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Dissection of a Redox Relay: H_2O_2-Dependent Activation of the Transcription Factor Pap1 through the Peroxidatic Tpx1-Thioredoxin Cycle

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SUMMARY

In fission yeast, the transcription factor Pap1 undergoes H_2O_2-dependent oxidation that promotes its nuclear accumulation and the activation of an antioxidant gene program. However, the mechanisms that regulate the sensitivity and selectivity of Pap1 activation by peroxides are not fully understood. Here, we demonstrate that the peroxiredoxin Tpx1, the sensor of this signaling cascade, activates the otherwise unresponsive Pap1 protein once the main cytosolic reduced thioredoxin, Trx1, becomes transiently depleted. In other words, Pap1 works as an alternative electron donor for oxidized Tpx1. We have trapped the very transient Tpx1-Pap1 intermediate in cells depleted in Trx1, as we show here using mass spectrometry. Recycling of Tpx1 by Trx1 is required for the efficient signaling to Pap1, suggesting that the complete cycle of H_2O_2 scavenging by Tpx1 and further recycling of oxidized Tpx1 by Trx1 is required for full downstream activation of the redox cascade.

INTRODUCTION

Cysteine (Cys) residues are essential mediators of catalytic reactions. Many enzymes, such as ribonucleotide reductase and glutathione peroxidases or thioredoxin peroxidases, undergo reversible oxidation of catalytic Cys, often forming intra- or intermolecular disulfides as part of their enzymatic activities. Their catalytic cycles are completed through disulfide reduction by electron donors of the thioredoxin and glutaredoxin families, which recycle the enzymes back to their reduced thiol stages (for reviews on thioredoxins and glutaredoxins, see Collet and Messens, 2010; Meyer et al., 2009).

Previously, thiol groups in Cys residues had been believed to be oxidized by hydrogen peroxide (H_2O_2) and other oxidants to higher oxidation states, such as sulfenic acid (SOH), disulfides, and sulfinic and sulfonic acids, leading to the inactivation of proteins containing the oxidized Cys residues. However, biochemical observations indicate that Cys in general are not very reactive to peroxides and that only highly reactive thiols in proteins are likely to respond to physiological fluctuations of H_2O_2 (for reviews, see Winterbourn, 2008; Winterbourn and Hampton, 2008). Therefore, the oxidation of these highly reactive Cys in so-called H_2O_2 sensors could constitute a signaling event that results in the activation of antioxidant cellular responses to peroxide stress.

Glutathione peroxidases and thioredoxin peroxidases (peroxiredoxins) seem to have highly reactive Cys residues to respond to H_2O_2. Kinetic parameters of reactivity to oxidants suggest that these enzymes, which are peroxide scavengers, are likely to be the only direct targets of H_2O_2 at moderate concentrations. This allows them to accomplish their primary function, the detoxification of peroxides, and also provides the basis for how they may participate in oxidative-stress-dependent signaling. Thus, glutathione peroxidases and peroxiredoxins have been proposed to mediate the oxidation/activation of the downstream components of antioxidant cascades and act as the molecular mechanism of sensitivity and specificity in H_2O_2 signaling.

The glutathione-peroxidase-like Gpx3/Orp1 of *Saccharomyces cerevisiae* was shown by Toledano and colleagues to initiate a signaling cascade in response to H_2O_2 (Delaunay et al., 2002). The authors proposed that in response to peroxide stress, a reactive thiol group in Gpx3 is oxidized to SOH, which then reacts with a Cys residue in the transcription factor Yap1. The intermolecular disulfide is then resolved by another Cys residue in Yap1, yielding an intramolecular disulfide bond that affects protein structure and induces its nuclear accumulation (Delaunay et al., 2000, 2002). Activation of Yap1 then triggers a gene expression program that mediates the cellular defense to oxidative stress. The protein Ybp1 is required for this SOH-to-disulfide relay between Gpx3 and Yap1, either by acting as a molecular scaffold holding both proteins together (Veal et al., 2003) or by promoting Yap1 stability (Patterson et al., 2013).

Similarly, *Schizosaccharomyces pombe* responds to moderate doses of H_2O_2 by activating the transcription factor Pap1 (*pombe* AP-1), which accumulates in the nucleus in response to stress. Pap1 is cytosolic prior to the onset of stress, and at least one disulfide bond is formed upon peroxide addition, which triggers a conformational change impairing Crm1/exportin-mediated

nuclear export (Castillo et al., 2002; Kudo et al., 1999; Vivancos et al., 2004). The transcription factor is then able to transiently bind and activate 40–80 genes, whose encoded proteins counteract peroxide stress and induce adaptation responses (Calvo et al., 2012; Chen et al., 2008). In this system, the peroxiredoxin Tpx1 is essential to transduce the H_2O_2 signal to Pap1, since in cells lacking Tpx1, Pap1 oxidation and subsequent gene expression does not occur (Bozonet et al., 2005; Vivancos et al., 2005).

As in any other signaling cascade, proper activation of the pathway also relies on negative feedback loops, both to maintain the initiators/sensors in the reduced conformation and to inactivate the cascade once the response has been triggered. In these particular Cys-mediated events, the thioredoxin system has been proposed to mediate the maintenance of the reduced protein conformations. Thus, in the budding yeast, cells lacking the thioredoxin system display partial basal activation of Yap1, and upon peroxide treatment the activity of the pathway is more sustained than in a wild-type background (Delaunay et al., 2000). In the case of fission yeast, deletion of the thioredoxin reductase gene leads to complete oxidation/activation of Pap1 and its gene expression program (Benko et al., 1998; Calvo et al., 2012; Vivancos et al., 2004).

In an attempt to dissect the redox relay driving the antioxidant response to H_2O_2 in a eukaryotic organism, we analyzed the effects of mutations on the Tpx1 and thioredoxin pathways with regard to Pap1 activation. Our experiments suggest that Tpx1 normally scavenges peroxides and uses thioredoxins to restart the cycle and that only when reduced thioredoxin becomes limiting does Pap1 become an alternative electron donor for oxidized Tpx1. Pap1 oxidation, which leads to a gain of function, can only be achieved by a peroxiredoxin such as Tpx1, and only massive overexpression of another thioredoxin-dependent peroxidase can substitute for Tpx1 as a transducer of the H_2O_2 signal. Importantly, we were able to isolate the transient Tpx1-Pap1 intermediate by overexpression of the transcription factor in a *Δtrx1* background and confirm its identity by mass spectrometry.

RESULTS

The Absence of Tpx1, Thioredoxin Reductase, or the Cytoplasmic Thioredoxin Trx1 Has Different Effects on Pap1 Activation

Both Tpx1 and the *S. pombe* thioredoxin reductase Trr1 have been shown to participate in the H_2O_2-dependent activation and basal inactivation, respectively, of Pap1 (Figure 1A). Thus, the conformational changes observed in Pap1 upon mild H_2O_2 stress using nonreducing electrophoresis of trichloroacetic acid (TCA) protein extracts and western blot analysis were not observed in cells lacking Tpx1, whereas cells lacking Trr1 displayed constitutive activation/oxidation of Pap1 (Figure 1B) (Vivancos et al., 2004). Consistently, activation of H_2O_2- and Pap1-dependent gene expression was completely abolished in *Δtpx1* cells and constitutively engaged in cells lacking Trr1 (Figure 1C). Thus, we next determined which thioredoxin mediates Pap1 reduction under basal conditions.

The *S. pombe* genome contains three thioredoxin-coding genes (Wood et al., 2002). Trx1 is the main cytoplasmic thioredoxin (Song and Roe, 2008), Trx2 is localized to the mitochondria (Song et al., 2008), and Trx3/Txl1 has been reported to be both present in the cytoplasm and associated with the proteasome as well as having importance in the antioxidant defense mechanism (Andersen et al., 2011; Jiménez et al., 2007; Kim et al., 2007). The contribution of these electron donors to normal tolerance to peroxides was analyzed in vivo by sequential spotting on H_2O_2-containing plates (Figure S1); cells lacking Trx1 are extremely sensitive to peroxides, whereas Trx2 and Trx3 appear to be dispensable for defense against H_2O_2 stress. We confirmed the mitochondrial localization of Trx2-HA (Figure 1D) as well as the dual cytoplasmic and nuclear localization of hemagglutinin (HA)-tagged Trx1 and Trx3 by immunofluorescence microscopy (Figure 1D). It is worth mentioning that the HA tag at the carboxy-terminal domain of Trx1 did not affect the sensitivity to peroxides of the wild-type protein, while tagging Trx3 at its carboxy-terminal domain altered its role as an electron donor toward some of its substrates (S.B. and E.H., unpublished data); therefore, we used an amino-terminal tagged HA-Trx3. In conclusion, either Trx1 or Trx3 could contribute to the reduction of Tpx1 and/or Pap1, because all four proteins display cytoplasmic subcellular localization under basal conditions (Figures 1D and 4A).

Deletion of the genes encoding Trx3 or Trx2 did not affect the oxidation of Pap1 (Figure 1E). Unexpectedly, depletion of Trx1 did not trigger constitutive oxidation of Pap1, according to the results of nonreducing electrophoresis. In fact, in *Δtrx1* cell extracts, the H_2O_2-dependent shift in the electrophoretic mobility of Pap1 was clearly impaired (Figure 1E). To rule out the possibility that Trx3 contributed to the reduction of Pap1 under basal conditions, we analyzed Pap1 activation in a *Δtrx1 Δtrx3* strain. As shown in Figure 1F, the pattern of Pap1 oxidation in *Δtrx1 Δtrx3* extracts did not significantly differ from that of *Δtrx1* extracts.

These biochemical data suggested that not only Tpx1 but also Trx1 is required for Pap1 oxidation. Therefore, we deleted *tpx1*, *trx1*, or both in the *Δtrr1* background. In all cases, Pap1 was fully oxidized (Figure S2A), constitutively bound to the promoters (Figure S2B), and its gene expression program engaged (Figure S2C). As we have recently shown, deletion of the *trr1* gene induces general disulfide formation in approximately 30% of the Cys-containing peptides of the *S. pombe* proteome. This massive thiol oxidation is likely dependent on the generation of oxidizing redox couples, namely oxidized Trx1, oxidized Trx3, and oxidized Tpx1 (García-Santamarina et al., 2013). In fact, only when the genes coding for these three proteins were deleted in the *Δtrr1* background did basal general thiol oxidation return to wild-type levels (S.G.-S. and E.H., unpublished data) and Pap1 become constitutively reduced (Figure S2A), never associated to chromatin (Esther Paulo and E.H., unpublished data), and its gene expression program inactivated (Figure S2C). Thus, depletion of Trr1 leads to constitutive activation of Pap1, irrespective of the presence of Tpx1 or Trx1, probably due to the generation of H_2O_2-independent disulfide stress in this strain background.

Trx1 Is the Main Electron Donor of Tpx1: Trapping of a Mixed Disulfide between Cys 30 of Trx1 and the Resolving Cys of Tpx1

The peroxiredoxin Tpx1 is not only a H_2O_2 sensor in the Pap1 pathway, but it is also essential to scavenge peroxides

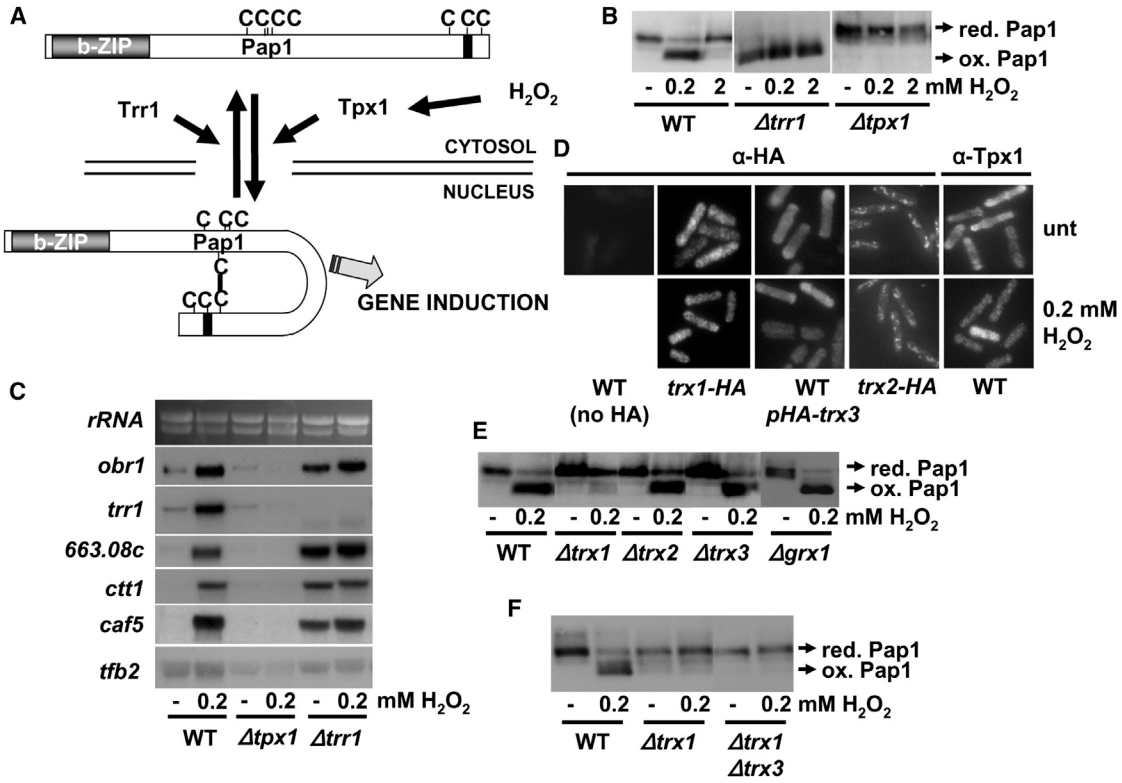

Figure 1. Role of Tpx1 and the Thioredoxin System in Pap1 Oxidation

(A) Schematic representation of Pap1 activation and inactivation. Upon H_2O_2, Tpx1 mediates oxidation of Pap1 and activation of the Pap1-dependent gene expression program. Thioredoxin reductase may mediate Pap1 reduction.

(B) In vivo oxidation of Pap1 in wild-type and mutant strains. Strains IC2 (WT), IC71 (Δ*trr1*), and MJ11 (Δ*tpx1*) were treated or not with 0.2 and 2 mM H_2O_2 for 5 min. TCA extracts were processed by nonreducing SDS-PAGE and analyzed by western blot with antibody against Pap1. Reduced/inactive (red. Pap1) and oxidized/active (ox. Pap1) Pap1 forms are indicated with arrows.

(C) Effect of Tpx1 and Trr1 in Pap1-dependent gene expression program. Cultures of strains as in (B) were treated or not with 0.2 mM H_2O_2 for 15 min. Total RNA was obtained and analyzed by northern blot with the indicated probes.

(D) Localization of Trx1, Trx2, Trx3, and Tpx1 by immunofluorescence. The localization of the indicated proteins of strains MJ3 (*trx1*-HA), AD2 (*trx2-HA),* IC2 + p442.3x (WT + p*HA-trx3*), and IC2 (WT) was analyzed by immunofluorescence with antibodies anti-HA or anti-Tpx1. As a control, the untagged wild-type strain was used.

(E and F) Trx1 is the only thioredoxin implicated in Pap1 oxidation. In vivo oxidation of Pap1 in strains IC2 (WT), MJ15 (Δ*trx1*), MJ16 (Δ*trx2*), SP2 (Δ*trx3*), IC38 (Δ*grx1*), and IC76 (Δ*trx1* Δ*trx3*) was analyzed as described in (B).

See also Figures S1 and S2.

during aerobic metabolism (Jara et al., 2007). To dissect the Tpx1-Pap1 signaling cascade, we first decided to analyze the Tpx1 cycle. Peroxiredoxins such as Tpx1 exist as dimers arranged head to tail, which upon reaction with H_2O_2 undergo oxidation of the peroxidatic Cys48 to SOH, disulfide formation with the resolving Cys169 of the antiparallel monomer, and release a molecule of H_2O. Reduced thioredoxins then act as electron donors to recycle the covalently linked peroxiredoxin dimers to the thiol form through the transient formation of a mixed disulfide.

To determine which thioredoxin mediates recycling of Tpx1 in vivo, we analyzed the basal levels of covalently linked Tpx1 dimers by nonreducing electrophoresis and western blot analysis of TCA extracts (Figure 2). As with many peroxiredoxins, Tpx1 is an abundant protein (Gunaratne et al., 2013) that is predominantly localized to the cytoplasm before and after stress (Figure 1D). Therefore, either Trx1 or Trx3, which are localized to both the cytoplasm and nucleus (Figure 1D), could recycle oxidized Tpx1. In wild-type cells, most Tpx1 is reduced under aerobic growth conditions and runs as a monomer in nonreducing denaturing gels, even though it is involved in detoxifying peroxides (Figure 2A and 2B, monomer). In extracts from H_2O_2-treated wild-type cultures, two slowly migrating bands are observed, which correspond to the covalently linked homodimers with one (lower band; 1 diS) or two (upper band; 2 diS) disulfides (Figure 2B). Cells lacking Trx2, Trx3, or Grx1 (the main cytoplasmic dithiol glutaredoxin in *S. pombe)* (Chung et al., 2005) showed a pattern of Tpx1 oxidation identical to that of the wild-type strain. However, in *Δtrx1* cells, most Tpx1 dimers contain one intradimer disulfide bridge under basal conditions, and H_2O_2 stress promotes the formation of two-disulfide-containing dimers (Figure 2B). Because Tpx1 is not fully oxidized in this strain background under basal conditions, we tested whether the other cytosolic thioredoxin, Trx3, could be an

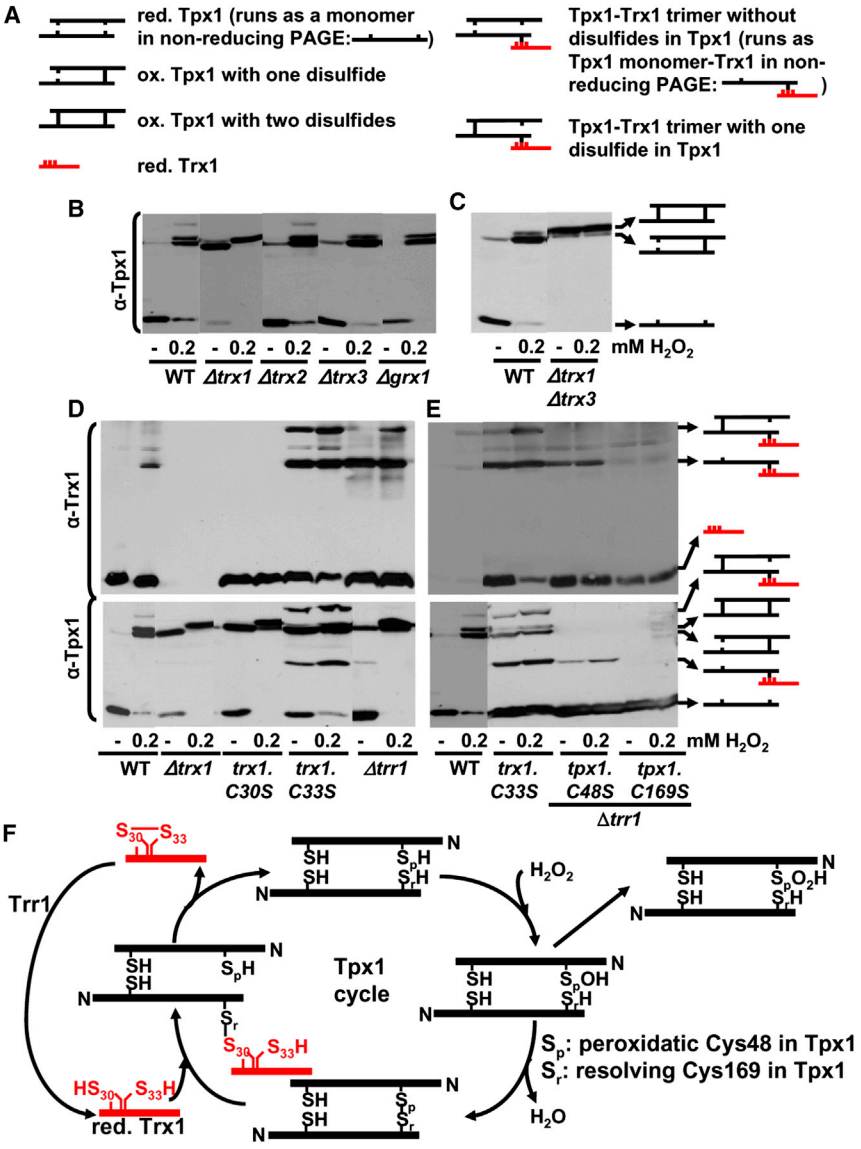

Figure 2. Trx1 Is the Main Electron Donor of Tpx1

(A) Scheme representing the Tpx1 (in black) dimer and Trx1 (in red) monomer, forming or not forming disulfides. Each vertical line represents the relative positions of all the Cys residues (two in Tpx1, three in Trx1).

(B) In vivo oxidation of Tpx1 in wild-type and mutant strains. The redox state of Tpx1 in extracts from Figure 1E was analyzed as described in Figure 1B using antibodies against Tpx1. Reduced and oxidized Tpx1 (with one or two disulfides) are indicated with arrows.

(C) Trx3 is an alternative electron donor for Tpx1 in the absence of Trx1. The redox state of Tpx1 in IC2 (WT) and IC76 (Δ*trx1* Δ*trx3*) was analyzed as described in Figure 1B, using antibodies against Tpx1.

(D and E) In vivo oxidation of Tpx1 and Trx1 in wild-type and mutant strains; trapping of a mixed disulfide between Tpx1 and Trx1. The different complexes are indicated by schemes.

(D) Strains IC2 (WT), MJ15 (Δ*trx1*), SG72.C30S (*trx1.C30S*), SG72.C33S (*trx1.C33S*), and IC71 (Δ*trr1*) were treated or not with 0.2 mM H_2O_2 for 5 min, and the oxidation status of Tpx1 and Trx1 was analyzed as described in Figure 1B, using polyclonal antibodies against Tpx1 or Trx1, as indicated.

(E) Same analysis as in (D) was performed from extracts of strains IC2 (WT), SG72.C33S (*trx1.C33S*), IC139 (Δ*trr1 tpx1.C48S*), and IC140 (Δ*trr1 tpx1.C169S*).

(F) Scheme of the Tpx1 cycle. Reduced Trx1 breaks the intramolecular disulfide of Tpx1 by disulfide exchange leading to a transient intermediate Trx1-Cys30-Cys169-Tpx1 to finally release oxidized Trx1 and reduced Tpx1. Cys169 is the resolving Cys of Tpx1.

See also Figure S3.

alternative electron donor for Tpx1. Indeed, cells lacking both Trx1 and Trx3 display full oxidation (two disulfide bonds per Tpx1 dimer) under basal conditions (Figure 2C). We conclude that Trx1 is the main electron donor of Tpx1 and that Trx3 can partially reduce Tpx1 disulfides in the absence of Trx1. Confirming the important role of Trx1 in Tpx1 recycling, the *Δtrx1* strain is the only deletion strain that exhibited a severe growth defect under H_2O_2 stress (Figure S1).

We then decided to trap the Trx1-Tpx1 intermediates by eliminating the resolving Cys residue in Trx1. Thioredoxins interact with their substrates through the N-terminal Cys of the thioredoxin fold, which contains the sequence Cys-X-X-Cys. The transient mixed disulfides are then resolved by nucleophilic attack with the thiol group of the C-terminal Cys of this sequence. We substituted the chromosomal *trx1* locus with the mutant alleles *trx1.C30S* or *trx1.C33S*, and demonstrated that strains expressing these mutant proteins were as sensitive to H_2O_2 as cells lacking Trx1 (Figure S3). As shown in Figure 2D, the pattern of Tpx1 oxidation was identical in the *Δtrx1* and *trx1.C30S* strains. However, two DTT-sensitive bands corresponding to Trx1-Tpx1 mixed disulfides, with or without an additional disulfide inside the Tpx1 dimer, were detected in extracts from *trx1.C33S* cells using antibodies against Trx1 or Tpx1 (Figure 2D). Interestingly, extracts from cells lacking thioredoxin reductase Trr1 also accumulated, although to a lesser extent, mixed disulfides between Tpx1 and Trx1 (Figure 2D). We postulated that in the *Δtrr1* background, in which Trx1 is fully oxidized, we could force the formation of this intermediate by expressing a form of Tpx1 lacking the Cys not involved in the formation of the bridge. Indeed, in extracts from *Δtrr1* cells expressing Tpx1.C48S, which lacks the peroxidatic Cys in Tpx1, we could clearly detect the Trx1-Tpx1 intermediate, whereas this mixed disulfide was not formed in *Δtrr1* cells expressing Tpx1.C169S (Figure 2E). We conclude that in the normal Tpx1 cycle, Trx1 breaks the disulfide between Cys48 and Cys169 of Tpx1 by dithiol-disulfide exchange and a Trx1-Cys30-Cys169-Tpx1 disulfide is

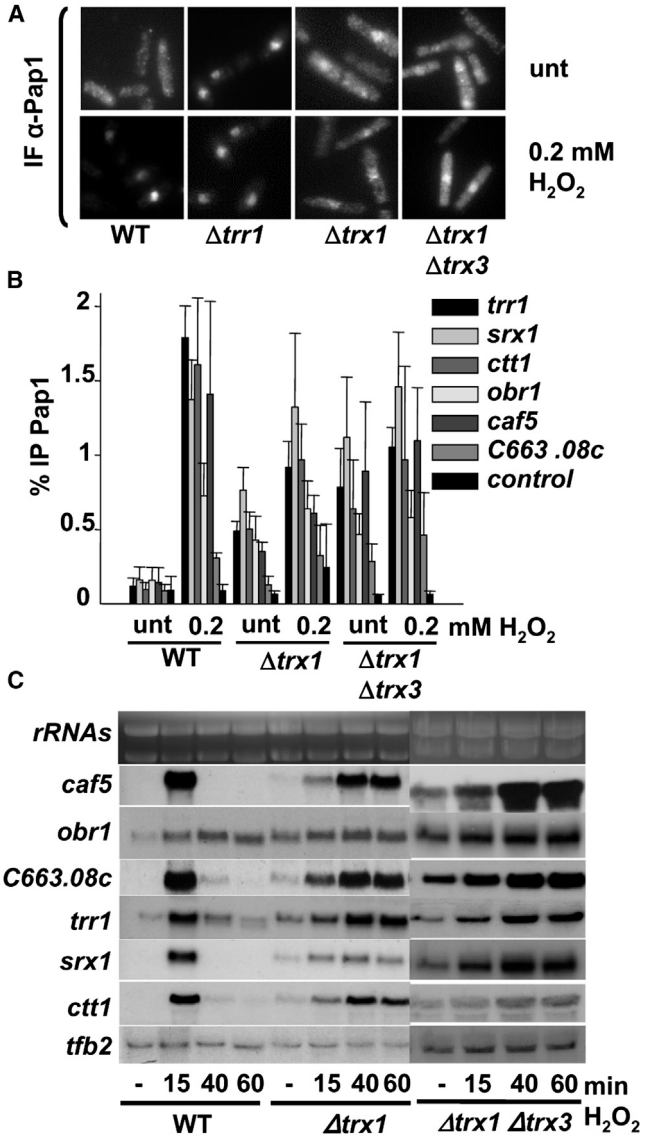

Figure 3. In the Absence of Trx1, Pap1 Basal Activity Is Higher and More Sustained after Stress Than in Wild-Type Cells

(A) Cellular localization of Pap1 in strains IC2 (WT), IC71 (Δ*trr1*), MJ15 (Δ*trx1*), and IC76 (Δ*trx1* Δ*trx3*) before and after 0.2 mM H_2O_2 for 5 min, as determined by immunofluorescence using polyclonal anti-Pap1.

(B) Pap1 is recruited to all Pap1-dependent promoters in strains Δ*trx1* and Δ*trx1* Δ*trx3* prior to stress. Cultures of IC2 (WT), MJ15 (Δ*trx1*), and IC76 (Δ*trx1* Δ*trx3*) strains were treated with 0.2 mM H_2O_2 for 5 min. ChIP experiments using anti-Pap1, coupled to quantification by real-time PCR, were performed using primers covering only promoter regions of *trr1*, *srx1*, *ctt1*, *obr1*, *caf5*, and *SPCC663.08c* genes. Primers of an intergenic region were used as a negative control (control). Error bars (SD) for all ChIP experiments were calculated from biological triplicates.

(C) H_2O_2-, Pap1-dependent transcription is maintained along the time in Δ*trx1* and Δ*trx1* Δ*trx3* backgrounds. Total RNA from strains as in (B) was analyzed by northern blot as described in Figure 1C before and after 0.2 mM H_2O_2 stress. See also Figure S4.

transiently generated to release oxidized Trx1 and reduced Tpx1 (Figure 2F).

Deletion of the Thioredoxin Gene Alters the Dynamics of Pap1 Oxidation by H_2O_2

Once the Tpx1 cycle was characterized at the molecular level, we analyzed the role of Trx1 in Pap1 activation. Mild oxidative stress induced transient oxidation of Pap1 as determined by nonreducing electrophoresis; the faster-migrating band could be detected in extracts from cells lacking Trx1 even prior to stress, and H_2O_2 treatment slightly enhanced its formation (Figures 1E, 1F, and S4A). Immunofluorescence of untagged Pap1 demonstrated that in a wild-type background, Pap1 fully accumulates in the nucleus upon H_2O_2 stress (Figure 3A), and the same change in localization was observed in cells lacking Trx2 or Trx3 (Figure S4B). Pap1 constitutive nuclear localization was observed in *Δtrr1* cells, with almost all of the fluorescence being detected in the nuclei (Figure 3A). An intermediate pattern was observed in cells lacking Trx1; a fraction of Pap1 could be visualized in the nucleus prior to stress (Figures 3A, S4B, and S4C). Further deletion of *trx3* in the *Δtrx1* background did not exacerbate either basal or stress-induced Pap1 nuclear localization (Figure 3A, *Δtrx1 Δtrx3*). Similar results were obtained with fluorescence microscopy using a GFP-Pap1 fusion protein, although Pap1 accumulation in *Δtrx1* cells was less pronounced (Figure S4D). Pap1 protein was bound to Pap1-dependent promoters prior to stress imposition in both *Δtrx1* and *Δtrx1 Δtrx3* background strains, as determined by chromatin immunoprecipitation (ChIP), and never reached the same levels as in wild-type cells upon H_2O_2 stress (Figure 3B). Furthermore, basal expression of Pap1-dependent gene expression was slightly upregulated in *Δtrx1* cells, and increased mRNA expression was delayed and sustained. A similar transcriptome profile was observed for the *Δtrx1 Δtrx3* strain (Figure 3C).

Slow and Sustained Activation of Pap1 in Cells Lacking Thioredoxins Is Fully Dependent on Tpx1

To verify that the role of *S. pombe* thioredoxins in Pap1 basal and H_2O_2-induced activity was directly linked to their role as Tpx1 electron donors, we analyzed Pap1 subcellular localization, chromatin association, and transcriptional activity in *Δtrx1 Δtpx1* and *Δtrx1 Δtrx3 Δtpx1* strains. As shown in Figure 4A, the absence of Tpx1 completely prevented Pap1 nuclear localization in all strains and conditions tested. Furthermore, the recruitment of Pap1 to the promoters (Figure 4B) and activation of the gene expression program (Figure 4C) were almost completely abrogated in these backgrounds.

Based on the above results, we hypothesized that an electron donor of the thioredoxin system, either Trx1 or Trx3, could be maintaining Pap1 in a reduced conformation and that the enhanced levels of Pap1-dependent gene expression in the *Δtrx1* and *Δtrx1 Δtrx3* strains could merely be a consequence of the depletion of this reducing power. In other words, Trx1 (with the help of Trx3) could be required to keep Pap1 thiols reduced under aerobic conditions, and H_2O_2 oxidation of Tpx1 could be required only to overcome this reducing power. This has already been proposed for the budding yeast Gpx3-Yap1 redox relay (Okazaki et al., 2007). However, our experiments

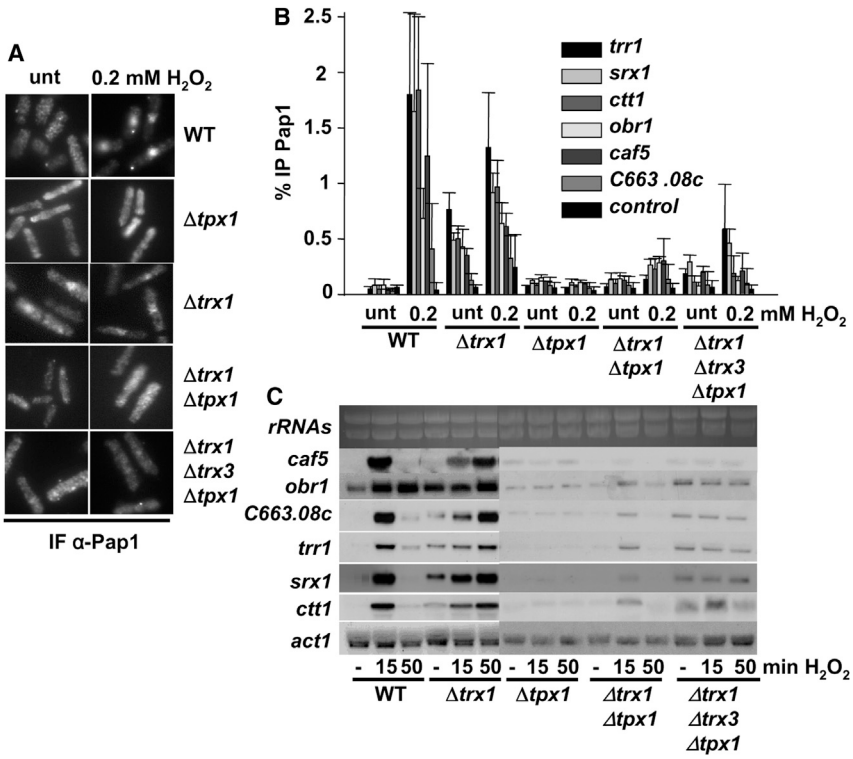

Figure 4. The Late Activation of Pap1 in *Δtrx1* and *Δtrx1 Δtrx3* Strains Is Tpx1 Dependent

(A) Localization of Pap1 by immunofluorescence in IC2 (WT), MJ15 (*Δtrx1*), MJ11 (*Δtpx1*), IC40 (*Δtrx1 Δtpx1*), and IC123 (*Δtrx1 Δtrx3 Δtpx1*) strains, before and after 0.2 mM H_2O_2 for 5 min, using polyclonal anti-Pap1.

(B) Pap1 recruitment to promoters is fully dependent on Tpx1 even in cells lacking thioredoxins. Cultures of strains as in (A) were treated with 0.2 mM H_2O_2 for 5 min, and ChIP experiments using anti-Pap1 were performed as described in Figure 3B.

(C) The activation of Pap1-dependent genes in *Δtrx1* and *Δtrx1 Δtrx3* strains is abolished upon *tpx1* deletion. Total RNA from cultures of strains as in (A), treated or not with 0.2 mM H_2O_2 for the times indicated, was analyzed as described in Figure 1C.

clearly show that Pap1 activation in a strain depleted of cytosolic thioredoxins requires Tpx1 both for basal activity and H_2O_2-dependent activation of the Pap1 pathway (Figures 4A–4C). Therefore, oxidation of Pap1 is dependent on Tpx1, even in the absence of thioredoxins.

Pap1 as an Alternative Electron Donor for Tpx1 upon Transient Oxidation of Trx1

As stated above, previous experiments suggested that Tpx1 transfers the redox signal to Pap1 by thiol-disulfide exchange. However, Tpx1 is scavenging peroxides during normal aerobic metabolism, since cells devoid of Tpx1 are unable to grow on aerobic plates (Jara et al., 2007). We hypothesized that Pap1 could be a secondary electron donor for disulfide-bridged oxidized Tpx1, but only when its natural electron donor, reduced Trx1, becomes transiently limiting. If this were the case, Tpx1 basal oxidation could be exacerbated in strains lacking both Trx1 and Pap1, similar to what we observed in *Δtrx1 Δtrx3* cells (Figure 2C). Indeed, we detected maximum oxidation of Tpx1 (two disulfide bonds per Tpx1 dimer) under basal conditions in extracts from the *Δtrx1 Δpap1* strain (Figure 5A). Similarly, the *Δtrx1* strain expressing the nonoxidizable mutant Pap1 proteins, Pap1.C278A or Pap1.C285S (Calvo et al., 2013), displayed the same pattern of Tpx1 oxidation as the *Δtrx1 Δpap1* strain (Figure 5B). Therefore, Pap1 can act as an electron donor to Tpx1 only when reduced Trx1 becomes limiting. As expected, in the case of cells expressing the Pap1.C278A and Pap1.C285S mutants, the absence of Trx1 was not sufficient to induce association of these Pap1 mutants with chromatin (Figure 5C) or trigger Pap1-dependent gene expression (Figure 5D).

The Role of Tpx1 in Pap1 Activation Can Be Bypassed by Overexpression of Another Thioredoxin-Dependent Peroxidase, Gpx1

Our experiments indicated that the peroxiredoxin Tpx1 senses and scavenges peroxides with Trx1 as the main electron donor in the Tpx1 cycle (Figure 2F). Only when reduced Trx1 becomes limiting is the disulfide of Tpx1 transferred to an alternative electron donor, Pap1. When added to the media, H_2O_2 is able to trigger reversible thiol oxidation in *S. pombe* (García-Santamarina et al., 2011); however, Pap1 cannot become oxidized/active in the absence of Tpx1, irrespective of the concentration of peroxides used (Bozonet et al., 2005; Vivancos et al., 2005) (S.B. and E.H., unpublished data). We hypothesized that this is because the Cys residues that become oxidized by H_2O_2 in Pap1 are masked by Pap1 tertiary structure and/or are not readily reactive to peroxides. Thus, only a redox transducer such as oxidized Tpx1 is able to catalyze this oxidation. We then tested whether Tpx1 activity persisted when another thioredoxin-based peroxidase able to sense and reduce H_2O_2 accumulated in the oxidized form upon peroxide addition. Gpx1, a glutathione peroxidase in fission yeast, has been reported to possess thioredoxin-dependent peroxidase activity (Kim et al., 2010). Endogenous Gpx1, due to either insufficient expression levels or its low peroxidatic activity, is not able to scavenge H_2O_2 produced during aerobic metabolism, because cells devoid of Tpx1 cannot grow on aerobic plates (Jara et al., 2007). Concomitantly, thioredoxin oxidation upon H_2O_2 stress should not occur in cells lacking Tpx1. We developed an assay based on the use of a heavy alkylating agent, AMS, to distinguish whether Trx1 has one, two, or three free thiol groups in acidic extracts. Trx1 contains three Cys residues at positions 16, 30, and 33, two of which (Cys30 and Cys33) engage in substrate recycling. Each AMS moiety, which reacts only with reduced Cys residues, enhances the size of the alkylated protein by 0.5 kDa, which can be detected by reducing electrophoresis (Figure 6A; see the Experimental Procedures). In wild-type cells, fully reduced Trx1, which is present under basal conditions,

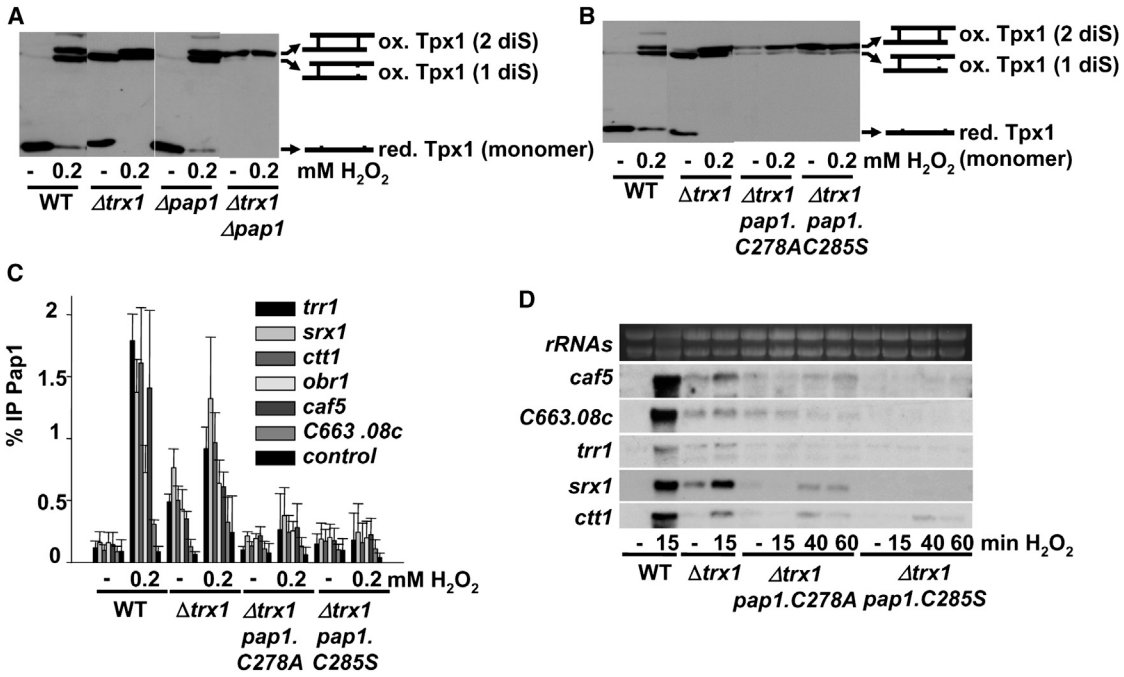

Figure 5. Pap1 Is an Alternative Electron Donor for Tpx1

(A and B) The redox state of Tpx1 in IC2 (WT), MJ15 (Δ*trx1*), IC1 (Δ*pap1*), SG69 (Δ*trx1* Δ*pap1*), IC75 (Δ*trx1 pap1.C278A*), and IC82 (Δ*trx1 pap1.C285S*) was analyzed as described in Figure 1B using polyclonal antibody against Tpx1.

(C) Pap1 recruitment to promoters in a Δ*trx1* strain depends on specific oxidation of Pap1 Cys residues. Cultures of strains as in (B) were treated with 0.2 mM H_2O_2 for 5 min, and ChIP experiments using anti-Pap1 were performed as described in Figure 3B.

(D) The activation of Pap1-dependent genes in strain *Δtrx1* is abolished in *pap1* mutants. Total RNA from cultures of strains as in (B), treated or not with 0.2 mM H_2O_2 for the times indicated, was analyzed as described in Figure 1C.

contains three AMS-modifiable thiol groups, and therefore migrates more slowly than the unmodified protein (Figure 6A). Upon mild oxidative stress, only one thiol group of Trx1 is modified by AMS and therefore the protein migrates faster, indicating that oxidized Trx1 (with a disulfide between Cys30 and Cys33) is the predominant form. In cells lacking Tpx1, oxidation of Trx1 upon H_2O_2 stress does not occur.

We then tested the effect of Gpx1 overexpression on cells lacking Tpx1. *Δtpx1* cells transformed with an episomal plasmid carrying the *HA-gpx1* gene were able to induce Trx1 oxidation upon H_2O_2 stress (Figure 6B, middle). In fact, HA-Gpx1 oxidation could be detected by nonreducing electrophoresis (Figure 6B, top). It is worth mentioning that Gpx1 can trigger significant Trx1 oxidation when H_2O_2 is added to the media at high concentrations (2 mM; Figure 6B), probably due to a higher K_m for H_2O_2 than Tpx1; at this concentration, Tpx1 cannot scavenge peroxides due to overoxidation of its peroxidatic Cys to sulfinic acid (Bozonet et al., 2005; Vivancos et al., 2005) (Figure 2F). In *Δtpx1* cells overexpressing HA-Gpx1, Pap1 is oxidized at concentrations of H_2O_2 that are sensed by Gpx1, as shown by nonreducing electrophoresis (Figure 6B, bottom) and analysis of Pap1-dependent gene expression (Figure 6C). Thus, we conclude that accumulation of the oxidized form of a thioredoxin-dependent peroxidase, either Tpx1 or Gpx1, can transduce redox signals to Pap1. Since Trx1 oxidation is concomitant to Gpx1 oxidation, we assessed the effect of deleting *trx1* in this background. As shown in Figure 6D, the absence of Trx1 enhanced the amount of oxidized HA-Gpx1 both before and after stress imposition (top). Even though Trx1 was absent, Pap1 oxidation could still be detected by nonreducing electrophoresis of TCA extracts (Figure 6D, *Δtpx1 Δtrx1* + o/e HA-Gpx1, bottom), and the Pap1-dependent gene expression profile was very similar to that of *Δtrx1* cells (compare the induction of genes in strain *Δtpx1 Δtrx1* + o/e HA-Gpx1 in Figure 6E with that in strain *Δtrx1* in Figure 3C).

Identification of the Tpx1-Pap1 Transient Intermediate by Mass Spectrometry

In redox biology, identification of the transient intermediates of a signaling redox relay is essential to verify the interdependence of each redox couple. In the case of the Tpx1-(Trx1)-Pap1 signaling cascade, we hypothesized that a direct signal transduction event occurs between the peroxiredoxin and the transcription factor; however, biochemical isolation of the disulfide-linked Tpx1-Pap1 intermediate proved to be extremely difficult. Taking into account that Tpx1 and Trx1 are abundant proteins, while Pap1 is not (Gunaratne et al., 2013) and that Pap1 can be considered an electron donor to Tpx1 when reduced Trx1 is transiently exhausted (Figure 5), we attempted to trap the elusive Tpx1-Pap1 transient intermediate by overexpressing Pap1 in a *Δtrx1* background. When HA-Pap1 was constitutively overexpressed from an episomal plasmid, the concentration of H_2O_2 required

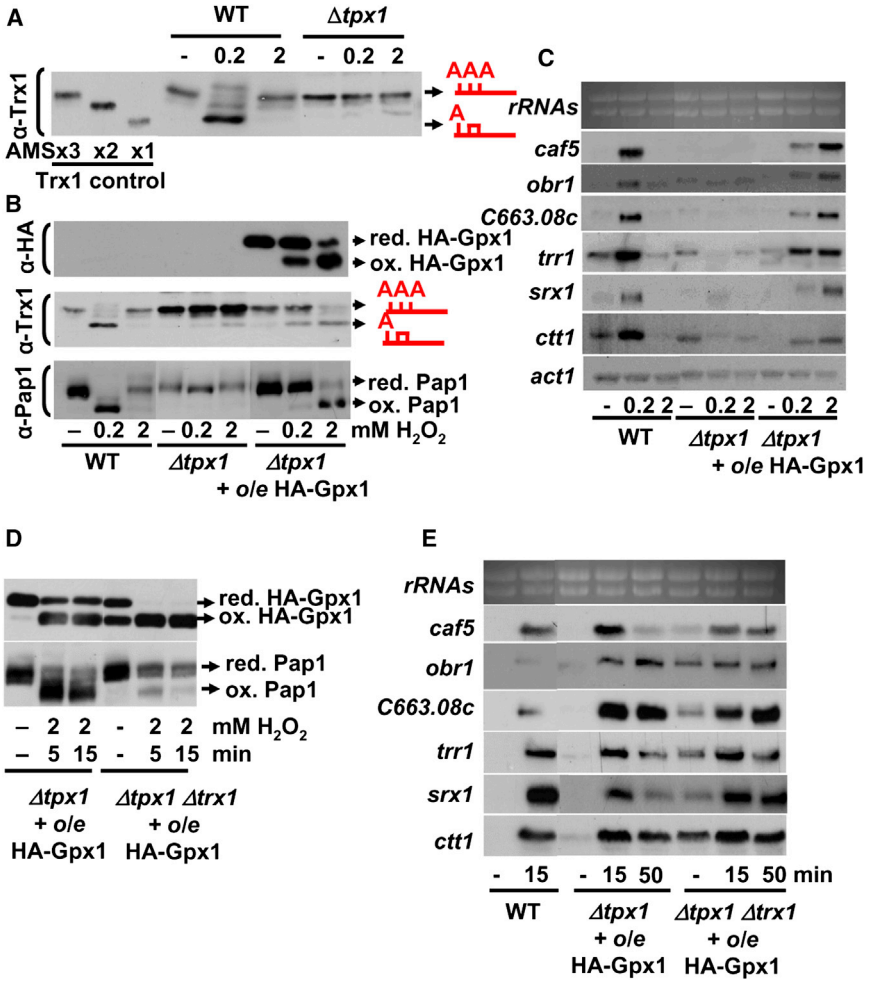

Figure 6. Overexpression of the Thioredoxin Peroxidase Gpx1 Induces H_2O_2-Dependent Oxidation of Pap1 in Cells Lacking Tpx1

(A) In vivo oxidation of Trx1. Free thiols in TCA extracts of cultures from strains IC2 (WT) and MJ11 (*Δtpx1*), treated or not with 0.2 mM or 2 mM H_2O_2 for 5 min, were labeled with AMS and separated using reducing electrophoresis. The relative positions of Trx1 alkylated with three (×3), two (×2), or one (×1) AMS molecules are indicated in the figure (Trx1 control). The relative positions of oxidized Trx1 (with only one AMS; "A") or reduced Trx1 (with three AMS moieties; "AAA") in extracts are indicated with arrows.

(B) Overexpression of HA-Gpx1 induces H_2O_2-dependent oxidation of Trx1 and Pap1 at high concentrations of H_2O_2. TCA extracts of cultures from strains IC2 (WT), SG5 (*Δtpx1*), and SG5 carrying plasmid p440.3x (*Δtpx1* + o/e HA-Gpx1), treated or not with 0.2 mM or 2 mM H_2O_2 for 5 min, were obtained and the redox state of Trx1 was analyzed as described in (A) (center), whereas the redox state of HA-Gpx1 (top) and Pap1 (bottom) was analyzed as described in Figure 1B using monoclonal anti-HA or polyclonal anti-Pap1, respectively.

(C) Overexpression of Gpx1 allows the activation of the Pap1-dependent gene expression program in cells lacking Tpx1. Total RNA from strains as in (B), treated or not with 0.2 mM and 2 mM H_2O_2 for 15 min, was analyzed as described in Figure 1C.

(D and E) The Gpx1-dependent activation of Pap1 in cells lacking Tpx1 is Trx1-independent.

(D) In vivo oxidation of Pap1 and HA-Gpx1 in strains SG5 + p440.3x (*Δtpx1* + o/e HA-Gpx1) and IC122 + p440.3x (Δ*tpx1* Δ*trx1* + o/e HA-Gpx1) was analyzed as described in (B).

(E) Total RNA from strains IC2 (WT), SG5 + p440.3x (*Δtpx1* + o/e HA-Gpx1), and IC122 + p440.3x (Δ*tpx1* Δ*trx1* + o/e HA-Gpx1) was analyzed as described in Figure 1C.

to oxidize Pap1 was higher than in wild-type cells but still dependent on the presence of Tpx1 and Cys285 of Pap1 (Figure 7A). In extracts from strain *Δtrx1* carrying the episomal vector, we detected a high-molecular-weight complex recognized by anti-HA(-Pap1) (Figure 7B) that was sensitive to the presence of reducing agents (Figure 7C). It was also recognized by anti-Tpx1 and was not present in extracts from *Δtpx1* cells (Figure 7B). To confirm the disulfide-linked Tpx1-Pap1 intermediate, we obtained TCA extracts from *Δtrx1* cells (and from *Δtpx1* as a negative control) overexpressing HA-Pap1, dialyzed the extracts to solubilize TCA-precipitated proteins, and immunoprecipitated HA-Pap1. As shown in Figure 7D, the procedure preserved the presence of slower-migrating bands present in whole-cell extracts. We excised the slowly migrating band from gels and performed protease digestion and mass spectrometry analysis (Figure 7D). The first two proteins identified by liquid chromatography tandem mass spectrometry were Tpx1 and Pap1, with coverage of 61% and 42%, respectively; other proteins identified in the analysis displayed significantly lower abundance and/or coverage, and most of them were very abundant cellular proteins or had a molecular mass around 100 kDa, corresponding to the area of the excised band (see Table S1 for a list of all the proteins detected in this band and Table S2 for the detailed peptides covering Pap1 and Tpx1). As expected, mass spectrometry analysis of a band excised from the *Δtpx1*-deriving extracts allowed the identification of only Pap1, used as bait, and of most of the highly abundant proteins identified in the *Δtrx1*-corresponding band (Table S3).

DISCUSSION

The presence of several redox couples in antioxidant redox relays, with interdependent cycles of oxidation-reduction, constitute the basis of redox signaling cascades. Here, we investigated the role of the main components of a simple antioxidant pathway consisting of the peroxiredoxin Tpx1 as the H_2O_2 sensor, Trx1 as the principal electron donor for the recycling of oxidized Tpx1, and Pap1 as the transcription factor, which acts as an alternative electron donor to Tpx1 that becomes activated by a thiol-disulfide exchange mechanism.

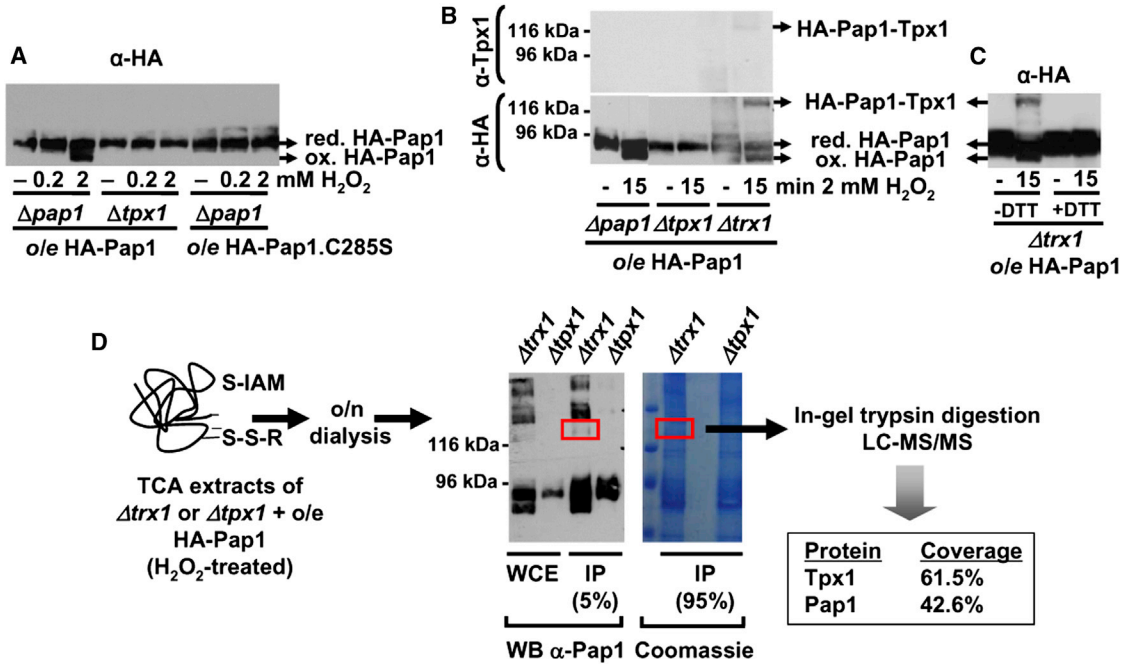

Figure 7. Trapping a Redox Transient Intermediate and Identification of Tpx1 and Pap1 by Mass Spectrometry

(A) Redox state of overexpressed HA-Pap1. Cultures from strains IC1 + p93.3x (Δ*pap1* o/e HA-Pap1), SG5 + p93.3x (Δ*tpx1* o/e HA-Pap1), and IC1 + p93.C285S.3x (Δ*pap1* o/e HA-Pap1.C285S) were treated or not with 0.2 mM H_2O_2 for 5 min or 2 mM H_2O_2 for 15 min and TCA extracts were prepared. The redox state of HA-Pap1 was analyzed as described in Figure 1B using α-HA antibody.

(B) A high-molecular-weight band in extracts from Δ*trx1* o/e HA-Pap1corresponds to Tpx1-Pap1. Cultures from strains IC1 (Δ*pap1*), SG5 (Δ*tpx1*), and MJ2 (Δ*trx1*) carrying p93.3x (o/e HA-Pap1) were treated or not with 2 mM H_2O_2 for 15 min, and TCA extracts were prepared and analyzed as described in Figure 1B using antibodies against Tpx1 (α-Tpx1) or HA (α-HA). The position of the molecular weight markers and of oxidized and reduced HA-Pap1, as well as the HA-Pap1-Tpx1 intermediate, are indicated.

(C) The high-molecular-weight complex is sensitive to dithiothreitol (DTT). Same as in (B), but adding 1 mM DTT to the samples prior to gel loading (right two lanes).

(D) Scheme depicting the strategy to identify by mass spectrometry the proteins present in the high-mobility weigh complex. Cultures of strains MJ2 + p93.3x (Δ*trx1* o/e HA-Pap1) and SG5 + p93.3x (Δ*tpx1* o/e HA-Pap1) were treated with 2 mM H_2O_2 for 15 min, and TCA extracts were prepared. Proteins in TCA extracts, with all the free thiol groups alkylated with iodoacetamide (IAM), were dialyzed. These renatured protein extracts (WCE) were immunoprecipitated with anti-HA crosslinked to Sepharose beads. A small percentage of the immunoprecipitates (IP) at the beads (IP 5%) were loaded in nonreducing gels together with 10 μg of WCE and processed by western blot with antibodies against Pap1 (left). In parallel, the majority of the IPs (IP 95%) were loaded in gels and visualized by colloidal Coomassie staining (right). The high-molecular-weight band was excised and digested with trypsin and analyzed by liquid chromatography tandem mass spectrometry. The coverage of the main two proteins present in the band slice, Tpx1 and Pap1, is indicated.

See also Tables S1, S2, and S3.

The unambiguous outcome of all the experiments described here is that Tpx1 directly transfers the H_2O_2 signal to Pap1. Tpx1 is precisely suited to sense and scavenge low concentrations of peroxides and is the primary enzyme responsible for H_2O_2 detoxification during aerobic growth on solid plates (Jara et al., 2007). Other peroxidases in *S. pombe*, such as Gpx1, BCP, or Pmp20, may contain Cys that are also able to react with H_2O_2 and transfer the redox signal to other Cys, but their low sensitivity and/or concentration may prevent them from becoming signal transducers. Our findings indicate that H_2O_2 can indiscriminately react with some reactive Cys residues in the thiol proteome leading to loss-of-function (García-Santamarina et al., 2011) in a peroxiredoxin-independent manner, because cells lacking Tpx1 (and multiple deletions in the other peroxiredoxin-coding genes) still exhibit accumulation of general disulfides after H_2O_2 treatment (S.G.-S, S.B., and E.H., unpublished data). However, the disulfides in Pap1 cannot be formed unless Tpx1 is present, suggesting that Tpx1-Pap1 protein interactions may provide specificity to this redox cascade and induce a gain of function in contraposition to unwanted inactivation. Only massive overexpression of another peroxidase, Gpx1, can overcome the absence of Tpx1. In other words, while general thiol oxidation may occur in response to H_2O_2, with a small fraction of each specific protein becoming oxidized and often inactivated, H_2O_2-dependent oxidation of Pap1 is a peroxiredoxin-driven event leading to full activation of the transcription factor. The thioredoxin system probably facilitates both Pap1 (Figure 3C) and general thiol reduction (S.G.-S., S.B., and E.H., unpublished data) after peroxide stress.

Prior to the experiments described here, three putative models could explain the role of Tpx1 and Trx1 in Pap1 activation: (1) H_2O_2 oxidizes Tpx1, which then oxidizes the pool of Trx1, and oxidized Trx1 directly activates Pap1; (2) oxidized Tpx1 directly oxidizes Pap1, although only after reduced Trx1 becomes

limiting; and (3) a mixed Trx1-Tpx1 intermediate in the Tpx1 cycle (Figure 2F) induces Pap1 oxidation. We have dismissed the last hypothesis because cells expressing Trx1.C33S still accumulate Trx1-Tpx1 intermediates (Figures 2D and 2E) and Pap1 follows the same activation kinetics as in cells lacking Trx1 (S.B. and E.H., unpublished data). We also eliminated the first model (oxidized Trx1 being the inducer of Pap1), because cells lacking Trx1 are still able to activate Pap1, as demonstrated by ChIP and transcriptome analysis (Figures 3B and 3C), whereas further deletion of Tpx1 on this background fully eliminates Pap1 activation (Figure 4). Therefore, we are left with the hypothesis that oxidized Tpx1 is the direct inducer of Pap1. The final confirmation of Tpx1 being the direct activator of Pap1 was confirmed by our isolation and identification of the transient intermediate between Tpx1 and Pap1 (Figure 7).

We also believe that Tpx1 activates Pap1 from the double disulfide form when reduced Trx1 becomes limiting (Figure 2F) and by thiol-disulfide exchange. A remarkable difference between budding and fission yeast signal transduction systems stems from the different mechanisms of peroxidase-mediated oxidation of the transcription factor. The system proposed for the Gpx3/Yap1 relay is based on the formation of one SOH in Gpx3, which would then react with a Cys residue in Yap1, forming a mixed disulfide intermediate. This transient intermolecular disulfide is then rearranged into an intramolecular bond in Yap1, which is the active transcription factor, with the concomitant release of reduced Gpx3 (Delaunay et al., 2002). Alternatively, in fission yeast, the system appears to undergo a thiol-disulfide exchange reaction, so that an internal disulfide in Tpx1, generated by resolution of an SOH in the peroxidatic active Cys with the thiol of the resolving one, can be transferred to Pap1 as part of a redox cascade. The other alternative, activation of Pap1 by Tpx1-S_{48}OH, is unlikely, because cells expressing Tpx1 with a mutation in the resolving Cys, Tpx1.C169S, which should allow the accumulation of Tpx1-S_{48}OH, are not able to activate Pap1 in response to peroxides (Vivancos et al., 2005). One could argue that the Tpx1-to-Pap1 redox relay takes places from a Tpx1 dimer with one Cys48-SOH and an intradimer Cys169-Cys48 disulfide; in that case, cells expressing only Tpx1.C169S would be unable to transfer the redox signal. However, we overexpressed Gpx1 lacking the resolving Cys (Cys-82-Ser) in *Δtpx1* cells, and this mutant protein was unable to transfer the H_2O_2 signal to Pap1 (I.A.C., S.B., and E.H, unpublished data), even though the glutathione-peroxidase-like Gpx1 does not form dimers. It is conceivable to imagine that the very unstable SOH group in one protein could only react with another free thiol in a different polypeptide if both Cys are very closely positioned and in a stable configuration. It has been proposed that a molecular scaffold, such as Ybp1 of *S. cerevisiae*, which is required for the Gpx3-to-Yap1 redox relay (Veal et al., 2003), could provide the proximity/optimal alignment/stability necessary between Gpx3 and Yap1 Cys residues as well as create an optimized micropocket for the exclusion of other potential thiol competitors, such as glutathione (Gutscher et al., 2009). In fact, another interesting feature of the budding yeast system is that the commonly used W303/Y700 strain background expresses a truncated form of Ybp1. In this strain, Yap1 activation depends on the abundant peroxiredoxin Tsa1, not on Gpx3, with both Cys residues of the peroxiredoxin being required for signal transduction (Okazaki et al., 2005; Tachibana et al., 2009). In the case of the Tpx1-to-Pap1 and Tsa1-to-Yap1 redox relays, both peroxidases participate in H_2O_2 scavenging and H_2O_2 signaling (Jara et al., 2007; Tachibana et al., 2009), while Gpx3 does not significantly contribute to peroxide degradation (Delaunay et al., 2002).

It is intriguing that in cells lacking Trx1, the oxidation of Pap1 is not easily detectable by nonreducing electrophoresis (Figures 1E, 1F, and S4A). It is likely that the formation of two disulfides are required for the activation of Pap1 as a transcription factor (Calvo et al., 2013). It may be that Trx1 participates in the isomerization of these disulfides. A second alternative is that Tpx1 has to be engaged in the catalytic cycle (reduction of which is mediated by Trx1) for Pap1 to be fully and rapidly activated, as has previously been described for the overoxidation of peroxiredoxins to their sulfinic form (Yang et al., 2002). Lastly, a third alternative is that Trx1 also works as a chaperone that holds Tpx1 and Pap1 together. However, we have been able to coimmunoprecipitate Pap1 and Tpx1 with Trx1 in a complex, but Pap1 and Tpx1 still coprecipitate in cells lacking Trx1 (Patricia García, I.A.C. and E.H., unpublished data).

It is important to point out that cells lacking thioredoxin reductase are able to generate an intracellular environment that fully disrupts wild-type thiol homeostasis (Figure S2). In fact, a proteome-wide approach indicates more than 30% of all Cys-containing *S. pombe* peptides are overoxidized by more than 2-fold in this strain background (García-Santamarina et al., 2013). In *Δtrr1* cells, several nonphysiological oxidized species accumulate, such as oxidized Trx1, Trx3 and Tpx1, and induce this massive, H_2O_2-independent thiol oxidation. Therefore, in this genetic background, H_2O_2 signaling is fully disrupted.

EXPERIMENTAL PROCEDURES

Details of the construction of strains and plasmids and of most methods can be found in the Supplemental Experimental Procedures.

Fluorescence Microscopy

Fluorescence microscopy and image capture were performed as described previously (Vivancos et al., 2004).

Preparation of *S. pombe* TCA Extracts and Immunoblot Analysis

To analyze the in vivo redox state of Pap1, Tpx1, Trx1, HA-Gpx1, and HA-Pap1, *S. pombe* cultures were obtained and total TCA extracts were prepared as previously described (Vivancos et al., 2005). We used already described specific polyclonal antibodies for Pap1 (Vivancos et al., 2004), for Tpx1 (Jara et al., 2007), and for Trx1 (García-Santamarina et al., 2011). We used house-made monoclonal anti-HA (12CA5) to detect HA-Gpx1 and HA-Pap1.

TCA Protein Extracts Labeled with AMS

To determine the number of free thiols in Trx1, TCA extracts were prepared as described elsewhere (Vivancos et al., 2005) with several modifications described in the Supplemental Experimental Procedures.

RNA Analysis

Total RNA was analyzed as described previously (Castillo et al., 2002).

Chromatin Immunoprecipitation

The in vivo binding of Pap1 to stress promoters was analyzed by ChIP as described previously (Calvo et al., 2012).

Immunofluorescence Assay

Subcellular localization of Pap1, Trx1, Trx2, Trx3, or Tpx1 was determined by immunofluorescence as described in the Supplemental Experimental Procedures.

Identification of Mixed Disulfides between Pap1 and Tpx1 and Mass Spectrometry Analysis

TCA extracts from cultures of strains overexpressing HA-Pap1 were obtained and dialyzed and HA-Pap1 was immunoprecipitated with anti-HA. The immunoprecipitates were separated by nonreducing electrophoresis, and after Coomassie staining, gel slices migrating slower than the HA-Pap1 protein (and therefore corresponding to the Pap1-Tpx1 mixed disulfide) were digested with proteases and analyzed by mass spectrometry. A detailed description of the procedure is provided in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental information includes Supplemental Experimental Procedures, four figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.11.027.

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