dichlorodihydrofluorescein (DCF) fluorescence (Fig. 4A). Little difference was found between WT and 8A cells in the absence of stress; however, ROS levels were 2-fold higher in multiple thiol peroxidase mutant cells following 1–5 mM H$_2$O$_2$ treatment (Fig. 4A). These data suggest that 8A...
cells were not in a state of severe peroxide stress that could preclude their response to H$_2$O$_2$ treatment. The increase in ROS levels in 8Δ cells after the addition of H$_2$O$_2$ could not explain the loss of the transcriptional response because 8Δ cells could transcriptionally respond to other stresses (Fig. 2B). In addition, in the presence of 0.1 mM H$_2$O$_2$, ROS were not increased in 8Δ cells, yet transcriptional response was inhibited (Fig. S4).

**Thioredoxin and Thioredoxin Reductase Null Cells Respond to H$_2$O$_2$ Treatment.** The reduced state of most or all thiol peroxidases in the yeast cytosol and nucleus is maintained by thioredoxins Trx1 and Trx2, which in turn are reduced by NADPH-dependent thioredoxin reductase Trr1. We tested if deletion of both Trxs or of Trr1 also disrupts regulation of gene expression by H$_2$O$_2$, but instead found that these mutants had an increased response to H$_2$O$_2$ (Fig. 4B). Stimulation of activation and repression of gene expression in response to H$_2$O$_2$ was also previously seen in the Trr1 mutant cells treated with lower concentrations of H$_2$O$_2$ (20). Thus, we observed an important difference between thiol peroxidases on the one side and their reductants on the other. The data suggest that deletion of Trxs or Trr1 leads to accumulation of oxidized forms of thiol peroxidases and therefore results in an increased response to H$_2$O$_2$.

**Thioredoxin and Thiol Peroxidase Mutants Show Similar Levels of Cysteine Oxidation.** Because Trxs, Trr1, and thiol peroxidases function in the same pathway to transfer electrons from NADPH to H$_2$O$_2$, further comparison of these mutant strains offered us an opportunity to better understand the unique role of thiol peroxidases in the peroxide-dependent transcriptional regulation. We examined levels of oxidized cysteine residues in protein extracts from unstressed 7Δ and Trx1,2Δ cells. Although both strains had somewhat elevated cysteine oxidation compared to WT cells, we found no significant differences between these mutant cells (Fig. 4C and D). These data further support the idea that redox stress cannot explain the specific block in the H$_2$O$_2$ response by thiol peroxidase null cells.

During the course of these studies, we made another interesting observation: Trx1,2Δ cells were more resistant to H$_2$O$_2$ treatment in the cell viability assay than even WT cells (Fig. 4E). It is possible that Trx1,2Δ cells had an increased capacity for H$_2$O$_2$ regulation due to elevation in oxidized thiol peroxidases. This finding is not contradictory with the current literature (20, 21). Indeed, the double thioredoxin mutant grows slowly in the presence of H$_2$O$_2$; however, its viability, determined as the number of colonies formed following the treatment and transfer of cells to a new medium, was not affected. Overall, our data support the idea that Trr1 and Trxs inhibit the transcriptional response to H$_2$O$_2$ (by reducing thiol peroxidases), whereas thiol peroxidases stimulate peroxide-dependent regulation of gene expression (by oxidizing target proteins).

**Antioxidant Compounds and Anaerobic Growth Do Not Restore the Ability of 8Δ Cells to Respond to Peroxide.** As an additional test, we examined the H$_2$O$_2$-dependent changes in gene expression of WT and 8Δ cells under anaerobic conditions (Fig. 2A), as well as aerobically in the presence of antioxidant compounds, 5 mM L-proline and 5 mM N-acetylcysteine (NAC) (Fig. S11). The difference in gene expression between WT and 8Δ cells (Fig. S11, Right) was significantly decreased by anaerobiosis and proline/NAC treatment; however, these conditions and treatments did not restore the ability of thiol peroxidase mutant cells to respond to H$_2$O$_2$. These data once again argue that peroxide stress is not a reason for the inability of the mutant strains to respond to H$_2$O$_2$.

**Antioxidants Inhibit Rather Than Stimulate Growth of 8Δ Cells.** We tested if expression of a bacterial thiol peroxidase, Escherichia coli 2-Cys peroxiredoxin BCP (Fig. S12), or treatment of the thiol peroxidase mutant strain with 5 mM NAC (Fig. S13) could decrease the elevated cysteine oxidation in multiple thiol peroxidase mutants and found that they could not. In addition, we examined if antioxidants (5 mM NAC and 2 mM DTT) could normalize the growth of thiol peroxidase mutant cells. Surprisingly, the growth of 8Δ cells was inhibited by both compounds (Fig. S14). For example, whereas the growth of WT cells was not affected by NAC, the NAC-treated 8Δ cells showed no growth until 20 h. Moreover, 8Δ cells did not grow in the presence of 2 mM DTT (Fig. S14), even though this compound did not affect viability of these cells (during brief exposure) to much higher concentrations (Fig. 1B and Fig. S2). Although 5 mM NAC did not change the overall levels of disulfides in WT and mutant cells (Fig. S13), our observations support the idea that DTT- and NAC-dependent inhibition of growth of 8Δ cells was due to the reduction of disulfides in regulatory proteins. It is possible that the increased levels of nonspecific cysteine oxidation in multiple thiol peroxidase mutant cells is a molecular response that protects cells from stress caused by deletion of thiol peroxidase genes.
Roles of Yap1, Skn7, Msn2, and Msn4 in the Global Response to H$_2$O$_2$.

In *S. cerevisiae*, four transcription factors, Yap1, Skn7, Msn2, and Msn4, are known to regulate gene expression in response to H$_2$O$_2$, but they can also be activated by other stresses. We deleted these genes individually or in combination and tested the transcriptional response of the resulting cells to H$_2$O$_2$. These mutant cells, including the mutant that lacked all four transcription factors (4Δ), were viable and responded to H$_2$O$_2$ similarly to WT cells (Fig. 5A and Fig. S15); however, sensitivity to H$_2$O$_2$ was higher in Yap1Δ and 4Δ cells compared to WT cells. For example, expression of ribosomal protein genes was inhibited by H$_2$O$_2$ in these cells (Fig. S16). Comparison of expression profiles between WT and cells lacking the four transcription factors showed little difference (Fig. 5B). Reversible cysteine oxidation in the mutant cells was also similar to that in WT cells under equivalent treatment conditions (i.e., with and without H$_2$O$_2$) (Fig. S17). These results suggest that the global response to H$_2$O$_2$ is not mediated exclusively by these four redox transcription factors. Our data also allowed us to better define the sets of genes dependent on these transcription factors (Fig. 5 C and D). In particular, we identified genes uniquely dependent on Yap1 as well as genes dependent on both Yap1 and Skn7 (Fig. 5C).

Expression of several, but not all, Yap1- and Yap1/Skn7-dependent genes was elevated in the untreated 8Δ cells compared to WT controls (Fig. 5D). It is known that Yap1 can be activated by general stresses, including treatment with diamide, and this regulation may be independent of thiol peroxidases (22). We examined changes in the expression of Yap1- and Yap1/Skn7-dependent genes in WT and 7Δ cells upon treatment with diamide, menadione, or DTT at different time points (Fig. S18). The analysis of the response to menadione and diamide suggested that Yap1/Skn7-dependent genes could still be fully regulated in 7Δ cells. On the other hand, the DTT response showed an opposite effect for WT and 7Δ cells. This observation suggested that disulfide bonds in Yap1/Skn7 transcription factors may have formed nonspecifically in the oxidative environment of 7Δ cells and that such disulfide bonds could not be reoxidized under experimental conditions, because formation of physiological disulfide bonds was not possible in the 7Δ mutant.

Hydrogen Peroxide-Sensing Cysteines Are Intact in 8Δ Cells. We tested the response of WT and 8Δ cells to the combined H$_2$O$_2$ and diamide treatment. H$_2$O$_2$ did not modify the response of WT and 8Δ cells to diamide (Fig. S19). Moreover, transcriptional responses to H$_2$O$_2$ and diamide significantly overlapped in WT cells and the response to H$_2$O$_2$ in WT cells also overlapped with the diamide response in 8Δ cells (Figs. S19, S20, and S21). Diamide can form disulfide bonds in proteins nonspecifically, and this observation explains the overlap between H$_2$O$_2$ and diamide responses and suggests that both compounds have a similar set of targets. These data support the hypothesis that 8Δ cells do not respond to H$_2$O$_2$ because they lack thiol peroxidases, which form regulatory disulfides in signaling proteins. Diamide can directly form such disulfides, and therefore the response to diamide in 8Δ cells is similar to those of H$_2$O$_2$ and diamide in WT cells.

A Model of Thiol Peroxidase-Dependent Regulation of Transcription. We showed that thiol peroxidase null cells are unable to sense H$_2$O$_2$ and carry out peroxide-dependent transcriptional reprogramming. The peroxide response was not observed at any time points following peroxide treatments nor at any peroxide concentrations. Neither anaerobic conditions nor antioxidants could restore it. Yet, thiol peroxidase mutant cells showed robust transcriptional responses to other redox treatments. We propose that these findings can be explained by disruption of the signaling network from peroxide to transcription factors. Thiol peroxidases emerge as global regulators of gene expression. Overall, the data suggest that thiol peroxidases exhibit two functions associated with H$_2$O$_2$ reduction: (i) They transmit oxidative signals to upstream (with respect to electron flow) effectors, such as transcription factors and signaling and regulatory molecules, and (ii) they provide antioxidant defense by reducing hydroperoxides. Previously, it has been difficult to distinguish these functions experimentally because by reducing peroxides, thiol peroxidases fulfill two roles: oxidation of target proteins and antioxidant defense.

Our model of gene regulation by H$_2$O$_2$ (Fig. 6) is illustrated using the example of the NADPH-dependent Trx system. In the absence of stress, thiol peroxidases are kept in the reduced state by Trxs and other thiol-disulfide oxidoreductases. Upon addition

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**Fig. 5.** Role of oxidative stress transcription factors in the H$_2$O$_2$ response. (A) Regulation of gene expression by H$_2$O$_2$ in cells lacking single and multiple transcription factors Yap1, Skn7, Msn2, and Msn4. (B) Comparison of gene expression between untreated WT and indicated mutant cells. The values of average changes in gene expression are shown under each column. (C) Changes in gene expression in response to H$_2$O$_2$ treatment of Yap1- and Yap1/Skn7-dependent genes in WT and transcription factor mutant cells. This set was generated by filtering the entire set of genes responsive to H$_2$O$_2$ treatment as described in Materials and Methods. (D) Changes in gene expression, in response to H$_2$O$_2$ treatment of Yap1- and Yap1/Skn7-dependent genes, in WT and thiol peroxidase mutant cells. D shows a filtered version of data from Fig. 5A. E same experiment performed with Trx1Δ and Trx1,2Δ cells and isogenic WT cells. E shows a filtered version of data from Fig. 4B. (F) Changes in gene expression of Yap1- and Yap1/Skn7-dependent genes between WT and indicated mutant cells. Yap1- and Yap1/Skn7-dependent genes are shown on the right.
(or intracellular generation) of low concentrations of H₂O₂, this compound specifically oxidizes thiol peroxidases, whereas oxidation of other cellular proteins by H₂O₂ is minimal. High specificity of these enzymes toward H₂O₂ (23) makes them excellent candidates for sensing H₂O₂ and other hydroperoxides and allows them to effectively compete for H₂O₂ with other proteins containing reactive cysteines. Thiol peroxidases then oxidize regulatory and signaling proteins, resulting in the transcriptional response and signaling programs. The examples of Gpx3-dependent activation of Yap1 in response to H₂O₂ (11, 24) and of Tsa1-dependent activation of a stress-activated MAP kinase (12, 13) are supportive of our model and illustrate a molecular mechanism, by which thiol peroxidases may transfer oxidative signals to regulate gene expression.

Although regulation due to a direct target oxidation by H₂O₂ also likely exists, the key difference between our model (Fig. 6) and previously suggested models of redox regulation is that H₂O₂ does not need to interact with other cellular proteins to a significant extent in order to regulate gene expression or other peroxide-dependent programs. The model also explains specificity of H₂O₂ transcriptional regulation and points to basic mechanisms of redox signaling and redox regulation of cellular processes. Finally, we found that thiol peroxidases regulate the H₂O₂ transcriptional response in S. cerevisiae. We suggest that thiol peroxidases, via thiol-based redox coupling with cellular proteins, may also control different signaling and regulatory programs in other organisms, including mammals.

Materials and Methods
A detailed SI Materials and Methods is located in SI Appendix.

Yeast Strains. The yeast strains used in this study are shown in Table S1. Single and multiple thiol peroxidase mutants were prepared by mating the strain lacking five peroxiredoxin genes (GY14) with the strain lacking three glutathione peroxidase genes (GY25, GY30) in BY4741 background.

Single and multiple thiol peroxidase mutants were prepared by a one-step gene disruption method.

Spot Assays. Overnight cultures were adjusted to OD₆₀₀ = 1, and 10 µL of serial dilutions (10-fold each) were spotted on SD solid medium that contained stressors at indicated concentrations. Cells were grown for 2 d.

Yeast Aging Assays. After growing for 2 d on fresh plates, 35 undivided daughter cells were collected and arranged on yeast YPD plates using a dissecting microscope. New buds from these original daughter cells were separated and discarded as they formed. This process continued until cells stopped dividing.

cDNA Microarray Analyses. Cells were grown to 0.3–0.5 OD₆₀₀ in 200 mL of YPD medium, treated for indicated times with indicated compounds, har-
Supporting Information

Materials and Methods

Strains, cells growth, and media. S. cerevisiae parental strain (referred to as WT) BY4741 (MATα his3Δ leu2Δ met15Δ ura3Δ) and its isogenic thiol peroxidase mutants were grown in YPD media (1% yeast extract/2% peptone/2% dextrose). SY2626 cells were used as a parental strain for Trx1,2 and Trr1 mutant strains.

Preparation and verification of thiol peroxidase mutant stains. The yeast strains used in this study are shown in Table S1. GY15 is a MATα derivative of BY4741. The mutant missing five peroxiredoxin genes (GY14) in BY4741 background was previously described (1). To isolate single peroxiredoxin mutants, GY15 and GY14 were mated and diploids were sporulated and dissected by a micromanipulator. Spores that were missing Tsa1, Tsa1, mPrx, nPrx and Ahp1 genes were selected on supplemented YNB minimal media by auxotrophic markers that were used to replace the original ORFs. The single and triple glutathione peroxidase mutants were previously described (2).

In order to isolate a mutant (GY29) missing seven peroxidase genes (it expresses a single thiol peroxidase Gpx2), GY14 and GY30 were mated and the resulting diploids were analyzed by tetrad dissection. GY30 is an isogenic strain of GY25 that was provided by Dr. S. Avery (U. Nottingham, UK). At the first stage of screening, several hundred spores were checked on YNB selection plates for histidine, leucine, uracil and methionine auxotrophy and for G418 resistance. The mutant missing all 8 genes (8Δ, GY40) was generated from GY29 by a one-step gene disruption method using a hygromycin marker and pAG32 (3). Since each of HIS3, URA3 and KAN markers were used to delete 2 genes in the GY29 mutant cells, we performed a second screening to confirm the deletion of each ORF and presence of locus-specific marker genes by PCR with amplification control for each strain used in our work. The presence/absence of a thiol peroxidase gene was also verified by quantifying microarray signals. The mutant lacking all eight thiol peroxidases was used to generate strains missing seven peroxidases genes by mating it with WT and selecting for cells containing single Gpx3 (GY110) and Tsa1 (GY111) thiol peroxidase genes.

A mutant strain missing both yap1 and skn7 (GY73) was prepared from GY70 Δyap1:KAN strain by a one-step gene disruption method using the LEU2 marker. The strain missing msn2 and msn4 (GY74) was similarly prepared from GY72 Δmsn2:KAN using the URA3 marker. The quadruple Δyap1:KAN Δskn7:LEU2, Δmsn2:His3 Δmsn4:URA3 mutant (GY75) was generated from GY73 by two consecutive gene disruption steps using msn2 (HIS3 marker) and msn4 (URA3 marker).

Spot assays. Resistance of WT and mutant strains to different stressors was determined with spot assays. For each strain, overnight cultures were adjusted to OD600=1 and 10 μl of serial dilutions (10-fold each) were spotted on SD solid medium that contained H2O2, diamide, menadione, or DTT at indicated concentrations. Cells were grown for two days at 30 °C and pictures were taken.

Yeast aging assays. After growing cells for two days on fresh plates, 35 undivided daughter cells were collected and arranged on YPD plates using a dissecting microscope. New buds from
these original daughter cells were separated and discarded as they formed. This process continued until cells stopped dividing. Lifespan was calculated as the total number of daughter cells that each cell generated. The aging experiment was performed twice for each strain and representative mortality curves are shown.

**cDNA microarray analyses.** Cells were grown to 0.3-0.5 OD<sub>600</sub> in 200 ml of YPD medium, treated for indicated times with indicated compounds, harvested by centrifugation, and kept at -80 °C. Total RNA was isolated by Ambion RiboPure™-Yeast Kit according to the manufacturer's instructions. Antisense RNA, prepared by Ambion MessageAmp™ aRNA Amplification Kit, was used to prepare cDNA probes by reverse transcription with incorporation of Cy3-dCTP or Cy5-dCTP (Amersham Biosciences). The cDNA-labeled probes were then hybridized onto an arrayed slide. Fluorescence was captured with a GenePix4000B Scanner (Axon Instruments, Foster City, CA) and fluorescent intensities were quantified by using IMAGENE 5.5 (BioDiscovery, El Segundo, CA). All signal values, which were lower than the background level were treated as background and were excluded from calculations.

**Clustering and visualization.** DNA microarray data were K-mean clustered with CLUSTER (http://rana.lbl.gov/manuals/ClusterTreeView.pdf) and visualized using TreeView (4).

**Cloning of E. coli peroxiredoxin Tsa1.** The gene coding for E. coli 2-Cys thiol peroxidase Ts1 was amplified with primers: 5’-gaagaacagttagtgcaccacaaccgcttcatttcag-3’, and 5’-agggcatgtcagcttatgtcgggtcagccagag-3’, and cloned into SpeI-SalI sites of p423 vector containing hygromycin resistance gene at the SacI site. The construct was transformed into WT cells and 7Δ cells, and the clones selected by resistance to 300 μg/ml hygromycin.

**Estimation of average levels of gene activation and repression.** Average values of gene activation and repression were estimated using the following equation:

\[
S = \frac{\sum I_i}{I} + \frac{\sum D_j}{D} \cdot \frac{1}{N};
\]

where N is the total number of yeast genes; I, the fold activation of genes that were induced at a minimum of two fold; n, the number of genes induced at a minimum of two fold; D, the fold repression for genes that were repressed at a minimum of two fold; and k, the number of genes repressed at a minimum of two fold. Fold activation and repression were calculated separately and their sum indicated the average value of gene expression changes. This equation was also used to calculate values for specific clusters and groups of genes; however, in that case, N was the number of genes in a cluster. This research and other computational analyses were completed utilizing the Prairiefire Beowulf cluster from Research Computing Facility of the University of Nebraska – Lincoln.

**Identification of Yap1- and Yap1/Skn7-dependent genes.** The dataset of gene expression response to H<sub>2</sub>O<sub>2</sub> observed in mutants lacking individual or multiple oxidative stress transcription factors Yap1, Skn7, Msn2 and Msn4 was searched for the following expression patterns: (i) Yap1-dependent genes. Expression changes in Yap1, Yap1/Skn7 and quadruple 4Δ mutants should be a minimum of two fold lower than the expression changes in WT, Skn7 or Msn2/Msn4 mutant cells. (ii) Yap1/Skn7-dependent genes. Expression changes in Yap1, Skn7, Yap1/Skn7 and quadruple 4Δ mutants should be a minimum of two fold lower than the expression changes in WT and Msn2/Msn4 mutant cells.
Detection of reversibly oxidized cysteine residues in WT and mutant yeast strains. Each strain was grown in 200 ml of YPD to OD\textsubscript{600} =1. 100 ml of each sample was removed, treated by 12% trichloroacetic acid (TCA), centrifuged, and washed with ice-cold 5% TCA. The remaining sample was treated with 0.5 mM H\textsubscript{2}O\textsubscript{2}, incubated for 1 min, and treated with TCA as described above. Each pellet was resuspended in 1 ml denaturing buffer (6 M urea, 200 mM Tris-HCl, pH 8.5, 10 mM EDTA, and 0.5% [w/v] SDS) containing 100 mM iodoacetamide. Iodoacetamide was used to alkylate all reduced cysteine residues. After 30 min of incubation at 25 °C, the reaction was stopped by the addition of ice-cold 12% TCA. The samples were incubated for 20 min on ice, centrifuged and washed with TCA as described above. The pellets were then dissolved in 1 ml of denaturing buffer containing 100 mM DTT and incubated for 30 min at 25 °C. Cysteine residues which were in the oxidized state in the initial sample and could not be alkylated by iodoacetamide were now in the reduced state. The reaction was stopped by addition of 12% TCA. After 20 min on ice, the samples were centrifuged and washed as described above. Three additional ice-cold acetone wash steps were performed to remove the remainder of DTT. The pellets were then dissolved in the denaturing buffer containing 0.5 µM biotinylated iodoacetamide (Molecular Probes) and incubated for 1 h at 25 °C. At this step, the cysteine residues which were oxidized in the initial sample were modified with biotin. Equal aliquots from each sample were resolved by SDS-PAGE, transferred onto a PVDF membrane, and proteins, in which cysteine residues were modified with biotin, were visualized by western blotting analysis using streptavidin-conjugated mouse antibodies.

Viability assays. Each strain was grown in 10 ml YPD to OD\textsubscript{600} =0.5 and treated with indicated stress agents for various time periods. Cells were centrifuged, washed with YPD medium and serial dilutions were plated on YPD agar plates. Colony numbers were counted after 3 days of growth.

ROS analyses. Yeast cells were grown to OD\textsubscript{600} 0.5, washed twice with PBS, resuspended in PBS to 10\textsuperscript{8} cells/ml, and loaded with 5 µM DCFDA (Sigma). Cells were treated with 1 mM or 5 mM H\textsubscript{2}O\textsubscript{2} for 30 min. DCF fluorescence was visualized by BD FACS Calibur (BD Biosciences) flow cytometer equipped with Sapphire Blue laser (488 nm) using 525 nm emission filter.

Genome sequence of \textit{8Δ} cells. Genomic DNA was isolated from \textit{8Δ} cells, fragmentized to 200 bp by sonication and sequenced on an Illumina platform at the University of Nebraska-Lincoln genomics facility. Reads were assembled into the genome with MAQ genome assembler.

References

Legends to Supporting Figures

**Figure S1.** Verification of thiol peroxidase mutant strains. Detection of thiol peroxidase genes in the mutant strains by PCR is shown. Genomic DNA fragments were amplified using each thiol peroxidase gene specific primers. DNA from a WT strain was used as a positive control. This figure is spliced from many agarose gels. All bands were of the expected size as indicated on the left.

**Figure S2.** Growth of WT and thiol peroxidase mutant cells in the presence of indicated concentrations of (A) H$_2$O$_2$, (B) DTT, (C) diamide, and (D) menadione. Series of 10x dilutions of each strain were incubated on plates in the presence of indicated stress inductors for 48 h and the images were taken. A difference in growth between two single columns in the figure implies a 10-fold difference in survival.

**Figure S3.** A graph showing average values of gene activation and repression for wild type and thiol peroxidase mutant cells in response to 0.5 mM H$_2$O$_2$ treatment for 30 min. WT refers to BY4741 (isogenic with thiol peroxidase mutants) and WT1 refers to SY2626 (isogenic with Trx1,2Δ and Trr1Δ). Average values of activation and repression were estimated as described in Methods. WTa and 8Δa refer to experiments performed under anaerobic conditions.

**Figure S4.** Changes in gene expression of WT and indicated mutant thiol peroxidase strains in response to treatment with 0.5 mM H$_2$O$_2$ (left panel) and 0.1 mM H$_2$O$_2$ (right panel) are shown for all genes that are either activated or repressed in at least one strain (WT or mutant) used in the study. Numbers below the columns show average values of changes in gene expression (the sum of activation and modular repression values divided by the total number of yeast genes) for each dataset.

**Figure S5.** Average changes in gene expression in WT and 8Δ cells in response to 30 min treatment with different concentration of H$_2$O$_2$. Average values of gene activation and repression were determined as described in Methods.

**Figure S6.** Regulation of gene expression in response to H$_2$O$_2$ treatment in cytochrome c peroxidase and sulfiredoxin mutant strains. Right panel shows changes in gene expression between indicated mutant and WT cells.

**Figure S7.** Time course of changes in gene expression of rRNA modification and protein synthesis genes in WT and 7Δ cells in response to 0.5 mM H$_2$O$_2$, 2.5 mM DTT and 2.5 mM diamide. Gray shows spots with no data.

**Figure S8.** Regulation of expression of rRNA modification and protein synthesis genes. Changes in gene expression in response to H$_2$O$_2$ treatment are shown for thiol peroxidases mutants and isogenic wild type cells (left panel) and for Trr1 and Trx1/Trx2 mutant and isogenic wild type cells (middle panel). Right panel shows changes in gene expression between indicated mutant and isogenic WT strains. Gray shows spots with no data.

**Figure S9.** Regulation of gene expression of mitochondrial ribosomal proteins in response to H$_2$O$_2$ treatment (shown for thiol peroxidase mutants and isogenic WT cells). Gray shows spots with no data.
Figure S10. Regulation of gene expression of the ubiquitin-dependent protein degradation system in response to H\(_2\)O\(_2\) (shown for thiol peroxidase mutant and isogenic WT cells). Gray shows spots with no data.

Figure S11. Regulation of gene expression in WT and 8Δ cells treated with 0.5 mM H\(_2\)O\(_2\) for 30 min in the presence or absence of 5 mM proline (Pro) or 5 mM NAC, or under anaerobic conditions (shown with extension ‘a’). Right panel shows changes in gene expression between indicated mutant and WT cells. Gray shows spots with no data.

Figure S12. (A) Detection of oxidized cysteine residues in yeast proteins in WT and indicated mutant strains. \textit{E. coli} 2-Cys peroxiredoxin BCP was overexpressed in wild type and 7Δ cells, however, it did not change overall cysteine oxidation. (B) Amido Black staining of the gel shown in A.

Figure S13. Cysteine oxidation in yeast proteins in WT and 8Δ cells in the presence or absence of 5 mM NAC and 0.1 mM H\(_2\)O\(_2\).

Figure S14. Growth curves of WT and 8Δ cells in YPD medium and in YPD supplemented with 3 mM NAC or 2 mM DTT.

Figure S15. Average values of gene induction and repression in response to 0.5 mM H\(_2\)O\(_2\) treatment for WT cells and cells lacking four transcription factors that regulate gene expression in response to oxidative stress. The right area of the graph shows changes in gene expression between indicated mutant and WT cells. Average values of gene induction and repression were estimated as described in Materials and Methods.

Figure S16. Regulation of gene expression of cytosolic ribosomal proteins in response to H\(_2\)O\(_2\) treatment. Left panel shows changes in gene expression for cytosolic ribosomal proteins upon treatment of WT cells and cells lacking four transcription factors with H\(_2\)O\(_2\). Right panel shows changes in gene expression for cytosolic ribosomal proteins between indicated mutant and WT cells. To the right of this panel, ribosomal protein genes are shown.

Figure S17. Analysis of cysteine oxidation in yeast proteins in WT and indicated mutant strains.

Figure S18. Changes in expression of Yap1- and Yap1/Skn7-dependent genes in WT and 7Δ mutant cells in response to menadione, diamide and DTT treatments. Unlike menadione and diamide responses, the DTT response of Yap1- and Yap1/Skn7-dependent genes is opposite in WT and 8Δ cells. Gray shows spots with no data.

Figure S19. Regulation of gene expression by H\(_2\)O\(_2\) and diamide. Changes in gene expression of WT and 8Δ strains in response to 0.5 mM H\(_2\)O\(_2\) treatment (30 min), 2.5 mM diamide (30 min) or combined treatment are shown for all genes in yeast proteome. Numbers below the columns show average values of changes in gene expression.

Figure S20. (A) Genes induced more than 2 fold in WT cells by H\(_2\)O\(_2\) (897 genes), and induced by diamide in WT cells (1439 genes). These two groups have 787 genes in common. (B) Genes
repressed more than 2 fold in WT cells by H\textsubscript{2}O\textsubscript{2} (923 genes), and repressed more than 2 fold by diamide in WT cells (1313 genes). These two groups have 790 genes in common.

**Figure S21.** (A) Genes induced more than 2 fold in WT cells by H\textsubscript{2}O\textsubscript{2} (897 genes), and induced by diamide in 8Δ cells (1498 genes). These two groups have 756 genes in common. (B) Genes repressed more than 2 fold in WT cells by H\textsubscript{2}O\textsubscript{2} (923 genes), and repressed more than 2 fold by diamide in 8Δ cells (1337 genes). These two groups have 767 genes in common.
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Figure S1

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Figure S3
Figure S4
Figure S5

The graph shows the average change in gene expression, fold, in response to varying concentrations of hydrogen peroxide. The x-axis represents the hydrogen peroxide concentration in mM, ranging from 0.5 to 5. The y-axis shows the average change in gene expression, with values ranging from -0.4 to 0.8. Four lines are plotted:

- Red line: WT average induction
- Green line: WT average repression
- Red line with black stars: 7Δ average induction
- Green line with black circles: 7Δ average repression
Figure S6
Figure S9
Figure S11
Figure S12

WT Δ (all PpxΔ+2 Gpx1,3Δ) + E. coli Ppx WT

WT Δ (all PpxΔ+2 Gpx1,3Δ) + E. coli Ppx BCP
Figure S13
Figure S14
Figure S15
Figure S17
Figure S18

Yap1, Skn7-dependent

Yap1-dependent
Figure S19
Figure S20

A

WT activated by H₂O₂ (897)

787

WT activated by diamide (1439)

B

WT repressed by H₂O₂ (923)

790

WT repressed by diamide (1313)
Figure S21

A

WT activated by $\text{H}_2\text{O}_2$ (897)

$8\Delta$ activated by diamide (1498)

756

B

WT repressed by $\text{H}_2\text{O}_2$ (923)

$8\Delta$ repressed by diamide (1337)

767