A Peroxiredoxin Promotes H₂O₂ Signaling and Oxidative Stress Resistance by Oxidizing a Thioredoxin Family Protein

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http://dx.doi.org/10.1016/j.celrep.2013.10.036
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SUMMARY

H₂O₂ can cause oxidative damage associated with age-related diseases such as diabetes and cancer but is also used to initiate diverse responses, including increased antioxidant gene expression. Despite significant interest, H₂O₂-signaling mechanisms remain poorly understood. Here, we present a mechanism for the propagation of an H₂O₂ signal that is vital for the adaptation of the model yeast, Schizosaccharomyces pombe, to oxidative stress. Peroxiredoxins are abundant peroxidases with conserved antiaging and anticancer activities. Remarkably, we find that the only essential function for the thioredoxin peroxidase activity of the Prx Tpx1(hPrx1/2) in resistance to H₂O₂ is to inhibit a conserved thioredoxin family protein Txl1(hTxnl1/TRP32). Thioredoxins regulate many enzymes and signaling proteins. Thus, our discovery that a Prx amplifies an H₂O₂ signal by driving the oxidation of a thioredoxin-like protein has important implications, both for Prx function in oxidative stress resistance and for responses to H₂O₂.

INTRODUCTION

Reactive oxygen species (ROS) such as H₂O₂ cause oxidative damage that is linked with many diseases and aging. Hence, cells use H₂O₂ as a signaling molecule to initiate transcriptional responses to limit this damage. For example, in fungi, following activation by H₂O₂-induced oxidation of specific cysteines, AP-1-like transcription factors promote expression of ROS defense and repair enzymes (reviewed in Veal et al., 2007). In pathogenic fungi, this is a vital response to ROS generated by host immune cells (Guo et al., 2011; Leal et al., 2012). As well as the activation of ROS defenses, H₂O₂ signals also regulate fundamental processes, including cell division, differentiation, migration, and death. In many cases, the H₂O₂-sensing/signaling mechanisms are poorly defined. However, these H₂O₂ responses also involve the oxidation of specific, redox-sensitive cysteine residues present in a variety of target proteins, including protein tyrosine phosphatases, kinases, and ubiquitin/ubiquitin-like pathway enzymes (for a review, see Veal et al., 2007, and also Cotto-Rios et al., 2012; Doris et al., 2012; Lee et al., 2013a).

2-Cys peroxiredoxins (Prxs) are highly abundant peroxidase enzymes that play important roles in responses to H₂O₂. Indeed, Prxs have crucial roles in maintaining genome stability, protecting against cancer and promoting longevity (for reviews, see Nyström et al., 2012; Rhee and Woo, 2011). Prxs use reversibly oxidized cysteines to catalytically break down peroxides (Figure S1) but have additional chaperone and signaling activities (Rhee and Woo, 2011). It is currently unclear how each of these activities contributes to Prx’s in vivo roles. There is also considerable interest in understanding how Prxs influence H₂O₂ signaling. Significantly, the peroxide-reacting (peroxidatic) cysteine in Prx is far more abundant and sensitive to H₂O₂ than redox-sensitive cysteines in H₂O₂-regulated-signaling proteins. Hence, it is not clear how H₂O₂ targets signaling proteins in cells rich in Prx (Winterbourn, 2008). Several mechanisms have been proposed to answer this question, as described below.

The thioredoxin peroxidase activity of eukaryotic 2-Cys Prx is susceptible to inactivation by hyperoxidation of the peroxidatic cysteine residue at relatively low levels of H₂O₂ (Wood et al., 2003) (Figure S1). It has been proposed that this inactivation may allow H₂O₂ to evade Prx-mediated breakdown and oxidize target-signaling proteins (Wood et al., 2003). Indeed, inhibition of the thioredoxin peroxidase activity of Prx by phosphorylation does promote certain ROS-signaling events (Woo et al., 2010). However, there is little evidence that the low levels of H₂O₂ sufficient to activate many signaling pathways cause significant Prx hyperoxidation (Woo et al., 2010). Moreover, in the model yeast Schizosaccharomyces pombe, the thioredoxin peroxidase activity of the 2-Cys Prx, Tpx1, is required for the H₂O₂-induced activation of the AP-1-like transcription factor Pap1. Accordingly, hyperoxidation of Tpx1 actually prevents Pap1 activation (Bozonet et al., 2005; Vivancos et al., 2005).

An alternative mechanism has been proposed to explain the increasing number of examples where Prxs are required for H₂O₂-induced signal transduction (Bozonet et al., 2005; Conway...
and Kinter, 2006; Delaunay et al., 2002; Fomenko et al., 2011; Jarvis et al., 2012; Ross et al., 2000; Veal et al., 2004; Vivancos et al., 2005). Pioneering studies in the yeast Saccharomyces cerevisiae revealed that the H$_2$O$_2$-induced oxidation of the AP-1-like transcription factor Yap1 (Pap1 homolog) is initiated by the formation of a disulfide complex between the Prx-related peroxidase, Gpx3(Orp1), and Yap1 (Delaunay et al., 2002). Thus, it has been proposed that Prxs positively transduce H$_2$O$_2$ signals by participating directly in the H$_2$O$_2$-induced oxidation of signaling proteins (Jarvis et al., 2012; Tachibana et al., 2009; Winterbourn, 2008). However, no Tpx1-Pap1 disulfide complex has been reported (Bozonet et al., 2005; Vivancos et al., 2005).

Our recent studies lead us to propose an additional mechanism by which Prxs can promote H$_2$O$_2$ signaling: by stimulating the oxidation of thioredoxin. Thioredoxin provides the electrons that reduce Prxs as part of their catalytic cycle (Figure S1). However, as broad specificity oxidoreductases, thioredoxins also reduce many other oxidized proteins (Lee et al., 2013b). Our studies revealed that, in S. pombe exposed to H$_2$O$_2$, Tpx1 disulfides are the major substrate for the single cytosolic thioredoxin, Trx1 (Figure S1). Accordingly, because thioredoxin reductase (Trr1) levels are limiting, Trx1 becomes completely oxidized, and other Trx1-dependent enzymes are inhibited (Day et al., 2012). Eukaryotic cells contain many thioredoxin-like enzymes that function as cofactors for particular enzymes and/or to regulate specific signaling proteins (Lee et al., 2013b). Hence, we proposed that the coupling of the peroxidase activity of 2-Cys Prx to thioredoxin provides a mechanism for the H$_2$O$_2$-dependent regulation of multiple thioredoxin substrates (Day et al., 2012).

Here, we test the hypothesis that Prxs promote H$_2$O$_2$ signaling by inhibiting thioredoxin family proteins. In support of this hypothesis, we show that the thioredoxin peroxidase activity of Tpx1 is essential for the H$_2$O$_2$-induced oxidation of the conserved, proteasome-associated thioredoxin-like protein Txl1 (homolog of human TRP32/Txnl1). Significantly, we identify Pap1 as an in vivo Txl1 substrate and show that, by reducing Pap1, Txl1 inhibits the accumulation of oxidized (active) Pap1. Thus, we describe an in vivo role for Txl1 as an inhibitor of H$_2$O$_2$ signaling. Moreover, we reveal that Pap1 is regulated by a mechanism in which a Prx propagates the H$_2$O$_2$ signal by inhibiting conserved thioredoxin family protein(s). This has significant implications for the role of Prx and thioredoxin reductase enzymes in H$_2$O$_2$ signal transduction. Remarkably, our data suggest that the key function for the thioredoxin peroxidase activity of Tpx1 in H$_2$O$_2$ resistance is to inhibit Txl1. This suggests that the regulation of thioredoxin family proteins may make a major contribution to the in vivo roles of Prxs.

**RESULTS**

**Tpx1 Regulates Pap1 by a Txl1-Independent Pathway**

The thioredoxin peroxidase activity of Tpx1 is important for the H$_2$O$_2$-induced oxidation and nuclear accumulation of Pap1, although the mechanism(s) involved is not clear (Bozonet et al., 2005; Vivancos et al., 2005). The prevalence of Tpx1 disulfide substrates in H$_2$O$_2$-treated cells causes severe depletion of reduced Trx1 (Day et al., 2012). This suggests that Tpx1 might promote Pap1 activation by preventing the reduction of oxidized, active Pap1 by Trx1 (Figure 1A). We reasoned that if Tpx1’s only role in Pap1 oxidation is to prevent Trx1 from reducing Pap1, then Tpx1 would not be required for Pap1 oxidation in Δtrx1 cells (Figure 1A). However, whereas Pap1 oxidation and nuclear accumulation were detected in unstressed Δtrx1 cells and increased following H$_2$O$_2$ treatment, Pap1 was reduced and cytoplasmic in both unstressed and H$_2$O$_2$-treated Δpx1Δtrx1 cells (Figures 1B, 1D, and 1E). Collectively, these data indicate a Trxl1-independent role for Tpx1 in Pap1 activation.

Pap1 is constitutively nuclear and oxidized in cells lacking the single thioredoxin reductase (Trr1) (Figures 1C–1E) (Vivancos et al., 2004) and substantially more oxidized compared with Δtrx1 cells (Figures 1B–1D). Furthermore, whereas the proportion of oxidized Pap1 in Δtrx1 cells increased following H$_2$O$_2$ treatment, Pap1 was maximally oxidized in Δtrr1 cells even prior to H$_2$O$_2$ treatment (Figures 1B–1D). These data raised the possibility that, in addition to Trx1, Trr1 reduces another Pap1-reducing protein(s). Strikingly, analysis of Δtxp1 and Δtxp1Δtrr1 cells revealed that Tpx1 was no longer required for oxidation and nuclear accumulation of Pap1 in the absence of thioredoxin reductase (Figures 1C–1E). These data suggest that the role of Tpx1 in Pap1 oxidation might be to promote the H$_2$O$_2$-induced oxidation of this second, unidentified, substrate of Trr1.

**The Redox State of the Thioredoxin-like Txl1 Is Regulated by Tpx1 and Trr1**

In addition to Trx1, the S. pombe genome encodes two other thioredoxin-like proteins: Trx2 (Lee et al., 2002), and Txl1(Trx3) (Jiménez et al., 2007). Because Trx2 localizes to mitochondria (Matsuyama et al., 2006), it was unlikely to reduce cytosolic or nuclear Pap1. Txl1 is larger than typical thioredoxins, consisting of an N-terminal thioredoxin domain and a PITH (C-terminal proteasome-interacting domain of thioredoxin-like) domain, which is important for interaction with the 26S proteasome (Andersen et al., 2009; Wiseman et al., 2009). Interestingly, loss of Txl1 delays the recovery of cells exposed to the peroxide t-BOOH, suggesting a link between Txl1 and oxidative stress responses (Jiménez et al., 2007). Hence, to investigate the relationship between Txl1 and oxidative stress responses, we first constructed a strain expressing Flag epitope-tagged Txl1 (Flag-Txl1) from its endogenous locus and confirmed that the Flag-Txl1 protein complemented the t-BOOH-induced growth delay of Δtxl1 cells (data not shown). In contrast to Trx1, which is excluded from the nucleus (Day et al., 2012; Figure 2A), as expected, immunostaining analysis revealed that Flag-Txl1 is distributed throughout the cytoplasm and the nucleus, with a punctate-staining pattern consistent with interaction with the proteasome (Figure 2A) (Andersen et al., 2009; Jiménez et al., 2007; Matsuyama et al., 2006). Unfortunately, Flag antibodies detected nonspecific (ns) bands present in cells expressing untagged wild-type Txl1 (txl1$^+$), including a band with a similar mobility to oxidized Flag-Txl1 (Figures 2B and 2D–2F). However, consistent with Txl1 being a thioredoxin reductase (Trr1) substrate, Flag-Txl1 was completely oxidized in Δtrr1 cells (Figure 2B). Our previous work revealed that in wild-type cells...
Immunostaining with Pap1 antibodies revealed the localization of Pap1 in wild-type (AD82), experiments. The mean percentage of oxidized Pap1 is shown, and the error bars indicate the SEM. The proportion of oxidized Pap1 was determined by densitometric analysis (ImageJ) quantification of oxidized and total Pap1 in at least three independent

upon H2O2 treatment, increased levels of Tpx1 disulfides (Figure S1) might indirectly promote Txl1 oxidation by increasing the levels of oxidized Trx1 and thus limiting the Trr1 available to reduce Txl1 (Day et al., 2012). However, the increased proportion of Tpx1 disulfides detected in (i) Tpx1 disulfides competitively inhibit the reduction of oxidized, active Pap1 by Txl1, and/or (ii) Tpx1 has another role in Pap1 oxidation. (B–D) Tpx1 is required for the oxidation of Pap1 in Δtxl1 but not Δtrr1 mutant cells as revealed by western blot analysis, using Pap1 antibodies, of IAA-treated proteins isolated from wild-type (WT; AD82), Δtpx1 (VX00), Δtxl1 (JB30), Δtrr1 (AD81), Δtpx1Δtxl1 (AD100), and Δtpx1Δtrr1 (AD138) cells treated with 0.2 mM H2O2 for the indicated times. Oxidized (Pap1ox) and reduced (Pap1red) Pap1 were separated on the basis of the slower mobility of Pap1 following modification of reduced cysteines with IAA.

See also Figure S1.

Txl1 Regulates the H2O2-Dependent Activation of Pap1

Having established that Txl1 is a Trr1 substrate and is oxidized in response to low levels of H2O2 by a Tpx1-dependent mechanism (Figure 2), we next examined whether Txl1 regulates Pap1. In contrast to either Δtxl1 or Δtrr1 cells, the oxidation and nuclear accumulation of Pap1 were similar in wild-type and Δtxl1 cells under normal growth conditions (compare Figures 1C, 3A, and 3B). However, there was some indication that the levels of nuclear Pap1 were increased in Δtxl1 cells following H2O2 treatment (Figure 3B). Indeed, although the general pattern of H2O2-induced expression of Pap1-dependent genes was similar in wild-type and Δtxl1 cells under normal growth conditions (compare Figures 1C, 3A, and 3B).

Please cite this article in press as: Brown et al., A Peroxiredoxin Promotes H2O2 Signaling and Oxidative Stress Resistance by Oxidizing a Thioredoxin Family Protein, Cell Reports (2013), http://dx.doi.org/10.1016/j.celrep.2013.10.036

Figure 1. Loss of the Thioredoxin Reductase, Trr1, but Not Trx1, Bypasses the Requirement for Tpx1 in the Oxidation and Nuclear Localization of Pap1

(A) The catalytic cycle of the Prx, Tpx1, involves reduction of Tpx1 disulfides by the thioredoxin, Trx1. Oxidized Trx1 is reduced by the thioredoxin reductase using NADPH. In cells treated with H2O2, Trx1 levels are limiting such that Trx1 becomes completely oxidized unless Tpx1 becomes hyperoxidized (Day et al., 2012). The question marks (‘?’) indicate that the role of Trx1 and Tpx1 in oxidation of Pap1 is unclear; it is possible that (i) Tpx1 disulfides competitively inhibit the reduction of oxidized, active Pap1 by Txl1, and/or (ii) Tpx1 has another role in Pap1 oxidation. (B–D) Tpx1 is required for the oxidation of Pap1 in Δtxl1 but not Δtrr1 mutant cells as revealed by western blot analysis, using Pap1 antibodies, of IAA-treated proteins isolated from wild-type (WT; AD82), Δtpx1 (VX00), Δtxl1 (JB30), Δtrr1 (AD81), Δtpx1Δtxl1 (AD100), and Δtpx1Δtrr1 (AD138) cells treated with 0.2 mM H2O2 for the indicated times. Oxidized (Pap1ox) and reduced (Pap1red) Pap1 were separated on the basis of the slower mobility of Pap1 following modification of reduced cysteines with IAA. Results in (B) and (C) are representative of at least three independent experiments. (D) The proportion of oxidized Pap1 was determined by densitometric analysis (ImageJ) quantification of oxidized and total Pap1 in at least three independent experiments. The mean percentage of oxidized Pap1 is shown, and the error bars indicate the SEM.

(E) Immunostaining with Pap1 antibodies revealed the localization of Pap1 in wild-type (AD82), Δtpx1 (VX00), Δtxl1 (JB30), Δtrr1 (AD81), Δtpx1Δtxl1 (AD100), and Δtpx1Δtrr1 (AD138) cells before and following treatment with 0.2 mM H2O2 for the indicated times. Each image was captured under identical conditions and exposure time.

See also Figure S1.
Pap1 was oxidized in Δtrx1Δtxl1 cells compared with un-stressed Δtrx1 cells (Figures 3A and 3B; data not shown). Moreover, consistent with the hypothesis that Txl1 and Trx1 independently repress Pap1-dependent gene expression, the levels of gst2+, srx1+, and obr1+ mRNA were all substantially higher in un-stressed Δtrx1Δtxl1 cells compared with either single mutant (Figure 4).

Notably, although the nuclear levels of Pap1 appeared similar in Δtrx1Δtxl1 and Δtrx1 cells (Figure 3B), a significantly smaller proportion of Pap1 was oxidized in Δtrx1Δtxl1 cells compared with either Δtrx1 or wild-type cells treated with H2O2 (Figures 3A and 3C). Moreover, in contrast to Δtrx1 cells, the proportion of oxidized Pap1 did not increase further in Δtrx1Δtxl1 cells following H2O2 treatment (Figures 3A and 3C; Table S2). Intriguingly, a substantial pool of reduced Pap1 that was resistant to H2O2-induced oxidation was also detected in Δtrx1Δtxl1Δtrx1 cells (Figure 5A). Thus, collectively, these data suggest that, although loss of Trx1 and/or Txl1 increases Pap1 activity under normal conditions, Trx1 and Txl1 are also required for H2O2-induced increases in Pap1 oxidation.

Trx1 and/or Txl1 Maintains Pap1 in a Soluble, H2O2-Responsive State

Although the oxidation of Pap1 was always (1) increased in Δtrx1Δtxl1 cells compared with either single mutant, and (2) non-inducible by H2O2, there was some variation in the proportion of Pap1 that was oxidized between individual experiments (Table S2). Furthermore, in contrast to Δtrx1, Δtxl1, and Δtrx1Δtxl1 cells, where the fold induction of gst2+, srx1+, and obr1+ was remarkably consistent between individual experiments (as reflected by the small error bars in Figure 4A), although consistently high, the fold increase in each mRNA in Δtrx1Δtxl1 cells varied considerably between experiments (Table S3). When we explored the basis for the variation in Pap1 activity between different cultures of the Δtrx1Δtxl1 strain, we discovered that, although pap1+ mRNA levels were similar in all of the other conditions explored, Pap1 activity was significantly increased when H2O2 stress was applied to cultures containing Δtrx1Δtxl1Δtrx1 cells in the presence of a non-inducible peroxiredoxin (prx1Δ) (Figure 5B). This was consistent with the hypothesis that, in the absence of Trx1 and/or Txl1, Pap1-dependent gene expression was induced by a non-redox-regulated mechanism. In support of this, we observed that transcription of the proapoptotic gene gad1 was increased in Δtrx1Δtxl1Δtrx1 cells compared with Δtrx1Δtxl1 cells (Figure 5C).

On the other hand, in the absence of H2O2 stress, Pap1 activity was only moderately increased in Δtrx1Δtxl1Δtrx1 cells compared with Δtrx1Δtxl1 cells (Figure 5D). This was consistent with the hypothesis that the increased stability of Pap1 in the absence of Trx1 and/or Txl1 was the result of a non-redox-regulated mechanism. In support of this, we observed that transcription of the proapoptotic gene gad1 was increased in Δtrx1Δtxl1Δtrx1 cells compared with Δtrx1Δtxl1 cells (Figure 5D).

Figure 2. The Thioredoxin-like Protein Txl1 Is Present in the Nucleus and Cytoplasm and Is Regulated by H2O2 in a Tpx1- and Trr1-Dependent Manner

(A) Immunolocalization with Flag antibodies of Flag epitope-tagged Txl1 (Flag-Txl1; JB95) and Trx1 (Flag-Trx1; JB35) expressed from their normal chromosomal loci, respectively, revealed that Flag-Txl1 is distributed throughout the cell, whereas Flag-Trx1 is excluded from the nucleus both before and following treatment with 0.2 mM H2O2. Nuclei were detected by DAPI staining.

(B) Txl1 is oxidized by H2O2 and reduced by Trr1. The redox state of Flag-Txl1 expressed from the normal chromosomal locus was analyzed by western blot using extracts prepared by acid lysis and AMS treatment from (A) wild-type (AD82), (B) Δtrx1 (JB30), Δtxl1 (EV75), and Δtrx1Δtxl1 (AD140) cells before and following treatment with 0.2 mM H2O2 for the indicated times. Oxidized (Txl1ox) and reduced (Txl1red) Flag-Txl1 were detected using Flag antibodies in cells expressing wild-type Txl1 (CHP429) are indicated, including a band with a similar mobility to oxidized Flag-Txl1 that is detected in cells before H2O2 treatment. (B and D–F) Oxidized (Txl1ox) and reduced (Txl1red) Flag-Txl1 were detected using Flag antibodies in cells expressing wild-type Txl1 (CHP429) are indicated, including a band with a similar mobility to oxidized Flag-Txl1 that is detected in cells before H2O2 treatment. (B and D–F) Oxidized (Txl1ox) and reduced (Txl1red) Flag-Txl1 were detected using Flag antibodies in cells expressing wild-type Txl1 (CHP429) are indicated, including a band with a similar mobility to oxidized Flag-Txl1 that is detected in cells before H2O2 treatment. (B and D–F) Oxidized (Txl1ox) and reduced (Txl1red) Flag-Txl1 were detected using Flag antibodies in cells expressing wild-type Txl1 (CHP429) are indicated, including a band with a similar mobility to oxidized Flag-Txl1 that is detected in cells before H2O2 treatment.
strains and not significantly affected by H$_2$O$_2$, pap1$^+$ mRNA levels were significantly higher in $\Delta$trx1$\Delta$txl1 cells and varied considerably between cultures (Figure 4B; Table S3). Indeed, the variation in the fold induction of Pap1-dependent gene expression observed between the individual experiments correlated directly with the variation in the level of pap1$^+$ mRNA in $\Delta$trx1$\Delta$txl1 cells (Table S3). Taken together, these data strongly suggest that increased levels of pap1$^+$ mRNA contribute to the increased levels of Pap1-dependent gene expression in $\Delta$trx1$\Delta$txl1 cells (Table S3). Given the constitutive activation of Pap1 in $\Delta$trx1$\Delta$txl1 cells, it was surprising to uncover a mechanism leading to increased pap1$^+$ gene expression (Figure 4; Table S3). Intriguingly, the presence of reduced Pap1 in $\Delta$trx1$\Delta$txl1 cells that is not oxidized following H$_2$O$_2$ treatment (Figures 3A, 3C, and 5A) suggested that these cells contain a pool of reduced Pap1 that is insensitive to oxidation. Indeed, although $\Delta$trx1$\Delta$txl1 cells contain similar total levels of Pap1 protein to other strains (Figure 5B, whole-cell lysates), when extracts were prepared under non-denaturing conditions (which do not preserve the in vivo thiol-redox state), Pap1 levels were markedly lower in $\Delta$trx1$\Delta$txl1 cells than in other strains (Figure 5B, soluble proteins). This suggests that the majority of Pap1 in $\Delta$trx1$\Delta$txl1 cells is insoluble and, hence, resistant to extraction under non-denaturing conditions (Figure 5B, compare soluble proteins with whole-cell lysates). These data suggest that Trxl1 and/or Tx1 is required to maintain Pap1 in a reduced, soluble form that can be activated by oxidation. Notably, wild-type levels of soluble Pap1 were maintained in $\Delta$trr1 cells (Figure 5B), in which Tx1 is completely oxidized (Figure 2B), and levels of oxidized Trx1 are increased (Figure S2). In contrast, a pool of nonoxidizable, insoluble Pap1 was present in $\Delta$trx1$\Delta$txl1$\Delta$trr1 cells (Figure 5A; data not shown). Although these data could indicate positive roles for oxidized Trx1 and Tx1 in Pap1 activation, $\Delta$trr1 cells also contain significant levels of reduced Trx1, which may be responsible for maintaining Pap1 in a soluble form that can be oxidized (Figure S2). Together, these data suggest that Trx1 and Tx1 have redundant roles in maintaining Pap1 in a soluble, oxidizable form (Figure 5) and feedback inhibition of pap1$^+$ gene expression (Figure 4B).

The Thioredoxin Peroxidase Activity of Tpx1 Is Required to Prevent Direct, Tx1-Mediated Reduction of Pap1

Cytoplasmic Trxs have recently been shown to be important for oxidative folding of certain mitochondrial proteins by preventing premature oxidation in the cytoplasm prior to mitochondrial import (Durigon et al., 2012). To test whether Trx1 or Tx1 might have a similar role in preventing inappropriate oxidation of Pap1, we looked for evidence that Trx1 and/or Tx1 directly regulates the oxidation/reduction of Pap1 in vivo. Tx1 has been demonstrated to reduce a model oxidized substrate in vitro, but in vivo substrates have not been established (Andersen et al., 2011). The oxidoreductase activities of thioredoxin family proteins such as Trx1 and Tx1 involve two cysteine residues within a CGPC motif (Lee et al., 1998; Miranda-Vizuete et al., 1998). The reduction mechanism involves the formation of an intermediate mixed disulfide between one of these cysteines and the oxidized substrate protein. Hence, to determine if Tx1 is directly involved in the reduction of Pap1 in vivo, we first examined whether Tx1 and Pap1 form a mixed disulfide complex(es). Although we could not detect potential Pap1-Tx1 mixed disulfide complexes when comparing extracts from wild-type and $\Delta$txl1 cells treated with H$_2$O$_2$ (data not shown), we reasoned that such complexes might be difficult to detect due to their likely transient nature. The peroxidatic and reducing catalytic cysteine residues of Tpx1 are both essential for normal in vivo thioredoxin peroxidase activity (Figure 2E; Day et al., 2012) and H$_2$O$_2$-induced activation of Pap1 (Bozonet et al., 2005; Vivancos et al., 2005). However, whereas the H$_2$O$_2$-induced oxidation of Pap1 is completely dependent on the peroxidatic cysteine residue, in cells expressing Tpx1C169S, where the resolving cysteine is substituted with serine, Pap1 still

Figure 3. Txl1 and Trx1 Have Overlapping Functions as Inhibitors of the H$_2$O$_2$-Induced Activation of Pap1

Txl1 and Trx1 have overlapping functions as inhibitors of Pap1 activation as revealed by (A and C) western blot analysis using Pap1 antibodies of IAA-treated protein extracts prepared from wild-type (AD82), $\Delta$trx1 (EV75), $\Delta$txl1 (JB30), $\Delta$trr1 (AD81), and $\Delta$trx1$\Delta$txl1 (AD140) cells treated with 0.2 mM H$_2$O$_2$ for the indicated times. Oxidized (Pap1$^{\text{ox}}$) and reduced (Pap1$^{\text{red}}$) Pap1 were separated on the basis of their different electrophoretic mobility following IAA modification of reduced cysteines. (B) The localization of Pap1 in midlog-phase-growing wild-type (AD82), $\Delta$txl1 (EV75), $\Delta$trx1 (JB30), $\Delta$trr1 (AD81), and $\Delta$trx1$\Delta$txl1 (AD140) cells treated with 0.2 mM H$_2$O$_2$ for the indicated times was examined by indirect immunofluorescence using Pap1 antibodies. Each image was captured under identical conditions and exposure time.

See also Table S2.
forms H₂O₂-induced HMW disulfide complexes (Figures 6A and 6B) (Vivancos et al., 2005). Despite considerable effort, we have found no evidence that these Pap1 HMW complexes contain either Tpx1 or Trx1 (Bozonet et al., 2005; Figure S3). To investigate whether any of these Pap1 HMW complexes represent mixed disulfide complexes with Tx1, we compared the mobilities of complexes formed in Tpx1C169S-expressing cells containing either wild-type Tx1 or Flag epitope-tagged Tx1 (Flag-Tx1). As expected, HMW complexes containing Pap1 were detected in cells expressing Tpx1C169S from the normal chromosomal locus, and these increased following H₂O₂ treatment (Figure 6A).

Strikingly, a complex with the predicted MW for a Tx1-Pap1 disulfide (asterisk [*] in Figure 6A) was observed in both untreated and H₂O₂-treated Tx1-containing cells. Consistent with the hypothesis that this was a Tx1-Pap1 disulfide, this band (*) was not present in cells expressing Flag-Tx1, and a new lower mobility band (#) was detected instead (Figure 6A). Unfortunately, the new putative Flag-Tx1-Pap1 band was of a similar mobility to a ns band, making it difficult to resolve the two bands. The new putative Flag-Tx1-Pap1 band was also less intense, perhaps reflecting that Flag-Tx1 levels are lower than levels of endogenous Tx1, although in the absence of a specific Tx1 antibody, this could not be confirmed. We also observed that the mobility of several HMW Pap1 disulfide complexes, induced following exposure to H₂O₂ (indicated by arrowheads), was very slightly decreased in cells expressing Flag-Tx1 (Figure 6A). These slight decreases in mobility were consistent with the effect of a Flag epitope tag, suggesting that these complexes may also contain Tx1 (Figure 6A).

These data suggest that Tx1 is directly involved in the oxidation/reduction of Pap1. To test this hypothesis, Pap1 oxidation was next examined in wild-type and Δtx1Δtrr1 cells expressing Tpx1C169S (Figure 5A). Notably, many of the HMW forms of Pap1 that were detected in cells expressing Tpx1C169S were no longer detected in Δtx1Δtrr1Δtxl1 cells, consistent with the hypothesis that Tx1 is necessary for the oxidation of Pap1.

Figure 4. Trx1 and Tx1 Inhibit pap1+ and Pap1-Dependent Gene Expression

Northern blot analyses of RNA extracted from midlog phase-growing wild-type (AD82), Δtx1 (EV75), Δtx1Δtrr1 (AD81), and Δtx1Δtxl1Δtrr1 (AD140) cells treated with 0.2 mM H₂O₂ for the indicated times. Gene-specific probes were used to detect RNA from the indicated genes. A gene-specific probe for leu1+ was used as a loading control (see also Table S1).

(A) A representative blot of the indicated Pap1-dependent genes and the graphs show the quantitative analyses of three independent experiments plotted as the mean fold induction of each mRNA relative to wild-type. Error bars represent the SEM.

(B) A representative blot is shown from three independent experiments illustrating the increased pap1+ mRNA levels detected in Δtx1Δtxl1 cells. For quantified experimental data from each experiment, See Table S3.

Figure 5. Trx1 and Tx1 Are Important for Maintaining Pap1 in a Soluble Form that Can Be Oxidized in Response to H₂O₂

(A) Western blot analysis of the oxidation state of Pap1 in wild-type (AD82), Δtx1 (JB30), Δtrr1 (AD81), Δtx1Δtxl1 (AD140), and Δtx1Δtxl1Δtrr1 (JB120) cells before and following treatment with 0.2 mM H₂O₂ for 10 min reveals that in the absence of Tx1 and Tx1, there is a pool of reduced Pap1 that is not oxidized following exposure to H₂O₂.

(B) Western blot analysis of Pap1 in extracts prepared under native (soluble) and denaturing (whole-cell lysate) conditions from the indicated strains and Δpap1 (TP108-3C), Δtrx1 (JB30), Δtrr1 (AD81), Δtrx1Δtxl1 (AD140), and Δtrr1 (EV75) reveals that, although total Pap1 levels are similar in each strain, Pap1 is undetectable in extracts from Δtx1Δtrr1 cells prepared under non-denaturing conditions. It should be noted that although, as indicated, oxidized and reduced Pap1 forms can be detected by both extraction methods, the absence of any precautions to prevent in vitro thiol exchange means that the relative levels of reduced and oxidized Pap1 in the soluble protein extracts are not a reliable indicator of the in vivo oxidation state of Pap1. See also Figure S2.
in Δtxl1 cells, including the band with the expected mobility of a Txl1-Pap1 disulfide present in untreated txl1+/+ cells (Figure 6B, compare lanes 3–6). Furthermore, loss of Txl1 restored wild-type H2O2-induced Pap1 oxidation to Tpx1C169S-expressing cells (Figure 6B, compare lanes 2 and 6). Excitingly, this suggests that Txl1 reduces Pap1, inhibiting the H2O2-induced formation of fully oxidized, active forms. Strikingly, it also suggests that the sole function of the resolving cysteine of Tpx1 in H2O2-induced activation of Pap1 is to promote Txl1 oxidation (Figure 2E) and, thus, prevent Txl1 from reducing Pap1 (Figures 6A and 6B). This raised the possibility that the failure to inhibit Txl1 underlies the increased H2O2 sensitivity of Tpx1C169S-expressing cells (Figure 6C). Collectively, these data suggest that the essential role for the thioredoxin peroxidase activity of Tpx1 in adaptation to H2O2 is to promote the H2O2-induced oxidation of Txl1 and, thus, allow H2O2 signal transduction (Figures 7 and S4).

**DISCUSSION**

Here, we present a mechanism for the sensing and propagation of an H2O2 signal that is mediated by the thioredoxin peroxidase activity of the 2-Cys Prx, Tpx1. Strikingly, we show that this signaling activity is dependent on the ability of Tpx1 to promote the H2O2-dependent oxidation of a conserved thioredoxin family protein, Txl1 (human TRP32/Txnl1). We have identified
the Pap1 transcription factor as an in vivo substrate for Txl1. We show that reduced Txl1 directly inhibits Pap1, preventing the accumulation of its active, oxidized form. We demonstrate that the critical role of the thioredoxin peroxidase activity of Tpx1 in the H2O2-dependent activation of Pap1 is to promote Txl1 oxidation and thus prevent Txl1 from reducing Pap1.

Thioredoxin family proteins, including Trx1 and Txl1, are highly conserved, acting as crucial cofactors for many enzymes and as redox-sensitive inhibitors of various signaling proteins (Lee et al., 2013b). Hence, as discussed below, our data have broader implications for the mechanisms mediating H2O2 signal transduction. For instance, our model suggests that the relative levels of the thioredoxin reductase Trr1, which reduces Trx1 and Txl1, and the 2-Cys Prx Tpx1, which drives Trx1 and Txl1 oxidation, are key in determining the H2O2 responsivity of Pap1 (Figure 7). Indeed, this model explains the observation that overexpression of Tpx1 increases the activation of Pap1 by H2O2 (Bozonet et al., 2005). Moreover, consistent with the idea that for effective adaptation to H2O2 it is important that levels of thioredoxin reductase are limiting, overexpression of Trr1 actually reduces the resistance of wild-type cells to H2O2 (Figure S5).

**Role of Trx1 and Txl1 in Regulation of Pap1**

The limited effect of loss of Txl1 on the activation of Pap1 compared with loss of both Trx1 and Txl1 (Figures 3 and 4) points to redundancy between Trx1 and Txl1 in the reduction of oxidized Pap1. Nonetheless, we have been unable to find any evidence that Txl1 directly reduces Pap1 in vivo (such as Trx1-Pap1 disulfides), even in cells lacking Txl1 (Figures S6B and S3). Moreover, because Trx1 is excluded from the nucleus, it is unlikely to be involved in reducing nuclear, oxidized Pap1 (Figure 2A) (Day et al., 2012). Previous studies have shown that Txl1 and the mammalian homolog, Tmx1/TRP52, associate with the proteasome (Andersen et al., 2009; Wiseman et al., 2009). Although this suggests that Txl1 might be involved in the degradation of oxidized proteins (Andersen et al., 2011), we did not detect any increase in Pap1 levels in unstressed or H2O2-treated cells lacking Txl1 (Figure 5). This suggests that mechanisms to target nuclear, oxidized Pap1 for proteasomal degradation are sufficient to compensate for loss of Txl1 (Kita-mur et al., 2011).

The partial oxidation (Figure 5A) and constitutive activity (Figure 4A) of Pap1 in Δtrx1Δtxl1 cells indicate that Trx1 and Txl1 are not required for the activation of Pap1. However, intriguingly, we have discovered that there is a significant pool of Pap1 that is insoluble (Figure 5B) and resistant to oxidation in Δtrx1Δtxl1 cells (Figure 5A) in which there is a complete absence of thioredoxin activity (e.g., Figure 2C). This suggests that Trx1 and Txl1 have semiredundant functions in maintaining Pap1 in a soluble form that is sensitive to oxidation. Hence, the most likely explanation for the increased *pap1*+ gene expression in Δtrx1Δtxl1 cells (Figure 4B; Table S3) is that a negative feedback mechanism, monitoring levels of thioredoxin and/or soluble Pap1, is disrupted in these cells (Figure S4).

In the evolutionarily distant yeast *S. cerevisiae*, the Pap1-related Yap1 transcription factor is also oxidized and activated in response to H2O2. Depending on the strain background, either the thioredoxin peroxidase Gpx3 or the Tpx1 ortholog, Tsa1, is essential for the H2O2-induced oxidation and activation of Yap1 (Delaunay et al., 2002; Ozakazi et al., 2005; Veal et al., 2003). Intermolecular disulfide bonds between either Gpx3 or Tsa1 and Yap1 have been detected and proposed to initiate the H2O2-induced oxidation of Yap1 (Delaunay et al., 2002; Tachibana et al., 2009). It is possible that, in addition to promoting the oxidation of Trx1 and Txl1, Tpx1 is also directly involved in the initiation of Pap1 oxidation. However, no intermolecular disulfide bond(s) has been detected between Pap1 and Tpx1 (Bozonet et al., 2005; Vivancos et al., 2005). Moreover, loss of Tpx1 does not prevent the constitutive oxidation and activation of Pap1 in cells lacking Trr1 (Figure 1). Thus, although Tpx1 is normally required for H2O2-induced activation, Pap1 can...
also be oxidized by a Prx-independent mechanism. We cannot rule out the possibility that another peroxidase directly transduces H$_2$O$_2$ signals to Pap1, similar to Gpx3-dependent regulation of Yap1 in S. cerevisiae. However, Pap1 is oxidized to a similar extent in Δtrr1 cells and in Δgpx1Δtpx1Δtrr1 cells, lacking both Tpx1 and Gpx1, the Gpx3 ortholog (data not shown). Thus, our data are consistent with a model whereby Pap1 can be directly oxidized, perhaps by a mechanism involving the initial formation of HMW Pap1-Pap1 disulfide complexes.

**The Role of Prxs as Regulators of Thioredoxin Family Proteins**

Prxs are multifunctional enzymes that make significant contributions to the oxidative stress resistance, genome stability, and longevity of unicellular eukaryotes, plants, and animals (for a review, see Nystro¨m et al., 2012). Although Prxs also have a chaperone activity that may be important for protein homeostasis, it is assumed that detoxification of peroxides by the thioredoxin peroxidase activity of these enzymes makes an important contribution to many of these biological roles (for reviews, see Nystro¨m et al., 2012; Rhee and Woo, 2011). Our previous discovery that the thioredoxin peroxidase activity of Tpx1 actually reduces the survival of cells exposed to acutely toxic levels of H$_2$O$_2$ by inhibiting thioredoxin-mediated repair processes (Day et al., 2012) led us to the hypothesis tested here that a major function of Prxs in response to low, signaling levels of H$_2$O$_2$ is to oxidize thioredoxin. Notably, there is evidence that Prx-mediated inhibition of a thioredoxin family protein is also important for signaling in plants (Dangoor et al., 2012). Strikingly, we find that an H$_2$O$_2$-signaling role, as a regulator of Tx1, is the major function for the thioredoxin peroxidase activity of Tpx1 in the oxidative stress resistance of S. pombe (Figure 6). These data raise the possibility that the function of Prxs, as peroxide-dependent regulators of thioredoxin family proteins, makes an important contribution to their roles in oxidative stress responses, cancer, and aging (Nystro¨m et al., 2012; Rhee and Woo, 2011).

**Broader Implications for H$_2$O$_2$ Signaling**

Thioredoxins are broad specificity oxidoreductases (Lee et al., 2013b). Consequently, it is likely that Tx1 reduces other protein substrates, in addition to Tpx1 and Pap1. Indeed, a recent study suggested that the human Tx1 homolog, TRP32, reduces a protein tyrosine phosphatase, PRL, that regulates various signaling pathways (Ishii et al., 2013). Hence, an important implication of our work is that the coupling of the redox state of Tx1 and Tx1 to the thioredoxin peroxidase activity of Tpx1 provides a means to regulate other Tx1 and Tx1 substrates in response to H$_2$O$_2$. This model predicts that the relative activities of thioredoxin reductase, which reduces thioredoxin family proteins, and Prxs, which promote their oxidation, play a vital role in determining cell responses to H$_2$O$_2$ (Figure 7).

H$_2$O$_2$ signals promote cell growth and diverse responses, such as cell migration and differentiation. ROS signals have also been shown to slow organismal aging (Yang and Hekimi, 2010; Zarse et al., 2012). However, the targets responsible for many of these ROS-mediated effects are unclear. Our study raises the exciting prospect that enzyme activities, not previously connected with H$_2$O$_2$ signal transduction, might be subject to Prx-dependent regulation in cells, such as S. pombe, where thioredoxin reductase activity is limiting. For example, Prx-mediated inhibition of thioredoxin may provide a mechanism to inactivate thioredoxin-dependent enzymes in response to H$_2$O$_2$.

It remains to be determined whether the mechanisms presented here contribute to the regulation of H$_2$O$_2$ signal transduction in multicellular eukaryotes. It is possible that inhibition of thioredoxin family proteins underlies some of the positive roles of mammalian Prx in H$_2$O$_2$ signaling. For instance, Prx-mediated inhibition of thioredoxin may promote the H$_2$O$_2$-induced activation of the many signaling proteins that are inhibited by thioredoxin (for a review, see Lillig and Holmgren, 2007). However, mammalian Prxs have also been shown to inhibit H$_2$O$_2$ signaling by removing H$_2$O$_2$ (Woo et al., 2010; Rhee and Woo, 2011). It is tempting to speculate that, in these cases, thioredoxin reductase levels are not limited, and hence, Prx activity has a greater effect on the levels of H$_2$O$_2$ available for signaling than on thioredoxin activity. Indeed, a further implication of this study is that regulating the relative abundance/activities of thioredoxin reductase and thioredoxin peroxidase enzymes within a particular cell/cell compartment could be an effective means to tailor the H$_2$O$_2$ sensitivity of specific cells/pathways. Accordingly, the mechanisms presented here provide a framework that will be important for fully understanding how cells/organisms utilize H$_2$O$_2$ as a signaling molecule.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth Conditions**

*S. pombe* strains are listed in Table S4. Plasmids and strains are described in the Supplemental Experimental Procedures. Strains were grown at 30°C with rich medium (YE5S) or minimal media (EMM) with essential supplements (Moreno et al., 1991). Kanamycin resistance was detected using YE5S media containing 200 µg/ml G418 disulfate (Melford).

**Analysis of Soluble Pap1 Levels and the Oxidation State of Pap1, Tpx1, and Tx1**

A total of 3.75–7.5 × 10$^5$ exponentially growing cells expressing Pap1, Tpx1, or Flag epitope-tagged Tpx1 (Flag-Tpx1) were harvested before or after exposure to the indicated concentration of H$_2$O$_2$. For analysis of soluble Pap1 levels, protein was extracted as described previously (Veal et al., 2002) using a non-denaturing procedure in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5 g/100 ml Nonidet P-40 with 2 µg/ml pepstatin A, 2 µg/ml leupeptin, 100 mg/ml phenylmethylsulfonyl fluoride, and 1 µl/100 ml aprotinin), and extracts were clarified. For determination of oxidation state, cells were harvested following addition of trichloroacetic acid (TCA) to a final concentration of 10%. Lysates were prepared essentially as described previously by Delaunay et al. (2000). Proteins were resuspended in either 200 mM Tris-HCl (pH 8.0), 1% w/v SDS, and 1 mM EDTA containing 4-acetamido-4′-(iodocarbamoyl)amino)stilbene-2,2′-disulfonic acid (AMS) (Invitrogen) (Flag-Tpx1 or Tpx1), or 100 mM Tris-HCl (pH 8.0), 1% w/v SDS, and 1 mM EDTA containing 75 mM iodoacetamide (IAA) (Pap1) as indicated. For Pap1, lysates were also treated with alkaline phosphatase (Roche Diagnostics).

Equivalent amounts of thiol-modified (AMS or IAA) and unmodified proteins were separated by nonreducing 8% (Pap1) or 10% (Flag-Tpx1 or Tpx1) SDS-PAGE and transferred to a Protran nitrocellulose membrane (Whatman) prior to incubation with monoclonal M2 Flag antibody (Sigma-Aldrich) for Flag-Tpx1, rabbit polyclonal Pap1 antibody for Pap1 (provided by Nic Jones and Caroline Wilkinson), or rabbit polyclonal Tpx1 antibody for Tpx1 (Day et al., 2012). Horseradish peroxidase-conjugated anti-rabbit (Pap1 or...
Tpx1) or anti-mouse (Flag-Txl1) IgG secondary antibodies were used together with the ECL detection system (Amersham Biosciences) to visualize proteins on film (Fuji) or using a Typhoon FLA9500. Quantitative densitometric analyses of western blots were conducted using ImageJ 1.44 or ImageQuant software.

Analysis of mRNA
RNA was extracted from 2.5-3 x 10^6 exponentially growing cells cultured in YESS harvested before and after treatment with H_2O_2 as previously described (Veal et al., 2002). RNA was analyzed using glyoxal gels and northern blotting with ^32P-dCTP-labeled gene-specific probes in QuikHyb (Stratagene). Phosphorimager analysis was conducted using a GE Typhoon FLA9500 and ImageQuant software.

Immunolocalization
To investigate cellular localization of Pap1, Flag-Trx1, and Flag-Txl1, exponentially growing cells were fixed and immunostained as described previously (Veal et al., 2002) using either polyclonal Pap1 or monoclonal Flag antibodies and either Alexa-conjugated anti-rabbit or anti-mouse IgG secondary antibodies, respectively (Veal et al., 2002). Wild-type and pap1 cells were stained with either Flag or Pap1 antibodies, respectively, as controls. Immunostained cells were mounted in VECTASHIELD containing DAPI (Vector Laboratories) to detect nuclei. Stained cells were analyzed using a Zeiss Axio Scope fluorescence microscope, and images were obtained using identical exposures within each experiment with the AxioVision imaging system.

Statistical Analysis of Data
All experiments were repeated at least three times on independently generated samples with similar results. Representative experiments or the quantitative densitometric analysis of several experiments are shown.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, five figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.10.036.

ACKNOWLEDGMENTS
We thank Janet Quinn, Viktor Koroichuk, Neil Perkins, and Simon Whitehall for comments, Caroline Wilkinson and Nic Jones for Pap1 antibodies, and Michelle Wray for technical assistance. The work was funded by the BBSRC (to A.M.D., J.D.B., L.E.T., B.A.M., and E.A.V.) and MRC (to S.R.T.).

Received: June 14, 2013
Revised: July 23, 2013
Accepted: October 21, 2013
Published: November 21, 2013

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