Abstract

Hydrogen peroxide (H$_2$O$_2$) plays important roles in cellular signaling, yet nonetheless is toxic at higher concentrations. Surprisingly, the mechanism(s) of cellular H$_2$O$_2$ toxicity remain poorly understood. Here, we reveal an important role for mitochondrial 1-Cys peroxiredoxin from budding yeast, Prx1, in regulating H$_2$O$_2$-induced cell death. We show that Prx1 efficiently transfers oxidative equivalents from H$_2$O$_2$ to the mitochondrial glutathione pool. Deletion of PRX1 abrogates glutathione oxidation and leads to a cytosolic adaptive response involving upregulation of the catalase, Ctt1. Both of these effects contribute to improved cell viability following an acute H$_2$O$_2$ challenge. By replacing PRX1 with natural and engineered peroxiredoxin variants, we could predictably induce widely differing matrix glutathione responses to H$_2$O$_2$. Therefore, we demonstrated a key role for matrix glutathione oxidation in driving H$_2$O$_2$-induced cell death. Finally, we reveal that hyperoxidation of Prx1 serves as a switch-off mechanism to limit oxidation of matrix glutathione at high H$_2$O$_2$ concentrations. This enables yeast cells to strike a fine balance between H$_2$O$_2$ removal and limitation of matrix glutathione oxidation.

Keywords cell death; hydrogen peroxide; hyperoxidation; mitochondria; peroxiredoxin

Subject Categories Autophagy & Cell Death; Membranes & Trafficking

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Introduction

Reactive oxygen species (ROS) are an unavoidable consequence of life in an oxygen-rich environment. Once considered solely as harmful molecules, which cells seek to remove as efficiently as possible, it is now accepted that some ROS have important physiological functions. In this regard, one of the best understood ROS is H$_2$O$_2$, which acts as a second messenger in several key cellular signaling pathways (Sundaresan et al, 1995; Delaunay et al, 2002; Sobotta et al, 2015; Stocker et al, 2018). On the other hand, it remains unequivocal that high concentrations of H$_2$O$_2$ are toxic and can lead to cellular dysfunction and cell death. Presumably therefore, cells strive to tightly regulate H$_2$O$_2$ to permit sufficiently large fluctuations in H$_2$O$_2$ concentration for signaling purposes, while simultaneously preventing accumulation of H$_2$O$_2$ to toxic levels.

Surprisingly, the exact mechanism(s) by which H$_2$O$_2$ leads to cell death remain poorly understood (Whittemore et al, 1995; Day et al, 2012; Uhl et al, 2015). The mediore reactivity of H$_2$O$_2$ with most biological molecules argues against direct oxidation of cellular biomolecules being a principal driver of cell death, at least at physiologically relevant H$_2$O$_2$ concentrations (Winterbourn & Metodiewa, 1999; Stone, 2004; Winterbourn, 2008). Other possible triggers of H$_2$O$_2$-induced cell death have been proposed, including induction of apoptosis (Greetham et al, 2013), depletion of reduced cytosolic thioredoxins (Day et al, 2012), and disruption of redox signaling pathways (Sies, 2017), but well-defined molecular mechanisms remain largely elusive. Therefore, in this study we sought to address the molecular underpinnings of cellular H$_2$O$_2$ toxicity, using budding yeast as a model system.

We hypothesized that mitochondrial matrix-localized processes may play an important role in H$_2$O$_2$ toxicity, for two reasons. First, the mitochondrial respiratory chain is thought to be a major source of cellular H$_2$O$_2$, and thus, the matrix is in close proximity to key sites of H$_2$O$_2$ production (Murphy, 2009; Quinlan et al, 2013). Specifically, the “leakage” of electrons from respiratory chain complexes to molecular oxygen leads to the generation of superoxide anions. Superoxide dismutases located in both the mitochondrial matrix (Sod2) and the cytosol/intermembrane space (Sod1) facilitate the subsequent rapid dismutation of superoxide to H$_2$O$_2$. Second, in comparison with the cytosol, the mitochondrial matrix appears to be poorly equipped with H$_2$O$_2$-detoxifying enzymes. The only enzymes in the yeast mitochondrial matrix that are known to be able to react efficiently with H$_2$O$_2$ are the 1-Cys peroxiredoxin, Prx1, and perhaps the glutathione peroxidase homolog, Gpx2 (Park...
et al., 2000; Ukai et al., 2011). Nonetheless, many aspects of H₂O₂ handling in the matrix remain poorly understood, for example, the efficiency of the matrix-localized H₂O₂ removal systems. Furthermore, the crosstalk between matrix H₂O₂ and different matrix redox couples is unclear, and often, conflicting results are present in the literature. For example, the exact reductive mechanisms for both Prx1 and Gpx2 remain a matter of debate, particularly regarding the specific roles of glutathione, glutaredoxins, thioredoxins and thioredoxin reductase (Pedrajas et al., 2000, 2010, 2016; Avery & Avery, 2001; Tanaka et al., 2005; Greetham & Grant, 2009).

To gain further insight into matrix H₂O₂ handling in general and more specifically into possible mechanisms of H₂O₂-mediated toxicity, we employed genetically encoded probes that enable subcellular compartment-specific measurement of H₂O₂, the glutathione redox potential (E₆GHs), and Prx1 oxidation, together with biochemical assessment of cysteine redox states, transcriptome analyses and cell death assays. We found that the matrix glutathione pool is significantly more sensitive to H₂O₂-induced oxidation than the cytosolic glutathione pool. However, we found that H₂O₂-induced matrix glutathione oxidation is completely dependent upon the presence of Prx1. Deletion of PRX1 eliminated H₂O₂-induced oxidation of matrix glutathione and elicited a transcriptional response that increased levels of the cytosolic catalase, Ctt1, showing that cells can recognize, and respond to, impaired matrix redox homeostasis. The loss of glutathione oxidation in the matrix and the improved Ctt1-dependent H₂O₂-handling capacity of the cytosol synergistically rendered cells more resistant to an acute H₂O₂ treatment. We subsequently generated a range of matrix-targeted thiol peroxidases and mutant variants thereof, with differing abilities to transfer oxidative equivalents from H₂O₂ to glutathione. By replacing endogenous Prx1 with these peroxiredoxin variants, we revealed a striking correlation between matrix glutathione oxidation and cell death. In wild-type cells, we found that the degree of cell death is limited by hyperoxidation-based inactivation of Prx1 at high H₂O₂ levels, which restricts oxidation of the matrix glutathione pool. In summary, Prx1 hyperoxidation allows cells to strike a fine balance between H₂O₂ removal and limitation of mitochondrial glutathione oxidation, which is strongly predictive of cell death.

Results

Exogenous H₂O₂ elicits compartment-specific E₆GHs and H₂O₂ responses

Little is known about the dynamic handling of H₂O₂ in the mitochondrial matrix of intact cells. We thus sought to characterize H₂O₂ flux inside the matrix and assess the impact of increased H₂O₂ on matrix reductive systems, particularly the glutathione pool. To this end, we made use of the genetically encoded fluorescent probes, roGFP2-Tsa2ΔCr and Grx1-roGFP2, which allow the real-time monitoring of the basal H₂O₂ level and E₆GHs, respectively, in specific subcellular compartments (Gutscher et al., 2008; Morgan et al., 2016; Fig 1A and B). Both probes comprise a redox-sensitive green fluorescent protein (roGFP2; Hanson et al., 2004) genetically fused with a specific redox enzyme. For roGFP2-Tsa2ΔCr, this is the Saccharomyces cerevisiae cytosolic typical 2-Cys peroxiredoxin, Tsa2, from which the resolving cysteine has been removed to increase the sensitivity of the probe to H₂O₂. In the case of Grx1-roGFP2, it is the human glutaredoxin, Grx1. The roGFP2-Tsa2ΔCr probe responds directly to H₂O₂, with the Tsa2ΔCr moiety serving to efficiently transfer oxidative equivalents from H₂O₂ to roGFP2. This probe is predominantly reduced by endogenous GSH/glutaredoxins, which directly reduce the roGFP2 moiety. RoGFP2-Tsa2ΔCr oxidation is therefore determined by rapid H₂O₂-driven oxidation and much slower GSH/glutaredoxin-driven reduction (Morgan et al., 2016; Roma et al., 2018). Conversely, Grx1-roGFP2 will predominantly only respond indirectly to H₂O₂, via H₂O₂-induced glutathione disulfide (GSSG) production and the concomitant increase in E₆GHs. The readout of roGFP-based probes is commonly presented as degree of probe oxidation to allow comparison between different experiments (OxD; for calculation, see Materials and Methods and Gutscher et al., 2008). OxDs of 0 and 1 therefore indicate fully reduced and fully oxidized roGFP2 moieties, respectively (Appendix Fig S1A; for more detailed discussion of the probe mechanisms, see, e.g., Roma et al., 2018).

We targeted each probe to either the cytosol or the mitochondrial matrix and monitored the probe response to the addition of exogenous H₂O₂ using a fluorescence plate reader-based system (Appendix Fig S1B, and Fig 1C and D). We observed that the matrix roGFP2-Tsa2ΔCr probe exhibited a significantly smaller response to exogenous H₂O₂, applied at initial concentrations of 0.1 and 1 mM, than the cytosolic roGFP2-Tsa2ΔCr probe (Fig 1C). In both subcellular compartments, the OxD of the roGFP2-Tsa2ΔCr probe decreased over time in the absence of H₂O₂, an observation explained by the depletion of oxygen in our plate reader-based assay (Morgan et al., 2016). In contrast, upon addition of exogenous H₂O₂, the matrix-targeted Grx1-roGFP2 probe exhibited a significantly larger response than the cytosol-localized Grx1-roGFP2 (Fig 1D). In summary, matrix E₆GHs is apparently more sensitive to perturbation by exogenous H₂O₂, even though the roGFP2-Tsa2ΔCr probe reports less H₂O₂ in the matrix than in the cytosol. We therefore sought to identify the cause(s) of these compartment-specific differences.

Cytosolic enzymes protect the matrix against both exogenous and mitochondria-derived H₂O₂

The smaller roGFP2-Tsa2ΔCr response in the matrix compared to the cytosol could be explained by either (i) efficient cytosolic scavenging systems as well as the two mitochondrial membranes limiting the amount of H₂O₂ that reaches the matrix or (ii) by more efficient scavenging of H₂O₂ in the matrix limiting the amount of H₂O₂ available to react with the probe.

An initial insight into these two possibilities came from monitoring roGFP2-Tsa2ΔCr and Grx1-roGFP2 probe responses to antimycin A treatment. Antimycin A is an inhibitor of respiratory chain complex III that results in release of superoxide anions on the IMS side of the inner mitochondrial membrane. Superoxide anions will be rapidly dismutated, both enzymatically and spontaneously, to H₂O₂. Antimycin A treatment led to a larger roGFP2-Tsa2ΔCr response in the matrix compared to the cytosol, i.e., the opposite of the situation following treatment with exogenous H₂O₂ (Fig 1E). Antimycin A treatment also induced a small deflection of E₆GHs in both the matrix and the cytosol. The response in the matrix appeared to be slightly larger than in the cytosol, although the overall response was very limited in both compartments (Fig 1F).
Currently, we do not understand why the comparatively strong roGFP2-Tsa2ΔCR probe response upon antimycin A treatment did not result in a respective $E_{GSH}$ deflection. A possible hint might be the antimycin A-specific response dynamics. Compared to addition of external $H_2O_2$, antimycin A induced a comparatively late oxidation of the roGFP2-Tsa2ΔCR probe without recovery. In conclusion, the observation of opposing compartment-specific responses to antimycin A and exogenous $H_2O_2$ treatment indicates that the subcellular localization of $H_2O_2$ production/influx is an important determinant of subcellular compartment-specific $H_2O_2$ levels. Likely, cellular $H_2O_2$ scavenging enzymes significantly limit the (sub)cellular diffusion of $H_2O_2$ leading to the generation of steep intracellular $H_2O_2$ gradients (Winterbourn, 2008; Lim et al, 2015; Travasso et al, 2017).

To further test this hypothesis, we monitored the response of cytosolic and matrix-localized roGFP2-Tsa2ΔCR probes to exogenous peroxide in either wild-type cells or cells deleted for the genes encoding the two cytosolic typical 2-Cys peroxiredoxins, Tsa1 and Tsa2. Tsa1, in particular, is a highly abundant protein and thought to be an important cytosolic scavenger of $H_2O_2$ (Iraqui et al, 2009). In Δtsa1Δtsa2 cells, we saw that cytosolic and matrix roGFP2-Tsa2ΔCR responses (although starting from a different initial steady state) to exogenous $H_2O_2$ were much more similar than in wild-type cells (Fig 1G). These data thus further support the hypothesis that cytosolic $H_2O_2$ scavenging enzymes, including Tsa1 and Tsa2, limit the amount of exogenous $H_2O_2$ that can diffuse through the cytosol to ultimately reach the mitochondrial matrix. Interestingly, we also observed that Tsa1 and Tsa2 are important for the...
detoxification of mitochondria-derived H₂O₂, as a matrix roGFP2-Tsa2ΔCₘ probe in Δsal1Δtsa2ΔCₘ cells responded more rapidly to antymycin A treatment than in wild-type cells (Fig 1H). Thus, release of H₂O₂ to the cytosol likely also constitutes a mitochondria H₂O₂ detoxification pathway.

We next tested whether transfer over the mitochondrial membranes contributes to a decreased roGFP2-Tsa2ΔCₘ response in the matrix compared to the cytosol. In other systems, it has been demonstrated that the velocity of H₂O₂ transfer over membranes is increased by the presence of specific transporters, e.g., aquaporin 8 in the NADPH oxidase 2-dependent signaling cascade (Bertolotti et al., 2016). The outer membrane of mitochondria (OMM) contains porins (in yeast Por1 and the less expressed Por2) that have been shown to facilitate small molecule transport (Kmita et al., 2004; Kojer et al., 2012). Indeed, we found that the response to 1 mM exogenous H₂O₂ of a matrix roGFP2-Tsa2ΔCₘ in a Δpor1 strain was decreased compared to a wild-type strain (Fig 1I). Conversely, following antymycin A treatment, the matrix roGFP2-Tsa2ΔCₘ probe responded more readily in Δpor1 cells than in wild-type cells, supporting the hypothesis that Por1 deletion decreases H₂O₂ transport to the cytosol (Fig 1J). In summary, Por1 appears to facilitate the bi-directional transport of H₂O₂ across the OMM. Collectively, our data indicate that cytosolic peroxiredoxins and porins in the OMM contribute a major line of defense for mitochondria from external H₂O₂ and support the efficient removal of mitochondria-derived H₂O₂ (Fig 1K).

Ctt1 upregulation constitutes an "adaptive response" in matrix redox enzyme mutants

We next investigated the contribution of matrix H₂O₂ scavenging enzymes toward regulation of matrix H₂O₂ level. The 1-Cys peroxiredoxin, Prx1, is likely the most important H₂O₂ scavenger in the yeast mitochondrial matrix. However, surprisingly, when we deleted this enzyme, the matrix roGFP2-Tsa2ΔCₘ response to exogenous H₂O₂ was decreased (Fig 2A). This is counterintuitive for an enzyme that has been implicated in efficient matrix H₂O₂ handling. Conversely, when we assessed the matrix roGFP2-Tsa2ΔCₘ response toward antymycin A treatment, the differences in response between wild-type and Δprx1 cells were lost (Appendix Fig S2A). Collectively, these results suggest that defective matrix H₂O₂ handling might result in compensatory responses in the cytosol. To test this hypothesis, we monitored cytosolic H₂O₂ handling in strains lacking the matrix redox enzymes Prx1 and Tvrx3, which have both previously been linked to efficient H₂O₂ handling in the matrix (Pedrajas et al., 2000; Greetham & Grant, 2009; Gostimskaya & Grant, 2016). Interestingly, in both deletion strains cytosolic roGFP2-Tsa2ΔCₘ responses were attenuated compared to the wild-type cells (Fig 2B and C). Complementation of gene loss by expressing the matrix redox enzymes from a plasmid saw cytosolic roGFP2-Tsa2ΔCₘ responses revert to a wild-type-like situation (Fig 2D).

To understand this apparent cytosolic adaptation, we performed RNA-Seq analysis on Δprx1 cells transformed either with an empty p416TEF plasmid (Δprx1 + empty) or with a pEPT-PRX1 plasmid (Δprx1 + Prx1-WT) for the expression of wild-type Prx1 (Fig 2E, and Appendix Fig S2B and C). We found that the transcript encoding the cytosolic catalase, Ctt1, was significantly upregulated, while transcripts encoding other redox enzymes were not significantly enriched above threshold (Fig 2E and Dataset EV1). In line with a role of Ctt1 in the cytosolic "adaptive response", the deletion of CTT1 in a Δprx1 background (Δctt1Δprx1) ablated the decreased response of cytosolic roGFP2-Tsa2ΔCₘ that was observed in Δprx1 cells (Fig 2F). In line with the loss of the adaptive response, Δprx1Δctt1 cells exhibit a prolonged lag phase compared to either wild-type, Δprx1, or Δctt1 cells during growth under chronic H₂O₂ stress (Appendix Fig S2D). Increased Ctt1 levels appear to be an "add-on" response as deletion of PRX1 in Δsal1Δtsa2ΔCₘ cells only conferred a small, although significantly, improved cytosolic H₂O₂ handling (Appendix Fig S2E). Thus, not only cytosolic redox enzymes protect the matrix from external H₂O₂ under “unperturbed” conditions, but also there appears to be a cytosol-based “adaptive response” if matrix redox enzyme systems are impaired.
This Ctt1-dependent, NADPH-independent, response decreases the amount of exogenous H₂O₂ that reaches the mitochondrial matrix in cells with compromised matrix H₂O₂ detoxification systems (Fig 2G).

Glutathione reductase activity is limiting in the matrix

The experiments described above indicate that cytosolic redox enzymes significantly limit the amount of exogenous H₂O₂ that...
reaches the mitochondrial matrix. Nevertheless, $E_{\text{GSH}}$ is still more responsive to exogenous $H_2O_2$ than cytosolic $E_{\text{GSH}}$, despite the lower concentration of $H_2O_2$ that reaches the matrix. We therefore wanted to gain a deeper understanding of the mechanistic basis of this difference. We reasoned that, in the matrix, either GSSG might be less efficiently reduced or $H_2O_2$ might more efficiently trigger glutathione oxidation in comparison with the situation in the cytosol. First, we assessed whether glutathione reductase (Glr1) levels are limiting in the matrix. Glr1 is dually localized to the cytosol and matrix. The two Glr1 variants are encoded by one gene and one mRNA that is translated from two different start codons, with the longer form encompassing a matrix-targeting sequence (Outten & Culotta, 2004). Deleting Glr1 resulted in a higher steady-state Grx1-roGFP2 oxidation in both compartments and a much larger response to exogenous $H_2O_2$. Cells were grown in Sgal (−Leu) medium and harvested at early exponential phase. The light gray curve in both panels represents the wild-type control, treated with the same concentration of exogenous $H_2O_2$. Cells were grown in Sgal (−Leu) medium and harvested at early exponential phase.

**Figure 2. Deletion of mitochondrial redox enzymes activates a cytosolic adaptive response.**

A The response of a mitochondrial matrix-localized roGFP2-Tsa2ΔC₆₈ probe, expressed in wild-type and Δprx1, to the addition of exogenous $H_2O_2$ at the indicated concentrations. Cells were grown in Sgal (−Leu) medium and harvested at early exponential phase. Lighter colored curves are controls showing the probe response to the addition of water.

B, C The response of a cytosolic roGFP2-Tsa2ΔC₆₈ probe, expressed in wild-type, Δatr3, and Δprx1 cells, to the addition of 0.1 mM (B) or 1 mM (C) exogenous $H_2O_2$. Cells were grown in Sgal (−Leu) medium and harvested at early exponential phase.

D The response of a cytosolic roGFP2-Tsa2ΔC₆₈ probe, in Δatr3 cells transformed with a Trx3 plasmid and Δprx1 cells transformed with a Prx1 plasmid, to the addition of 0.1 mM exogenous $H_2O_2$. Cells were grown in Sgal medium lacking the appropriate amino acids for plasmid selection and harvested at early exponential phase.

E The profile of mRNA expression of the yeast redox or redox-related enzymes in Δatr3 + empty vector cells, compared to Δatr3 + Prx1-WT cells, both grown to an early exponential phase in Sgal (−Lra) medium (n = 3 biological replicates, with cells obtained from three independent cultures). Cutoff was set at a log2 fold change (FC) ≥ 0.32. Raw data are presented in Dataset EV1.

F The response of a cytosolic roGFP2-Tsa2ΔC₆₈ probe, in Δatr3 cells or Δctl1Δatr3x cells transformed with either an empty plasmid or a Prx1-WT plasmid to the addition of 0.1 mM exogenous $H_2O_2$. Cells were grown in Sgal medium lacking the appropriate amino acids for selection and harvested in early exponential phase.

G Model. Levels of the cytosolic catalase Ctt1 increase when activity of mitochondrial redox enzymes is impaired, leading to an increased cytosolic capacity for $H_2O_2$ removal.

Data information: In all panels, OXD refers to the degree of sensor oxidation. Error bars represent the standard deviation (n = 3 biological replicates, with cells obtained from three independent cultures for each biological replicate, three technical replicates were performed). Significance was assessed with Student’s 2-tailed, unpaired, t-test. **p < 0.01; ***p < 0.001; and ****p < 0.0001.

**Prx1 catalyzes the glutathione-dependent reduction of $H_2O_2$ in the matrix**

To gain further insight into how $H_2O_2$ affects $E_{\text{GSH}}$ in the matrix, we monitored the response of matrix $E_{\text{GSH}}$ to exogenous $H_2O_2$ in Δatr3, Δatr2, and Δprx1 strains, and compared it with wild-type, Δtsa1Δtsa2, and Δapor1 cells (Fig 3A). As expected, Δtsa1Δtsa2 cells exhibited an increased response of matrix Grx1-roGFP2 compared to wild-type because more $H_2O_2$ reaches the matrix in these cells (Fig 1G). Conversely, and in line with the roGFP2-Tsa2ΔC₆₈ data (Fig 1I), Δapor1 cells exhibited a decreased response of the $E_{\text{GSH}}$ sensor. Intriguingly, in the Δatr3, Δatr2, and Δprx1 strains we observed either no $E_{\text{GSH}}$ response (Δprx1) or an $E_{\text{GSH}}$ response that was greatly decreased compared to wild-type cells (Δatr3, Δatr2), following the addition of exogenous $H_2O_2$ at an initial concentration of 1 mM (Fig 3A). To assess whether the cytosolic adaptive response described above (Fig 2) might explain the decreased matrix $E_{\text{GSH}}$ response, we applied exogenous $H_2O_2$ at the higher initial concentration of 2.5 mM. While the matrix $E_{\text{GSH}}$ response in the Δatr3x and Δatr2 strains increased to almost the same level as that observed in wild-type cells, we still observed no matrix $E_{\text{GSH}}$ response in Δprx1 cells (Fig 3B). Importantly, at 2.5 mM exogenous $H_2O_2$, the matrix roGFP2-Tsa2ΔC₆₈ response in Δprx1 cells was clearly increased compared to the respective response in the wild-type to 1 mM exogenous $H_2O_2$ (Fig 3C). This indicates that despite the increased $H_2O_2$-handling capacity of the cytosol due to increased Ctt1 levels, Prx1 is crucial in mediating the $E_{\text{GSH}}$ response to $H_2O_2$. Consistent with the enzymatic activity of Prx1 being required for the $H_2O_2$-induced oxidation of glutathione, we observed no $E_{\text{GSH}}$ response in Δprx1 cells transformed with a plasmid encoding a Prx1 peroxidatic cysteine mutant, Prx1-C91A (Fig 3D). However, transformation of a plasmid encoding a wild-type Prx1 (Prx1-WT) into Δprx1 cells fully restored the $E_{\text{GSH}}$ response (Fig 3D). In summary, the most likely explanation for our data is that glutathione is involved in the reduction of Prx1 following its reaction with $H_2O_2$, although it is not possible to say whether glutathione is directly or indirectly reducing Prx1 (Greetham & Grant, 2009; Pedrajas et al., 2016, 2010; Fig 3E).

**Prx1 hyperoxidation protects matrix glutathione from $H_2O_2$-induced oxidation**

Given that Prx1 appears to efficiently catalyze the transfer of oxidative equivalents from $H_2O_2$ to glutathione, we next asked about the consequences of acute $H_2O_2$ challenges for the mitochondrial matrix glutathione pool. To this end, we developed an acute stress-washout assay. In this experiment, Δprx1 cells transformed with a plasmid for the expression of wild-type Prx1 (Δprx1 + Prx1-WT) and
expressing matrix Grxl-roGFP2 were incubated with increasing amounts of H₂O₂. The H₂O₂ was then removed, and in a subsequent readout experiment, the response of the matrix Grxl-roGFP2 probe toward the addition of 1 mM exogenous H₂O₂ was monitored (Fig 4A and B). Counterintuitively, we observed a strong negative correlation between the concentration of H₂O₂ used in the pre-treatment and the response of the matrix Grxl-roGFP2 to the subsequent bolus of 1 mM exogenous H₂O₂ (Fig 4B; for data with wild-type cells, see Appendix Fig S4A). When we repeated the experiment with Δapr1 cells transformed with a plasmid encoding an enzymatically inactive Prx1-C91A mutant, we observed no response to the 1 mM bolus of exogenous H₂O₂, irrespective of the concentration of H₂O₂ used in the initial pre-treatment (Fig 4C). To test whether this attenuation of the EₐGSH response after preceding H₂O₂ treatment could also be caused by matrix-originating H₂O₂, we employed a matrix-targeted D-amino acid oxidase (DAO; Matlashov et al., 2014). Upon addition of D-alanine but not L-alanine, this enzyme locally produces H₂O₂. When we compared the EₐGSH response toward the addition of 1 mM H₂O₂, we found that upon pre-treatment with D-alanine but not L-alanine, the EₐGSH response was indeed attenuated (Fig 4D and Appendix Fig S4B). Thus, both pre-treatment of wild-type cells with exogenous H₂O₂ and matrix-specific generation of H₂O₂ induce a Δapr1-like mitochondrial matrix EₐGSH response upon subsequent exogenous H₂O₂ treatment. 

High levels of H₂O₂ can lead to hyperoxidation of peroxidatic cysteine thiol groups to sulfenic or sulfonic acids, which render the peroxiredoxin enzymatically inactive. We thus asked whether our H₂O₂ pre-treatments lead to hyperoxidation of the Prx1 peroxidatic cysteine. We therefore employed two different assays to test whether Prx1 in the mitochondrial matrix can be hyperoxidized at the concentrations of H₂O₂ used in our acute stress assays. First, we made use of a fusion construct between Prx1 and roGFP2, which we targeted to the matrix. RoGFP2-based fusion constructs have recently been shown to be well suited for monitoring peroxiredoxin activity and hyperoxidation in vivo (Staudacher et al., 2018). In the roGFP2-Prx1 probe, Prx1 will directly interact with H₂O₂ and transfer oxidation onto roGFP2. Interaction of roGFP2 with matrix Grx2/glutathione will reduce roGFP2 again. We observed an increasing roGFP2-Prx1 sensor response with increasing concentrations of exogenous H₂O₂ up to 2.5 mM (Fig 4E). However, at 5 mM exogenous H₂O₂, we observed no further increase in maximum probe oxidation.
and an initial more rapid recovery as compared to 2.5 mM H$_2$O$_2$ (Fig 4E). This is indicative of hyperoxidation of the Prx1 moiety (Staudacher et al., 2018). As a control, a matrix-targeted unfused roGFP2 did not exhibit a lowered response with increasing H$_2$O$_2$ concentrations and showed a similar response to the roGFP2-Prx1 probe at 5 mM H$_2$O$_2$, further supporting Prx1 inactivation (Fig 4E). Thus, hyperoxidation-based inactivation of the fused peroxiredoxin results in a more reduced roGFP2 (Morgan et al., 2016; Roma et al., 2018; Staudacher et al., 2018). In line with these results, matrix Grx1-roGFP2 responses in a Δtsa1Δtsa2 strain became attenuated upon application of exogenous H$_2$O$_2$ at lower concentrations than those used in assays with the wild-type strain (Appendix Fig S4C).

As a second approach to test for hyperoxidation, we used an SDS–PAGE-based redox shift assay to monitor the redox state of the single cysteine in mature endogenous Prx1, i.e., the peroxidatic cysteine C91 (Fig 4F). We used the maleimide compound mmPEG24, which upon modification of a cysteine thiol results in an increased mass of the modified protein that can be detected on SDS–PAGE (Kojer et al., 2015). Maleimides react with reduced cysteine thiols but not hyperoxidized cysteine residues or cysteine residues involved in disulfide bonds. Samples were either (i) treated with

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**Figure 4.**
mmPEG_{24} (steady state), (ii) treated with the reducing agent Tris(2-carboxyethyl)phosphine (TCEP) (unmodified), or (iii) treated with both TCEP and mmPEG_{24} (TCEP-reduced). TCEP reduces disulfide bonds and sulfenic acids but not sulfenic or sulfonic acids (Reisz et al., 2013). Under unperturbed conditions, the cysteine of Prx1 was found to be predominantly in the thiol/thiolate form and can therefore be modified by maleimide (Fig 4G, lane 3, compare Prx1-WT to Prx1-C91A). Next, we assessed the Cys91 redox state following treatment of cells with H$_2$O$_2$. We observed that upon treatment with 1 mM H$_2$O$_2$, half of the Prx1 pool became unreactive toward mmPEG$_{24}$ (Fig 4H, lane 3). This can only be partially reverted by addition of TCEP (Fig 4H, lane 2) indicating partial hyperoxidation of Prx1 already at this H$_2$O$_2$ concentration. Upon treatment with 10 mM H$_2$O$_2$, Prx1 is rendered completely unreactive toward mmPEG$_{24}$ (Fig 4H, lane 3), even after subsequent treatment with TCEP, indicative of hyperoxidation of the peroxidatic cysteine. Treatment of matrix DAO-expressing cells with d-alanine but not t-alanine also rendered Prx1 partially unreactive toward mmPEG$_{24}$ (Appendix Fig 4D and E). Thus, exposure of Prx1 to high concentrations of H$_2$O$_2$ renders its active-site cysteine maleimide inaccessible (likely hyperoxidized) and results in a lower capacity to elicit an H$_2$O$_2$-dependent $E_{GSH}$ response.

In the cytosol, hyperoxidation in the form of a sulfonic acid (but not a sulfonic acid) can be reverted by sulfiredoxin. However, sulfiredoxin is not thought to be present in the matrix of yeast mitochondria. We thus tested whether recovery of Prx1 activity after oxidative shock relies on de novo Prx1 translation rather than reduction of Cys91. To this end, we allowed cells to recover after acute H$_2$O$_2$ challenge in the presence or absence of cytosolic translation, while partial recovery of the $E_{GSH}$ response was observed within 2 h in the absence of cytosolic translation (Fig 4J and Appendix Fig S4F). In summary, these results strongly support the hypothesis that hyperoxidation of Prx1 prevents H$_2$O$_2$-induced oxidation of the matrix glutathione pool. The logical next step was therefore to ask whether Prx1 hyperoxidation and the consequent protection of the mitochondrial glutathione pool have any influence on cell viability under H$_2$O$_2$ stress.
Prx1 increases cell sensitivity to acute H₂O₂ stress

We assessed the importance of Prx1 for cell viability under both chronic and acute H₂O₂ stresses. Prx1 has previously been shown to be important for maintaining cell viability under chronic oxidative stress (Greetham & Grant, 2009). We confirmed this result using halo assays (Appendix Fig S4G–I). We observed a significant detrimental effect of Prx1 deletion for growth in the continuous presence of H₂O₂ (Fig 4I, left panel), while we observed no impact of Prx1 deletion for viability of cells growing in the continuous presence of the thiol oxidant N,N,N’,N’-tetramethylethylcarboxamide (diame) (Fig 4I, right panel). Expression of the Zea mays aquaporin ZmPip2.5 wild-type but not an inactive mutant, ZmPip2.5 ΔH199K, leads to an increased zone of growth inhibition in our halo assays (Appendix Fig S4H). These results support the idea that aquaporin can mediate the transport of H₂O₂ across membranes and suggest that in laboratory yeast strains, which typically harbor inactive aquaporins (Laize et al., 2000; Sabir et al., 2017), H₂O₂ influx across the plasma membrane is limited. This may partly explain why high concentrations of H₂O₂ are frequently required to observe effects in yeast assays. We next asked whether Prx1 deletion also rendered cells more sensitive to an acute H₂O₂ stress. To this end, we determined the percentage of viable cells following 30-min incubation with H₂O₂ at concentrations ranging from 0 to 25 mM (Appendix Fig S4I). As a control, we show that expression of the aquaporin ZmPip2.5 wild-type but not the inactive mutant, ZmPip2.5 ΔH199K, led to decreased viability upon acute H₂O₂ stress (Appendix Fig S4K). Surprisingly, when we compared wild-type and Δprx1 cells in these assays, we observed that Δprx1 cells were not more sensitive than wild-type cells at H₂O₂ concentrations below 10 mM, while at higher H₂O₂ concentrations, the presence of Prx1 was even found to be significantly detrimental (Appendix Fig S4L and M). Thus, Prx1 is not required for cell viability under acute H₂O₂ stress, and at higher H₂O₂ concentrations, its presence is detrimental.

The loss of Prx1 causes an adaptive response in the cytosol characterized by increased levels of Ctt1. To test whether these increased Ctt1 levels might explain the superior survival of Δprx1 compared to wild-type cells during acute stress, we analyzed a Δprx1Δcct1 strain in the presence or absence of Prx1 complementation (Appendix Fig S4N). Here, we also observed that cells lacking Prx1 performed better than cells containing Prx1 despite the absence of Ctt1 in both strains. Thus, the absence of Prx1 is specifically protective during acute H₂O₂ stress. Nonetheless, given that Prx1 hyperoxidation at high H₂O₂ concentration in wild-type cells should effectively mimic a Δprx1-like state, it could be reasonably argued that at high H₂O₂ concentrations, the acute stress experiment is, in effect, comparing Δprx1-like cells with Δprx1 cells. Thus, with these experiments it is not possible to determine the full extent of the protective effect of Prx1 hyperoxidation against acute H₂O₂ stress-induced cell death.

Prx1 hyperoxidation protects against H₂O₂-induced cell death

A rigorous assessment of the role of Prx1 hyperoxidation in acute H₂O₂ stress-induced cell death requires the development of matrix-targeted Prx1 variants, which are more resistant to hyperoxidation but nonetheless retain their capacity to oxidize glutathione. We were able to generate just such a Prx1 variant, a truncation mutant of Prx1, Prx1-P233stop (Fig 5A and Appendix Fig S5A). Indeed, we observed an H₂O₂ concentration-dependent increase in the response of a roGFP2-Prx1-P233stop probe, up to 5 mM exogenous H₂O₂, in contrast to the decreased response of a roGFP2-Prx1 probe above 2.5 mM exogenous H₂O₂ (compare Figs 4E and 5B). We further tested this increased resistance of Prx1-P233stop using our gel-based redox shift assay to probe for hyperoxidation. This confirmed that Prx1-P233stop was resistant to hyperoxidation after addition of up to 10 mM exogenous H₂O₂ (Fig 5C). In the same experiment, we observed hyperoxidation of wild-type Prx1 from 1 mM exogenous H₂O₂ (Fig 5C). Importantly, by monitoring the response of a matrix-localized Grx1-roGFP2 probe, we observed that the Prx1-P233stop variant was capable of transferring oxidation from H₂O₂ to glutathione to a similar extent as wild-type Prx1 (Fig 5D, black lines). Furthermore, when we repeated this experiment after an initial pre-treatment of our cells with 10 mM H₂O₂ for 10 min, we observed that the Grx1-roGFP2 response in Prx1-P233stop-expressing cells was larger than without H₂O₂ pre-treatment, opposite to what is observed in cells expressing wild-type Prx1 (Fig 5D, red lines). These results are consistent with the strongly decreased hyperoxidation sensitivity of the Prx1P233stop variant, which means that it remains active following the H₂O₂ pre-treatment. The increased Grx1-roGFP2 response after H₂O₂ pre-treatment is possibly due to hyperoxidation of cytosolic peroxiredoxins, meaning that more H₂O₂ reaches the mitochondrial matrix. Additionally, depletion of the matrix NADPH pool in the presence of a non-hyperoxidizable peroxiredoxin might contribute to a stronger Grx1-roGFP2 response. Importantly, we again observed that cells expressing functional Prx1 constructs were significantly more sensitive to acute H₂O₂ stress-induced cell death than cells lacking Prx1 or expressing an inactive Prx1 variant. The Prx1-P233stop variant also appeared to be more sensitive than Prx1-WT although this difference was statistically significant only at 10 mM H₂O₂ (Fig 5E and Appendix Fig S5C).

Mitochondrial glutathione oxidation correlates with cell death under acute H₂O₂ stress

To further investigate the relationship between matrix glutathione oxidation and cell death, we turned to the peroxiredoxin, PfAOP, from the parasite Plasmodium falciparum. PfAOP is known to efficiently transfer oxidation to glutathione and has a well-characterized mutant, L109M (Staudacher et al., 2015, 2018; Appendix Fig S6A). In vitro, L109M has an increased activity and decreased susceptibility to hyperoxidation compared to wild-type PfAOP. Under the conditions of our assay, PfAOP-L109M indeed more efficiently oxidized EsGSH than wild-type PfAOP. However, like the wild-type cells it became inactivated by an acute H₂O₂ pre-incubation (Appendix Fig S6B). Consistent with our previous results, we observed that cells expressing PfAOP-L109M were significantly more sensitive to acute H₂O₂ treatment-induced cell death than cells expressing Prx1 and much more sensitive than cells with no matrix-localized thiol peroxidase (Fig 6A and Appendix Fig S6C). PfAOP-L109M-expressing cells also appeared to be more sensitive to acute H₂O₂ treatment-induced cell death than wild-type PfAOP-expressing cells although these differences were not statistically significant.

Finally, we turned to targeting Tsa1, human PRDX3, and human PRDX5 (which are the two 2-Cys peroxiredoxins residing in the matrix of human mitochondria) as well as human PRDX6 (a 1-Cys peroxiredoxin found in the cytosol of human cells) to the
mitochondrial matrix of Δprx1 cells (Appendix Fig S6A). We found that Tsa1 only very inefficiently transferred oxidation to glutathione, consistent with thioredoxin being the preferred reductive partner for this protein (Tairum et al., 2012). A similar result was observed for PRDX3 and PRDX5 (Appendix Fig S6B). In all three cases, a slightly increased Grx1-roGFP2 response was observed in cells pre-treated with 10 mM H2O2, implying that at least a fraction of these proteins remains in a non-hyperoxidized state (Appendix Fig S6B). In contrast, PRDX6 led to efficient oxidation of glutathione based on the matrix Grx1-roGFP2 response. Furthermore, H2O2 pre-treatment further increased this response, again likely due to hyperoxidation-based inactivation of cytosolic peroxiredoxins (Appendix Fig S6B). Strikingly, cells expressing PRDX6 were significantly more sensitive to acute H2O2 stress-induced cell death compared to cells expressing Prx1 and particularly compared to Δprx1 cells transformed with PRDX3, Tsa1, or an empty plasmid (Fig 6A and Appendix Fig S6C). Overall, while cells expressing mitochondria-localized Tsa1, PRDX3, PRDX5, or only containing an empty plasmid remained up to 60% viable after 30-min treatment with 10 mM H2O2, cells expressing Prx1 were ~25% viable, while those expressing PfAOP-L109M or PRDX6 were only ~10% viable.

Eg50 Responses and acute stress assays indicated that PRDX6 is hyperoxidation-resistant. To directly test this, we performed the maleimide shift assay with PRDX6 in comparison with Prx1 (Appendix Fig S6D–F). We observed that PRDX6 cysteines became inaccessible to mmPEG modification at comparatively low H2O2 concentrations. However, by treating with TCEP before maleimide modification, we found that a large fraction of PRDX6 oxidation was
reversible and could therefore conclude that PRDX6 was not hyper-oxidized, indicating that PRDX6 is strongly resistant against hyper-oxidation. In conclusion, a lack of hyperoxidation sensitivity, which means that a peroxiredoxin remains active under acute H₂O₂ stress, leads to increased cell death probably due to oxidation of matrix glutathione (Fig 6B).

Mitochondrial glutathione oxidation is the predominant determinant of cell death following acute H₂O₂ stress

Is glutathione oxidation the main determinant for cell death or just a proxy for depletion of matrix NADPH levels? To answer this question, we first assessed the impact of GLR1 deletion on matrix E_GSH, with and without concomitant deletion of PRX1. In Δglr1 cells, GSSG reduction is impaired, thereby preserving the NADPH pool (at the expense of a more oxidized E_GSH), while in Δprx1 cells, GSH oxidation is impaired (preserving both the NADPH pool and E_GSH). In line with this, we observed a strongly increased matrix Grx1-roGFP2 response to exogenous H₂O₂ in Δglr1 cells compared to wild-type cells, which could not be rescued by expression of only the cytosolic form of Glr1. However, upon additional deletion of PRX1, to generate Δglr1Δprx1 cells expressing only cytosolic Glr1, this increased matrix Grx1-roGFP2 response was almost completely absent (Fig 7A). We next assessed the growth of these cells. Intriguingly,
we observed that Δglr1 cells had an extended lag phase, while the additional deletion of PRX1 (Δglr1Δprx1 cells) rescued this growth delay (Fig 7B). Lastly, we tested these cells in the acute H2O2 stress assay. We found that Δglr1 cells were severely impaired in their survival after acute stress (Fig 7C, 1 and 5 mM H2O2). However, the additional deletion of PRX1 improved survival during acute H2O2 stress.

Figure 7. Mitochondrial matrix GSSG accumulation drives cell death during acute H2O2 stress.

A The response of a mitochondrial matrix-localized Grx1-roGFP2 probe to 1 mM H2O2 in BY4742 wild-type cells with an empty vector, in Δglr1 cells transformed either with an empty vector or with a vector encoding wild-type Glr1 or the cytosolic form of Glr1, where the MTS-encoding region was removed, or in Δglr1Δprx1 cells transformed either with an empty vector or with a vector encoding the cytosolic form of Glr1. Cells were grown to exponential phase in SGal (−Leu, −Ura) medium. Error bars represent the standard deviation (n = 3 biological replicates, with cells obtained from three independent cultures for every strain and probe combinations. For each biological replicate, three technical replicates were performed).

B Growth curve of wild-type, Δprx1, Δglr1, and Δglr1Δprx1 cells in SD medium complemented with all amino acids (n = 3 biological replicates). Significance for the difference in the time the cultures reach 50% of their maximal OD600 was assessed with the t-test. Error bars (as ribbon) represent the standard deviation (n = 4 biological replicates, with cells taken from independent cultures for each individual biological replicate).

C H2O2 “acute stress” assay. Wild-type, Δprx1, Δglr1, and Δglr1Δprx1 cells pre-grown in SGal medium complemented with all amino acids to early exponential phase. Cells were treated with H2O2 at the indicated concentrations for 30 min. Subsequently, cells were diluted, and a fixed volume was plated on YPD plates. The number of viable colonies was counted after 2 days growth at 30°C, here represented as a percentage relative to the 0 mM pre-treatment. Error bars represent standard deviation (n = 3 biological replicates, with cells taken from independent cultures for each individual biological replicate). An inset with a different scale on the y-axis is presented for the 1 and 5 mM concentration to allow better interpretation.

D H2O2 “acute stress” assay. Δprx1Δglr1 cells co-transformed with an empty plasmid or with a plasmid encoding either wild-type Prx1, the P233stop or the C91A variants were grown in SGal (−Ura) to early exponential phase. Cells were treated with the indicated amounts of H2O2 for 30 min. Subsequently, cells were diluted, and a fixed volume was plated on YPD plates. The number of viable colonies was counted after 2 days growth at 30°C, here represented as a percentage relative to the 0 mM treatment. Error bars represent standard deviation (n = 3 biological replicates, with cells taken from independent cultures for each individual biological replicate).

E Model. Prx1 drives oxidation of glutathione upon exposure to hydrogen peroxide. During acute H2O2 stress, Prx1 becomes hyperoxidized, which effectively uncouples the glutathione pool from H2O2. Prx1 hyperoxidation thus helps to limit GSSG accumulation and contributes to cell survival.

Data information: Significance was assessed with Student’s 2-tailed, unpaired, t-test. *P < 0.05; **P < 0.01; and ***P < 0.001.
stress. Complementation of this double deletion strain with different Prx1-WT, Prx1-C91A, and Prx1-P233stop variants confirmed that an increased capacity to promote glutathione oxidation during acute H₂O₂ stress resulted in decreased cell survival (Fig 7D). Collectively, these data thus support the conclusion that matrix glutathione oxidation specifically promotes cell death (Fig 7E).

Discussion

Prx1 activity leads to the oxidation of glutathione

Prx1 is a 1-Cys peroxiredoxin in the Prx6 subfamily (Nelson et al., 2011). The physiological reductive mechanism of such peroxiredoxins remains unclear. Studies on Prx1 from S. cerevisiae have differentially concluded that: (i) GSH is part of the reductive mechanism of Prx1 but is not oxidized in the process, i.e., GSH serves as a “resolving cysteine”, forming a disulfide bond with the Prx1 peroxidatic cysteine, which is reduced by Trx3 (Pedrajas et al., 2016); (ii) GSH forms a transient mixed disulfide bond with the Prx1 peroxidatic cysteine that is subsequently reduced by Grx2, ultimately leading to GSSG formation (Pedrajas et al., 2010); (iii) GSH forms a transient mixed disulfide with the Prx1 peroxidatic cysteine that is subsequently reduced by Trr2, leading to the formation of a transient intermolecular disulfide between Prx1 and Trr2 that is reduced by GSH leading to GSSG formation (Greetham & Grant, 2009); and (iv) in vitro, the mitochondrial thioredoxin system, Trx3 and Trr2, can efficiently reduce Prx1 (Pedrajas et al., 2000).

While the study of the Prx1 reductive mechanism was not a primary objective of our study, our data unequivocally show that, inside living cells, Prx1 activity very efficiently drives GSSG formation. While it is not possible to conclude whether the involvement of GSH in Prx1 reduction is direct or indirect, our data nonetheless allow us to exclude models that suggest no involvement of GSH in Prx1 reduction. The involvement of GSH in the reduction of Prx6-type peroxiredoxins appears not to be restricted to yeast Prx1 as human PRX6, targeted to the yeast mitochondrial matrix, also very efficiently elicited GSSG formation upon treatment of cells with H₂O₂. This observation is interesting as previously PRDX6 was suggested to require a GST-Pi to mediate its enzymatic activity (Manevich et al., 2004; Zhou et al., 2013). Our observations show that GST-Pi cannot be essential for PRDX6 activity as there is no GST-Pi in the yeast matrix, although it cannot be completely excluded that other enzymes in the yeast matrix may fulfill a similar role.

The observation that H₂O₂-induced GSH oxidation requires Prx1 also supports the more general assertion that H₂O₂-induced oxidation of most cellular thiols requires enzymatic catalysis. Indeed, cytosolic thioredoxin oxidation under acute H₂O₂ stress was also shown to be dependent on the presence of cytosolic peroxiredoxins (Day et al., 2012). Furthermore, it seems increasingly likely that H₂O₂-induced oxidation of many, if not most, H₂O₂-sensitive protein thiols requires enzyme catalysis, namely by thiol peroxidases (Delaunay et al., 2002; Bozonet et al., 2005; Jarvis et al., 2012; Sobotta et al., 2015; Stocker et al., 2018). Together, these studies underline the importance of kinetics and kinetic (un)coupling in determining the relative poise of different cellular redox species and redox couples and underline the conclusion that there is no such thing as a general cellular or subcellular “redox state”.

Mitochondrial glutathione oxidation leads to cell death

In this study, we have demonstrated that oxidation of the mitochondrial glutathione pool, driven almost exclusively by Prx1 activity, is an important determinant of cell death upon exposure to an acute H₂O₂ stress (Fig 7E). The groups of Elizabeth Veal and Chris Grant have previously reported that oxidation of cytosolic thioredoxins in fission yeast and oxidation of the mitochondrial thioredoxin in budding yeast, respectively, correlate with H₂O₂-induced cell death (Day et al., 2012; Greetham et al., 2013). These studies are in line with our findings and suggest that depletion of cellular reductive systems is an important determinant of cell death upon an acute H₂O₂ stress and not, as might have previously been expected, an accumulation of “oxidative damage”. Nonetheless, the conclusions from these different studies raise the question of which, if any, redox couples are directly leading to cell death, i.e., is oxidation of thioredoxins or glutathione important, or are both perhaps simply markers of NADPH depletion. Furthermore, it may also be asked whether oxidation in the cytosol or mitochondrial matrix is more important for triggering cell death.

To address the above questions, we have employed several assays to allow us to distinguish between the relative importance of different matrix redox couples. First, by employing an matrix “redox engineering” approach in which we targeted different peroxiredoxins, with differing sensitivities to hyperoxidation and differing abilities to transfer oxidation to glutathione, we revealed a strong correlation between matrix glutathione oxidation and cell death upon acute H₂O₂ stress. The use of engineered peroxiredoxins (even from other species and compartments) in this study also argues against Prx1 exerting a specific intrinsic cell death signaling function. Second, to enable us to distinguish between glutathione oxidation and other possible causes of cell death, for example, NADPH depletion, we turned to strains deleted for glutathione reductase. Specifically, we observed that deletion of the matrix form of glutathione reductase leads to matrix glutathione oxidation, even under “unperturbed” conditions, and leads to a delayed entry to exponential growth. Intriguingly, we found that concomitant deletion of PRX1 in this background restored matrix GSH to a wild-type-like value and completely rescued the delayed cell growth. Furthermore, we found that Aglr1 cells fared much worse that wild-type cells at 1 and 5 mM exogenous H₂O₂ in an acute H₂O₂ stress assay. Importantly however, the additional deletion of PRX1 at least partially rescued the increased sensitivity of a Aglr1 strain. As deletion of GLR1 would not be expected to decrease NADPH levels, these assays support the hypothesis that matrix glutathione oxidation specifically is detrimental for cell fitness. Nevertheless, it is important to emphasize that we cannot completely exclude that NADPH depletion may also be an important determinant of cell death upon acute H₂O₂ stress. Perhaps both matrix GSSG accumulation and NADPH depletion synergistically lead to cell death under many circumstances. However, our evidence does clearly support a role for matrix GSSG accumulation in driving cell death that is additional to, and independent of, any role of NADPH depletion. This conclusion is also consistent with a previous study that reported that the increased oxidant sensitivity of yeast cells lacking glutathione reductase can be ascribed to the loss of matrix glutathione reductase activity (Gostimskaya & Grant, 2016).
Is Prx1 hyperoxidation physiologically relevant?

In wild-type cells, it was proposed that Prx1 hyperoxidation represents an important protective mechanism to mitigate against oxidation of the matrix glutathione pool in conditions of acute H₂O₂ stress. However, this leads to the question of whether peroxiredoxin oxidation is physiologically relevant, i.e., will cells ever encounter conditions severe enough to induce peroxiredoxin oxidation during their “normal” lifestyle?

It is our contention that the almost universal conservation of hyperoxidation sensitivity-associated GGLG and YF motifs among eukaryotic typical 2-Cys peroxiredoxins argues that there is a strong evolutionary selective pressure to evolve or maintain hyperoxidation sensitivity (Wood et al., 2003; Hall et al., 2009). Indeed, many bacterial typical 2-Cys peroxiredoxins are robust against hyperoxidation, without any apparent detrimental impact upon catalytic efficiency (Ferrer-Sueta et al., 2011; Perkins et al., 2014). One possible reason for maintaining hyperoxidation sensitivity is that hyperoxidized peroxiredoxins serve specific functions within the cell, for example, recruitment of chaperones to protein aggregates (Hanzen et al., 2016) or prevention of excessive oxidation of important cellular redox couples and therefore maintenance of cell viability, as shown in this study and in others (Day et al., 2012; Veal et al., 2018). Other conserved motifs were recently shown to further fine-localization, and the typical H₂O₂ concentrations that it will likely serving to tailor each peroxiredoxin to its specific function(s), example, recruitment of chaperones to protein aggregates (Hanzen et al., 2016) or prevention of excessive oxidation of important cellular redox couples and therefore maintenance of cell viability, as shown in this study and in others (Day et al., 2012; Veal et al., 2018). Other conserved motifs were recently shown to further fine-localization, and the typical H₂O₂ concentrations that it will encounter. In the specific case of Prx1, the possibility to generate a hyperoxidation-insensitive variant, without any apparent loss in catalytic prowess, by removal of part of the C-terminus (P233STOP) implies that specific motifs also exist in Prx6-family, 1-Cys peroxiredoxins that confer sensitivity to hyperoxidation. However, the identity of such motifs was beyond the scope of this study and remains completely unclear. For Prx1, it remains to be demonstrated to what extent hyperoxidation occurs under “normal” physiological conditions in yeast cells grown in standard laboratory conditions. However, it is important to note that our data show a progressive increase in Prx1 hyperoxidation with increasing H₂O₂ concentration. We observed Prx1 hyperoxidation with as little as 1 mM exogenous H₂O₂: it is unclear what this means in terms of mitochondrial matrix H₂O₂ levels, but likely they are at most in the low micromolar range in these conditions. Thus, Prx1 hyperoxidation is not a “digital” all-or-nothing switch but rather an “analog” switch, implying that hyperoxidation of even a fraction of Prx1 may be beneficial for cell survival and therefore arguing strongly in favor of the physiological relevance of this mechanism. Investigation of the possible biological reasons for the wide range of peroxiredoxin hyperoxidation sensitivities will undoubtedly be an interesting area for future research.

As a final note, caution should always be applied when interpreting results obtained with laboratory yeast strains, grown in a carefully controlled environment. It is unclear what “normal physiological conditions” means for a yeast strain living in its wild environment. The impact of environmental stresses on cellular redox homeostasis, for example, heat, desiccation, UV radiation, limitation of key nutrients and exposure to xenobiotic compounds, is barely understood. Furthermore, certain potentially H₂O₂ relevant proteins, for example, aquaporins, are typically inactive in many laboratory yeast strains (Laize et al., 2000; Sabir et al., 2017). This suggests that adaptation, likely to frequent freezing and thawing, might explain an increased resistance to externally added H₂O₂. In line with this, artificial expression of aquaporins in the yeast plasma membrane (Appendix Fig S4) rendered these cells more sensitive to chronic and acute H₂O₂ stresses. It is thus tempting to speculate that hyperoxidation may be a highly relevant phenomenon for true wild-type cells in their native environment.

Compartment-specific NADPH-independent systems to regulate GSH

GSH is robustly maintained in most investigated compartments (Meyer et al., 2007; Kojer et al., 2012; Morgan et al., 2013; Elbaz-Alon et al., 2014). In the cytosol and the matrix, NADPH-dependent glutathione reductase provides the major mechanism of GSH maintenance. Impairment of glutathione reductase in the cytosol is compensated by crosstalk between the glutathione and thioredoxin redox systems, and a conserved NADPH-independent GSH maintenance pathway, i.e., the ABC transporter-dependent removal of GSSG from the cytosol (Minich et al., 2006; Morgan et al., 2013). In the matrix, GSSG accumulation is prevented under conditions of acute stress by switching off Prx1 activity, which appears to be the exclusive source of GSSG upon H₂O₂ exposure. Inactivation of Prx1 is achieved by apparently irreversible hyperoxidation that can only be reversed by synthesizing and importing new Prx1. This indicates that under acute stress conditions, the prime imperative for the matrix seems to be avoiding excessive oxidation of the glutathione pool rather than the immediate removal of H₂O₂, thereby ensuring cell viability.

A cytosolic adaptive response occurs upon matrix redox enzyme perturbation

Restricting the amounts of H₂O₂, which reach the matrix by efficient cytosolic H₂O₂ removal, appears to be a further strategy of mediating compartment-specific redox homeostasis. Under unperturbed conditions, the 2-Cys peroxiredoxins Tsa1 and Tsa2 perform this task. By employing transcriptome analyses, we also found evidence for a cytosolic adaptive response, which is induced under conditions of impaired function of matrix redox enzymes. This adaptive response specifically involves upregulation of the catalase, Ctt1, thereby providing an additional NADPH-independent system of H₂O₂ handling to further decrease the amount of H₂O₂ reaching the matrix. It will be extremely interesting to identify the signal that allows for a nuclear transcriptional response upon disruption of matrix redox enzymes. It is tempting to speculate that there is a specific target protein involved in the signaling mechanism whose activity is regulated by a Prx1-dependent post-translational redox modification.

Materials and Methods

All reagents were purchased from Sigma-Aldrich unless otherwise stated.

Generation and growth of yeast strains

All experiments were performed in a BY4742 strain background (EUROSCARF, Frankfurt, Germany). Strains are listed in...
Appendix Table S1. Gene deletion strains were constructed using a PCR-based standard homologous recombination technique (Janke et al., 2004). Gene deletions were confirmed by PCR using primers designed to anneal ~ 100 bp up- and downstream of the gene of interest. Yeast strains were grown as described previously (Kojer et al., 2012). Briefly, for the roGFPS2-based experiments and the redox shift experiments, the strains were grown in synthetic medium (S-medium) lacking the appropriate amino acids for plasmid selection (given in the standard three-letter code, e.g., S-medium—Leu) with 2% galactose (Gal) as carbon source at 30°C. The medium used for growth of strains is given for each experiment in the Figure Legends. As an example, “S-Gal-Leu” indicates synthetic medium with galactose as carbon source and lacking the metabolic marker amino acid leucine. For the acute stress experiments, the strains were grown in synthetic medium lacking the appropriate amino acids for plasmid selection with 2% glucose (D) or 2% galactose (Gal) as carbon source. After H2O2 exposure, cells were plated on rich media plates with glucose as carbon source (YPD). Growth to assess cell viability following the stress assays took place at 30°C for 2 days.

For 1 l synthetic medium (S-medium), 1.7 g yeast nitrogen base (without amino acids) and 5 g ammonium sulfate were dissolved in water at pH 5.5. In the final medium, as carbon source either 2% glucose (D), 2% galactose (Gal), or 2% glycerol (G) was present. In addition, according to the auxotrophic selections, the following amino acids were either present or excluded: adenine (0.15 mM), lysine (0.20 mM), leucine (0.23 mM), histidine (0.10 mM), tryptophan (0.07 mM), and uracil (0.18 mM). For 1 l rich medium (yeast–peptone, YP-medium), 10 g yeast extract and 20 g bacto-peptone were dissolved in water at pH 5.5. In the final medium, as carbon source either 2% glucose (D), 2% galactose (Gal), or 2% glycerol (G) was present. All plates have been prepared following the recipes described above and adding agar 20 g/l.

Primer and plasmid construction

All roGFPS2 sensors were constructed as previously described (Gutsch et al., 2008; Morgan et al., 2016). All roGFPS2-fusion proteins used in this study were constitutively expressed from the low copy-number (CEN) p415 (LEU2 marker) or p416 plasmids (URA3 marker) under the control of a constitutive TEF promoter (from translation elongation factor 1 a gene, TEF2, yeast). For mitochondrial matrix targeting, indicated constructs were genetically fused with the N-terminal mitochondrial targeting sequence (MTS) from subunit 9 of the F0-ATPase (Su9) from Neurospora crassa (encoding amino acids 1–69).

The mitochondrial peroxidereoxin PRX1 gene was amplified by PCR including endogenous promoter and terminator from yeast genomic DNA preparations and cloned into the low copy-number (CEN) p416 (URA3 marker) vector using standard molecular cloning procedures, removing the TEF promoter and CYC terminator from yeast genomic DNA preparations and cloned into the low copy-number p416 vector (URA3 marker) with TEF promoter. To allow mitochondrial targeting, the TSA1 gene was genetically fused with the N-terminal Su9 MTS.

The AOP constructs from P. falciparum were constructed as previously described (Staudacher et al., 2018). They were genetically fused with the N-terminal Su9 MTS and expressed in the low copy-number p416 vector (URA3 marker) with TEF promoter.

The genes of Homo sapiens PRDX3, PRDX5, and PRDX6 were amplified by PCR from HEK293 cell cDNA preparations and cloned, respectively, into the low copy-number p416 (URA3 marker), p415 (LEU2 marker), and p413 (HIS3 marker) vectors with TEF promoter and C-terminal triple hemagglutinin (HA), hexahistidine (His6), or FLAG tags. Only the PRDX6 gene was genetically fused with the N-terminal Su9 MTS, while for PRDX3 and PRDX5 genes, their endogenous MTS was used for mitochondrial targeting.

The constructs for the yeast-codon-optimized aquaporin Pip25 wild-type and H199K variants from Z. mays were previously described (Bienert et al., 2014). They were expressed in the multi-copy prS426-pTPIu (URA3 marker) vector with TPI promoter (from triosephosphate isomerase gene, TPI1, yeast).

The gene of D-amino acid oxidase (DAO) from Rhodotorula gracilis (red yeast) was amplified by PCR from the “HyPer-D-amino acid oxidase” plasmid generated and characterized in Matlabosh et al. (2014). To allow mitochondrial targeting, the PCR product was genetically fused with the N-terminal Su9 MTS and C-terminal FLAG tag; subsequently, it was cloned into the low copy-number p413 (HIS3 marker) vector under the control of the strong constitutive GPD promoter (from glyceraldehyde-3-phosphate dehydrogenase gene, TDH3/GPD, yeast). For a detailed list of primers and plasmids, see Appendix Table S2.

Antibodies

The antibody against yeast Prx1 was generated in this study. Mature Prx1 without the MTS was subcloned into pRSET-A plasmid. Prx1 was expressed for 4 h at 30°C in Escherichia coli BL21 (DE3) cells, purified via its N-terminal hexahistidine (His6) tag and used for in-house immunization of rabbits. The serum was confirmed in immuno-blot analysis using the purified antigen as well as comparing yeast cells lacking Prx1 with wild-type cells. The serum was used in a 1:1,000 dilution in 5% Milk-TBS for Western blot detection (Milk powder 5%, NaCl 150 mM, Tris–HCl 10 mM, adjusted to pH 7.5).

The antibody against PRDX6 was purchased from Sigma (ID: P0058) and used 1:2,000 dilution in 5% milk–TBS for Western blot detection.

Fluorescence measurement of roGFPS2-sensor oxidation

Fluorescence measurements were performed with a CLARIOStar (BMG Labtech) fluorescence plate reader as described previously (Morgan et al., 2011). Fluorescence was recorded using filter optics at excitation wavelengths 410 ± 5 and 482 ± 8 nm and an emission wavelength of 530 ± 20 nm. For measurements, yeast cells were grown to mid-log phase in synthetic medium lacking the appropriate amino acids for plasmid selection. The cells were harvested by centrifugation at 1,500 × g for 3 min at room temperature and subsequently resuspended in an isosmotic buffer (sorbitol 0.1 M, Tris–HCl pH 7.4 0.1 M, NaCl 0.1 M) to a final concentration of 1.5 OD600 units/ml (where 1 OD600 unit represents 1 ml of culture with
an OD$_{600} = 1$). This process was repeated one more time. Subsequently, each cell solution was transferred to a flat-bottomed 96-well imaging plate (BD Falcon) with 180 µl solution per well. To one well, 20 µl of the oxidant diamide was added (final concentration of 20 mM), serving as fully oxidized sensor control. To a second well, 20 µl of the reductant DTT was added (final concentration of 100 mM), serving as fully reduced sensor control. Cells not expressing the roGFP2-based sensors, yet treated with diamide, DTT, or buffer, were used as blanks. The cells were pelleted by centrifugation at 20 × g for 5 min at room temperature and placed in the instrument, kept at 30°C. A “steady state” was measured for 10 min, then the sample cells wells were subjected to experimental treatments adding 20 µl of a 10× experimental solution (e.g., containing H$_2$O$_2$), and the response followed for 120 min. Water was used as negative control for H$_2$O$_2$, and ethanol with final concentration 0.1% was used as negative control for antimycin A treatments. The degree of sensor oxidation (OxD) was calculated as in Equation (1) (Meyer & Dick, 2010; Morgan et al, 2011):

$$\text{OxD} = \frac{R_{\text{Sample}} - R_{\text{RED}}}{R_{\text{RED}}} \times \left(1 + \frac{R_{\text{RED}} - R_{\text{Sample}}}{R_{\text{Sample}}} \right)$$

with \( R = \frac{I_{482}}{I_{482}} \)

\( I_n \) = intensity at a given wavelength \( n \); \( R_{482} \), and \( R_{\text{RED}} \) = intensities at 482 nm upon complete oxidation by diamide or reduction by DTT. All experiments were performed in three independent biological replicates unless stated otherwise.

**“Acute stress-washout” experiment with genetically encoded sensors**

Yeast cells were grown to mid-log phase in synthetic medium lacking the appropriate amino acids for plasmid selection. The cells were harvested by centrifugation at 1,500 × g for 3 min at room temperature and resuspended in isosmotic buffer to a final concentration of 1.5 OD$_{600}$ units/ml. This process was repeated one more time. A bolus of either d-Alanine or l-Alanine (final 0.15 M) was added to the cell suspension. The cells were kept shaking at 30°C for the indicated time. d-/l-Alanine was removed with two centrifugation steps at 1,500 × g for 3 min at room temperature and following resuspension in isosmotic buffer to a final concentration of 1.5 OD$_{600}$ units/ml. Subsequently, each cell solution was transferred to a flat-bottomed 96-well imaging plate, and the experiment followed the classical fluorescence measurement of roGFP2-sensor oxidation.

**“Acute stress-washout” experiment with genetically encoded sensors and cytosolic translation inhibition experiment**

Yeast cells were prepared as in the “Acute stress-washout” experiment until the removal of H$_2$O$_2$ with two centrifugation steps at 1,500 × g for 3 min at room temperature. They were resuspended in the proper selective medium in the presence of either 50 µg/ml of the ribosome inhibitor cycloheximide (CHX) or dimethyl sulfoxide (DMSO) as control. After 2-h shaking at 30°C, cells were harvested and underwent two centrifugation steps at 1,500 × g for 3 min at room temperature, followed by resuspension in isosmotic buffer to a final concentration of 1.5 OD$_{600}$ units/ml. Subsequently, each cell solution was transferred to a flat-bottomed 96-well imaging plate, and the experiment followed the classical fluorescence measurement of roGFP2-sensor oxidation. Controls at time zero, before the addition of cycloheximide or DMSO, but after the pre-treatment with H$_2$O$_2$, were also measured.

**Matrix DAO-based “Acute stress-washout” experiment with genetically encoded sensors**

Yeast cells expressing Su9-DAO were grown to mid-log phase in synthetic medium lacking the appropriate amino acids for plasmid selection. The cells were harvested by centrifugation at 1,500 × g for 3 min at room temperature and resuspended in pre-heated fresh medium to a final concentration of 1 OD$_{600}$ units/ml. This process was repeated one more time. A bolus of either d-Alanine or l-Alanine (final 0.15 M) was added to the cell suspension. The cells were kept shaking at 30°C for the indicated time. d-/l-Alanine was removed with two centrifugation steps at 1,500 × g for 3 min at room temperature and following resuspension in isosmotic buffer to a final concentration of 1.5 OD$_{600}$ units/ml. Subsequently, each cell solution was transferred to a flat-bottomed 96-well imaging plate, and the experiment followed the classical fluorescence measurement of roGFP2-sensor oxidation.

**RNA preparation, sequencing, and analysis**

Yeast cells were grown to early-log phase in synthetic medium lacking the appropriate amino acids for plasmid selection. 5 OD$_{600}$ units of cells were harvested by centrifugation at 1,500 × g for 3 min at room temperature and resuspended in isosmotic buffer, and a second centrifugation step was necessary to retrieve pellets. For the Illumina library preparations, total RNA was first extracted from collected pellets using the RiboPure™ RNA Purification Kit, yeast (Invitrogen), according to the manufacturer’s protocol. RNA-Seq libraries were prepared from total RNA using poly(A) enrichment of the mRNA (mRNA-Seq) and later analyzed on an Illumina HiSeq 4000 with a read-length of 2 × 75 base pairs. The sequencing data were uploaded to the Galaxy web platform (usegalax y.org), and bioinformatics analysis was performed using the tools available on the public server (Afgan et al, 2016). Reads were aligned using the HISAT2 tool (Kim et al, 2015) and mapped using the S. cerevisiae R64-1-1.91 genome reference GTF file. The counting was performed using the featureCount tool. Statistical analysis was performed for three independent biological replicates, with the final differential expression between strains calculated using the package DESeq2 (Love et al, 2014). All tools were used with the default settings. The differential expression output data were represented in a volcano plot using the program OriginLab. The GO term analysis was performed using the online tool Gorilla (Eden et al, 2009).

**In vivo redox state analyses of Prx1 using mmPEG$_{24}$-based alkylation**

Mature Prx1 without its MTS contains only one cysteine, the active-site cysteine at position 91 (C91). To distinguish the redox states of this cysteine, a mmPEG$_{24}$-based alkylation was applied, yielding a shift of ~2.4 kDa in the migration behavior of reduced Prx1 in a Western blot analysis (Koje et al, 2012). Briefly, yeast cells were
grown to mid-log phase in synthetic medium lacking the appropriate amino acids for plasmid selection. Cells were harvested by centrifugation at 1,500 × g for 3 min at room temperature and resuspended in isosmotic buffer to a final concentration of 1.5 OD_{600} units/ml. This process was repeated. Pellets of 2 OD_{600} units were resuspended in 100 μl SDS-loading buffer containing either 10 mM mmPEG24 (steady state), DMSO (unmodified), or 10 mM TCEP (maximally reduced) and boiled for 5 min at 96°C. The cells were then disrupted by vortexing with glass beads for 15 min in the dark. In the reduced sample, mmPEG24 was added to a final concentration of 10 mM. After 40 min in the dark, all samples were analyzed in Western blots against Prx1.

Cells were also exposed to a pre-treatment as explained for the “Oxidation shock” experiment. In that case, after the pre-treatment 2 OD_{600} units of cells were centrifuged at 20,000 × g for 30 s at room temperature and directly resuspended in 100 μl SDS-loading buffer containing 10 mM mmPEG24. They were boiled, disrupted, and analyzed by Western blot as described above.

To test for irreversibility and the type of inactivation occurring in Prx1 after the hydrogen peroxide pre-treatment, 2 OD_{600} units of cells were resuspended in 100 μl SDS-loading buffer containing either DMSO (unmodified), 10 mM TCEP (TCEP reduces disulfides and sulfenic acids only), or 10 mM mmPEG24 (steady state), and boiled for 5 min at 96°C. The cells were then disrupted vortexing with glass beads for 15 min in the darkness. In the TCEP-reduced samples, mmPEG24 was added to a final concentration of 10 mM. After 40 min in the darkness, all samples were analyzed in Western blots against Prx1.

To identify the redox state of Prx1 in the samples used in the Matrix DAO-based “Acute stress-washout” experiment, an aliquot of 2 OD_{600} units of yeast for each time point was acquired at the steps at 1,500 × g for 3 min at room temperature and resuspended in isosmotic buffer to a final concentration of 1.5 OD_{600} units/ml. The 2 OD_{600} units of yeast were centrifuged at 20,000 × g for 30 s at room temperature and directly resuspended in 100 μl SDS-loading buffer containing 10 mM mmPEG24. They were boiled, disrupted, and analyzed by Western blot as described above.

"Acute stress" viability assay

Yeast cells were grown to mid-log phase in synthetic medium lacking the appropriate amino acids (S-medium) for plasmid selection or in YP-medium if no plasmid was present both with either 2% glucose or 2% galactose as carbon source. 10 OD_{600} units of cells were harvested by centrifugation at 1,500 × g for 3 min at room temperature and then resuspended in water to a final concentration of 10 OD_{600} units/ml. Subsequently, the cell solution was split into five tubes and centrifuged at 1,500 × g for 3 min at room temperature. The five 2 OD_{600} pellets were then resuspended in 1 ml of either water or H2O2 to the final concentration. After shaking at 30°C for 30 min, 3.5 μl of cells solution was added to 25 ml water to a final concentration of approximately 0.00025 OD_{500}/ml. Next, 200 μl was plated onYPD plate (ca. 500 cells), and after growth for 5 days at 30°C, the number of viable cells was determined by the counting the colonies using the software ImageJ.

“Halo” assay

Yeast cells were grown to mid-log phase in YP-medium with 2% glucose (YPD) as carbon source overnight and then diluted in YPD medium to a final concentration of 0.25 OD_{600}/ml. The culture was incubated at 30°C for 4 h and afterward diluted to a final concentration of 0.01 OD_{600}/ml in water. Subsequently, the suspension was used as inoculum and 100 μl spread on YP-medium 1% agar plates with either 2% glucose (YPD), 2% galactose (YPGal), or 2% glycerol (YPG). A 6-mm disk of cellulose loaded with 15 μl of 1 M diamide or 1 M H2O2 was placed at the center of the plate. From there, the reagent diffused into the plate. The plates were incubated at 30°C for 72 h, and then, the halo of growth around the disk was measured.

Statistical analysis

In all figures, error bars represent mean ± standard deviation. P-values were determined using the t-test.

Expanded View for this article is available online.

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Author contributions

GC, MD, BM, and JR designed the project, analyzed results, and wrote the article. GC, PSA, MNH, MM, TR, GPB, and EP performed the experiments and analyzed results.

Conflict of interest

The authors declare that they have no conflict of interest.

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