A genetic approach to study H_2O_2 scavenging in fission yeast – distinct roles of peroxiredoxin and catalase

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SUMMARY

The main peroxiredoxin in *Schizosaccharomyces pombe*, Tpx1, is important to sustain aerobic growth, and cells lacking this protein are only able to grow on solid plates under anaerobic conditions. We have found that deletion of the gene coding for thioredoxin reductase, *trr1*, is a suppressor of the sensitivity to aerobic growth of Δ*tpx1* cells, so that cells lacking both proteins are able to grow on solid plates in the presence of oxygen. We have investigated this suppression effect, and determined that it depends on the presence of catalase, which is constitutively expressed in Δ*trr1* cells in a transcription factor Pap1-dependent manner. A complete characterization of the repertoire of hydrogen peroxide scavenging activities in fission yeast suggests that Tpx1 is the only enzyme with sufficient sensitivity for peroxides and cellular abundance as to control the low levels produced during aerobic growth, catalase being the next barrier of detoxification when the steady state levels of peroxides are increased in Δ*tpx1* cells. Gpx1, the only glutathione peroxidase encoded by the *S. pombe* genome, only has a minor secondary role when extracellular peroxides are added. Our study proposes non-overlapping roles for the different hydrogen peroxide scavenging activities of this eukaryotic organism.

INTRODUCTION

Reactive oxygen species such as superoxide and hydrogen peroxide (H_2O_2) are produced during aerobic growth mainly as by-products during the transfer of electrons in mitochondrial respiration. A battery of cellular activities scavenge superoxide and H_2O_2, so that an equilibrium between production and detoxification is achieved, reaching physiological, non-toxic steady-state levels of these reactive oxygen species.

Regarding H_2O_2 detoxification, at least three families of enzymes cooperate to reach nanomolar intracellular levels: catalases, glutathione peroxidases (Gpxs) and peroxiredoxins (Prxs) (for reviews, see (Flohe *et al.*, 2011; Kirkman and Gaetani, 2007; Low *et al.*, 2008; Rhee *et al.*, 1994; Zamocky *et al.*, 2008). Catalases dismutase two molecules of H_2O_2 to generate water and oxygen at the expense of the reversible oxidation-reduction of iron at their heme group. Most Gpxs ant Prxs decompose H_2O_2 to water with the concomitant oxidation of two cysteine residues to a disulfide bond; reduction of the disulfide to the thiol form will require the participation of glutathione or thioredoxin, respectively, at the expense of reduced cofactor. It is worth pointing out that some Prxs, such as bacterial AhpC, can use electron donors other than thioredoxin (Poole *et al.*, 2000).

The *Schizosaccharomyces pombe* genome contains three genes coding for Prx isoforms (Tpx1, Pmp20/SPCC330.06c and BCP/SPBC1773.02c), one gene coding for a Gpx (Gpx1), and another one coding for catalase, Ctt1. While cells lacking *ctt1* (Mutoh *et al.*, 1999), *gpx1* or *pmp20* (Vivancos *et al.*, 2005) do not seem to display growth defects on aerobic plates, strain Δ*tpx1* cannot grow on solid agar unless anaerobic conditions are used (Jara *et al.*, 2007; Vivancos *et al.*, 2005), which prompted us to hypothesize that Tpx1 is the main H_2O_2 scavenger during aerobic growth in fission yeast. It is worth pointing out that growth on aerobic plates is always more extreme to microbial cells than growth on liquid cultures: cells lacking Tpx1 are able to duplicate in liquid, while single cells cannot, at least efficiently, initiate colony formation on solid plates.

Gpxs and, especially, Prxs have been proposed to have an additional role in transmitting the H_2O_2-dependent oxidizing signal to transduction pathways, so that antioxidant responses can be triggered (for reviews, see (Fourquet *et al.*, 2008; Rhee *et al.*, 2012). In these

signalling events, reversible oxidation of thiols in pathway components as an activation step cannot be achieved directly by H_2O_2, since reactivity of peroxides towards most cysteine residues is rather limited. Only proteins such as Prxs and Gpxs contain cysteine residues with very high sensitivity for H_2O_2, and may be able not only to sense and react with peroxides but also to transmit the signal and oxidize other secondary targets in signal transduction pathways (for a review, see (Winterbourn and Hampton, 2008). In *S. pombe*, upon mild extracellular oxidative stress Tpx1 is an upstream activator of the transcription factor Pap1 (Bozonet *et al.*, 2005; Vivancos *et al.*, 2005), which then accumulates in the nucleus to induce up to 80 genes and develop an adaptation response (Chen *et al.*, 2008). Among the many genes triggered by oxidized Pap1 is *ctt1,* whose transcription is activated 17-fold in response to mild extracellular oxidative stress (Chen *et al.*, 2008). The activation of Pap1 can also be achieved by genetic ablation of the thioredoxin system in a H_2O_2-independent manner: cells lacking thioredoxin reductase, Trr1, display massive disulfide stress in an oxidized thioredoxin-dependent manner, and Pap1 is fully active under these conditions (Garcia-Santamarina *et al.*, 2012; Vivancos *et al.*, 2004).

Due to the prevalent role of Tpx1 not only in aerobic H_2O_2 scavenging but also in signalling towards the Pap1 pathway, we decided to confirm the first one by searching for suppressors of the aerobic growth defects of cells lacking Tpx1. We show here that over-expression of catalase, Ctt1, either after constitutive activation of the Pap1 pathway by deletion of the Trr1-coding gene or from a heterologous promoter, is able to rescue the limitation to grow on solid plates of cells lacking Tpx1. We also show that the ability of Δ*tpx1* to grow on anaerobic plates depends on the presence of Ctt1, which is probably able to sense the enhanced steady state levels of peroxides in this strain background. Furthermore, Ctt1 is the main H_2O_2 scavenger when supplied extracellularly, while the other three putative peroxide detoxification activities, Gpx1, Pmp20 and BCP, hardly display a phenotype when multiple deletions accumulate. Our experiments demonstrate that in fission yeast Tpx1 is the first and only line of defence to control H_2O_2 generated during aerobic growth, while catalase has a major role controlling high levels of peroxides. Surprisingly, over-expression of catalase is sufficient to fully suppress the H_2O_2 sensitivity of strains lacking the transcription factor Pap1 or Atf1, essential to mediate the complex gene expression programs which response to peroxides.

RESULTS

Deletion of *trr1* suppresses the aerobic growth defects of cells lacking Tpx1

We had previously demonstrated that cells lacking Tpx1 are able to grow aerobically in liquid media, even though they display some growth defects which are more pronounced under respiratory-prone conditions (Fig. S1). These phenotypes are exacerbated when Δ*tpx1* cells are plated on solid media under normal aerobic conditions (Jara *et al.*, 2007), possibly due to the oxygen-dependent photochemical formation of H_2O_2 on the surfaces of plates by flavins present in the yeast media. The role of Tpx1 in the activation of the transcription factor Pap1 is not the cause of the aerobic phenotype of Δ*tpx1* cells, since cells lacking Pap1 do not display any growth defect on aerobic plates (Jara *et al.*, 2007) (Fig. 1A). Cells deficient in *trr1,* the only *S. pombe* gene coding for a thioredoxin reductase, are able to grow on aerobic plates (Fig. 1A), but they display pronounced sensitivity to the presence of extracellular peroxides (Fig. 1A). Surprisingly, cells deleted in both the Tpx1- and the Trr1-coding genes do not longer display defects to grow on solid plates, which indicates that deletion of *trr1* suppresses the phenotypic defect of Δ*tpx1* cells (Fig. 1A).

Tpx1 and Trr1 had been reported to antagonistically regulate the activity of the antioxidant transcription factor Pap1: Δ*trr1* cells display constitutive oxidation/activation of Pap1 (Vivancos *et al.*, 2004), while in cells lacking Tpx1 the transcription factor Pap1 cannot sense H_2O_2 stress (Bozonet *et al.*, 2005; Vivancos *et al.*, 2005). Constitutive oxidation/activation of Pap1 in *S. pombe* strains can be easily tested through their resistance to caffeine (Benko *et al.*, 1998; Calvo *et al.*, 2009). The double mutant Δ*tpx1* Δ*trr1* displays all the hallmarks of constitutive Pap1 activation: it is resistant to caffeine (Fig. 1A), Pap1 is constitutively oxidized according to non-reducing electrophoresis (Fig. 1B), the transcription factor is bound to promoters even prior to stress as determined by chromatin immuno-precipitation (Fig. 1C) and the Pap1-dependent gene expression program is engaged in the absence of peroxides (Fig. 1D). Antioxidant activities such as catalase, encoded by the *ctt1* gene, are therefore up-regulated in Δ*tpx1* Δ*trr1* cells.

We then tested whether constitutive Pap1 activation is required for the suppression effect of *trr1* deletion on Δ*tpx1* strain. As shown in Figure 1E, a triple mutant Δ*tpx1* Δ*trr1* Δ*pap1*

is no longer able to grow on solid plates in the presence of oxygen. This suggests that one or several of the Pap1-dependent gene products is able to rescue the aerobic growth defects of cells lacking Tpx1.

Over-expression of catalase is sufficient to support aerobic growth in cells lacking Tpx1

We then speculated that one or several of the four putative H_2O_2-scavenging activities left in cells lacking Tpx1, namely catalase/Ctt1, Gpx1 or the Prxs Pmp20 and BCP/SPBC1773.02c, could be over-expressed in Δ*tpx1* Δ*trr1* in a Pap1-dependent manner, and that would suppress the aerobic growth defect of cells lacking Tpx1. According to transcriptomic studies of cells exposed to H_2O_2, only the *ctt1* gene responds to sub-toxic doses of peroxides, those known to activate Pap1 (Chen *et al.*, 2008), which pointed to catalase as the activity overcoming the lack of Tpx1. We first attempted to obtain a triple Δ*tpx1* Δ*trr1* Δ*ctt1* strain by tetrad analysis, without success (Fig. S2A), indicating that cells lacking all three genes are not viable. We further demonstrated that these three genes are synthetic lethal by constructing a triple Δ*tpx1* Δ*trr1* Δ*ctt1* strain carrying an episomal plasmid containing the *ctt1* gene under the control of the thiamine-repressible *nmt* promoter (*pnmt::ctt1*, Fig. 2AB). As shown by Northern blot in Figure 2A, the levels of *ctt1* in this conditional strain were undetectable after the addition of thiamine. The viability of this strain in thiamine-containing plates was severely compromised, as shown in Figure 2B.

We then transformed wild-type cells and cells lacking Tpx1 with the same plasmid, *pnmt::ctt1*. As shown in Figure 2C, upon thiamine depletion both plasmid-containing cells types (wild-type and Δ*tpx1*) express the *ctt1* mRNA. These levels of expression were sufficient to enhance the tolerance of wild-type cells to grow in the presence of H_2O_2 (Fig. 2D). As we expected, those high catalase levels accomplished after thiamine withdrawal were also sufficient to suppress the aerobic growth defects of Δ*tpx1* cells on solid plates (Fig. 2D). It is worth pointing out that when thiamine is added, the catalase-expressing plasmid is not able to sustain the aerobic growth of Δ*tpx1* cells, nor to allow gain of tolerance to H_2O_2 of wild-type cells (Fig. 2D); importantly enough, the levels of *ctt1* mRNA upon thiamine repression of Δ*tpx1* + *pnmt::ctt1* cells are significantly lower than those constitutively reached in a Δ*tpx1* Δ*trr1* strain

(Fig. 2C). As expected, this catalase-expressing plasmid is also able to sustain the growth on aerobic plates of the triple mutant Δ*tpx1* Δ*trr1* Δ*pap1* (Fig. S2B).

Only Tpx1 and Ctt1 have significant roles in H_2O_2 scavenging

Given the previous experiments, Tpx1 and catalase having specific roles in H_2O_2 detoxification, we decided to determine whether the other three putative peroxide scavengers, namely Gpx1, Pmp20 and BCP, also had a role in H_2O_2 scavenging. According to recent proteomic studies, Tpx1 is a very abundant cellular protein, while catalase is not (relative values of 1 to 0.04, respectively; Table S1). We compared the growth of each one of the five deletion strains on aerobic plates containing or not H_2O_2. As shown in Figure 3B, only Tpx1 is required for aerobic growth, whereas deletion of *ctt1* renders cells extremely sensitive to the presence of exogenously added H_2O_2. Regarding Gpx1, Pmp20 and BCP, only Δ*gpx1* cells display a mild sensitivity to H_2O_2. Unlike catalase, *nmt-driven* over-expression of Gpx1, Pmp20 or BCP from episomal plasmids was not sufficient to sustain aerobic growth of cells lacking Tpx1 (Fig. S3A).

Tpx1 seems to be the only enzyme able to detoxify peroxides arising from aerobic metabolism. However, the enhanced levels of H_2O_2 of Δ*tpx1* cells can be partially scavenged by either Ctt1 or combination of Gpx1, Pmp20 and BCP, as observed by the enhanced anaerobic growth defects of Δ*tpx1* Δ*ctt1* and Δ*tpx1* Δ*gpx1* Δ*pmp20* Δ*BCP* strains (Fig. 3C, 3D and 3E). In conclusion, while cells lacking or over-expressing Pmp20, Gpx1 or BCP behave as wild-type cells in response to H_2O_2 stress, these proteins may have some peroxide scavenging activity which may be restricted to specific sub-cellular localizations or to non-exponentially growing conditions, and which may slightly contribute to the fitness of cells lacking Tpx1. It is worth pointing out that Pmp20 is a very abundant protein (Table S1), and that the *S. cerevisiae* ortholog has peroxisomal localization; similarly, Gpx1 has been proposed to have a role at stationary phase (Lee *et al.*, 2008).

We used a fluorescent dye, DHR123 (dihydrorhodamine 123), to measure relative levels of peroxides in different mutant strains. As shown in Figure S4, a small but significant increase in the levels of intracellular peroxides could be measured in cells lacking Tpx1, but not Ctt1. The increase, however, was almost 3-fold in cells lacking both Tpx1 and Ctt1, which

confirms the idea that in the absence of the Prx Tpx1 the enhanced levels of peroxides are partially scavenged by catalase.

We then measured peroxidase activity of whole cells (Fig. 4A). In this assay, we measured extracellular H_2O_2 leftovers as an indicator of the peroxide scavenging by intracellular activities. Again, catalase was fully responsible for the scavenging of 1 mM extracellular peroxides (Fig. 4A, left panel). However, extracellular concentrations of 10 µM are scavenged in both a Tpx1- and Ctt1-dependent manner, since only cells lacking both scavengers are unable to deplete these doses of peroxides (Fig. 4A, right panel).

We also measured the activity of Tpx1 and Ctt1 in total extracts from wild-type and mutant strains, and showed that the sensitivity of the method (2 µM H_2O_2 in the assay) allowed us to follow the H_2O_2 scavenging activity of catalase. Thus, extracts from both wild-type and Δ*tpx1* cells were able to scavenge those concentrations of peroxides, while addition of the catalase inhibitor azide to wild-type extracts, or the use of Δ*ctt1* extracts fully prevented H_2O_2 scavenging (Fig. 4B, left panel). We then decided to highlight Tpx1 activity by enhancing the amount of total protein in our assays (from 0.25 to 1 µg/µl), using extracts from cells lacking catalase and providing the corresponding electron donor for Tpx1 recycling. Tpx1 recycling is performed by thioredoxin (Trx1), thioredoxin reductase (Trr1) and NADPH (Jara *et al.*, 2007), and at least Trx1 is partially depleted from our native extracts according to Western blot analysis (data not shown; Table S1). As shown in Fig. 4B (right panel), extracts from cells lacking Ctt1 were proficient to scavenge peroxides when the complete thioredoxin system (recombinant Trx1 and Trr1, as well as reduced cofactor, NADPH) was provided to the assay. Importantly enough, this scavenging activity was dependent on the presence of Tpx1. Similarly, we could also exacerbate Tpx1-dependent H_2O_2 scavenging activity of wild-type extracts in which catalase activity had been inactivated by azide (Fig. 4C, left panel). Further evidence for the role of the thioredoxin system in Tpx1 recycling and H_2O_2 scavenging arose from the use of the thioredoxin reductase inhibitor 1-chloro-2,4-dinitrobenzene (DNCB) in our *in vitro* assay (Fig. 4C, right panel).

Over-expression of catalase is the main cellular strategy for the adaptation to H_2O_2 stress

Our data confirms the role of Tpx1 in general H_2O_2 homeostasis during aerobic growth and in activation of signalling cascades. Catalase, on the contrary, only participates in peroxide scavenging when the levels of H_2O_2 arise in a Δ*tpx1* background or upon addition of peroxides to the growth media. Up-regulation of the *ctt1* gene seems to be a common theme in the cellular adaptation to peroxides. In fission yeast, mild concentrations of H_2O_2 activate the Tpx1-Pap1 pathway, which triggers transcription of around 50-80 genes meant to mount an adaptive response to peroxides, one of them being *ctt1* (Chen *et al.*, 2008) (Fig. 5A). Higher doses of peroxides, however, temporarily halt Pap1 activation while maximally trigger a cascade of phosphorylations which end up activating the MAP kinase Sty1 and its transcription factor Atf1/Pcr1; the transcription of up to 300 genes is then activated, and again *ctt1* is at the top of the list (Chen *et al.*, 2008) (Fig. 5A).

To test the prevalent role of catalase up-regulation in the response of fission yeast to peroxides, we tested whether over-expression of Ctt1 using a strong and constitutive promoter fused to the *ctt1* open reading frame (ORF) in an integrative (one copy of the chimeric gene) or an episomal (8-9 copies/cell) plasmid could be sufficient to complement some of the H_2O_2 defects of cells mutated in components of either the Sty1-Atf1 or the Pap1 pathways. As shown in Figure 5B, we reached different levels of constitutive *ctt1* expression using these two vectors (*pctt1* epi. and *pctt1* int. in Fig. 5B), and these levels were lower than those accomplished by the *nmt*-driven plasmid (*pnmt::ctt1* in Fig. 2B and 5B). Both plasmids were able to complement the H_2O_2 sensitivity of Δ*ctt1* cells (Fig. 5C), and were also sufficient to sustain aerobic growth of cells lacking Tpx1, and to improve to different extends the tolerance of wild-type cells to peroxides, in a thiamine independent manner (Fig. S5). High levels of expression of catalase were able to suppress to a great extent, but not fully, the peroxide sensitivity of cells lacking Pap1 (Fig. 5D), while they fully recovered wild-type tolerance to H_2O_2 of cells lacking Atf1 (Fig. 5E). These levels of catalase, achieved from the episomal plasmid *pctt1*, enhanced the survival to peroxides of cells lacking both Pap1 and Atf1 to the same extend as Δ*pap1* transformed with the same plasmid (data not shown), while over-expression of Gpx1, Pmp20 or BCP could not (Fig. S3B). Moderate levels of catalase expression, lower than those reached by wild-type cells after exposure to H_2O_2 (compare lane 2 with lane 9 in Fig. 5B), were sufficient to enhance cell

survival upon a severe dose of peroxides in wild-type, Δ*pap1* and Δ*atf1* liquid cultures (Fig. 5F), which highlights the importance of catalase in scavenging high levels of H_2O_2.

DISCUSSION

Aerobic organisms express a battery of antioxidant activities to counteract the toxic effects of reactive oxygen species. Often, several enzymes seem to be redundant in terms of their antioxidant activity. In an attempt to provide an integrative view of H_2O_2 scavenging in fission yeast, we have performed an *in vivo* analysis of cells lacking each one of the five proteins encoded by the fission yeast genome meant to scavenge peroxides: catalase (Ctt1), the glutathione peroxidase Gpx1, and the Prxs Tpx1, Pmp20 and BCP.

We have confirmed here the important role of Tpx1 not only in signalling towards the Pap1 pathway, but specially in aerobic H_2O_2 scavenging. Searching for suppressors of the aerobic growth defects of cells lacking Tpx1, we have found that deletion of the *trr1* gene, coding for thioredoxin reductase, is able to restore growth on aerobic plates due to the constitutive activation of Pap1 and concomitant over-expression of catalase, Ctt1. The defects of cells lacking Tpx1 to grow on aerobic plates can be suppressed over-expressing only Ctt1 (Fig. 2D, Fig. S5), while Pap1-dependent oxidation/activation (Fig. S6A) and transcription (Fig. S6B) is not recovered in these cells. This result, together with the fact that Δ*pap1* cells are not sensitive to grow in the presence of oxygen (Fig. 1A), confirms that the aerobic growth defects of strain Δ*tpx1* are due to deficient H_2O_2 scavenging rather that to null signaling towards Pap1, as previously published (Jara *et al.*, 2007).

Furthermore, Ctt1 is the main H_2O_2 scavenger when peroxides are supplied extracellularly, while cells lacking any of the other three putative peroxide detoxification activities, Gpx1, Pmp20 and BCP, do not display sensitivity to peroxides. Our experiments demonstrate that in *S. pombe* the Prx Tpx1, with high sensitivity for peroxides and very high abundance (Table S1), is the first line of defence to control H_2O_2 generated during aerobic metabolism, while catalase has a major role controlling high levels of peroxides. Probably the activity of Tpx1 can be saturated upon high doses of H_2O_2 (either by temporary depletion of NADPH reducing power, required for its recycling, or by over-oxidation and inactivation of its peroxidatic cysteine to sulfinic acid) (Bozonet *et al.*, 2005; Vivancos *et al.*, 2005). Under these circumstances of severe H_2O_2 stress, catalase may be better suited to become the predominant peroxide scavenger. This hierarchical model of peroxide scavenging is supported by the fact

that *ctt1* expression is triggered by Pap1 only when Tpx1 becomes fully oxidized/temporarily inactivated. Indeed, over-expression of Ctt1 seems to be a critical strategy to survive a severe threat of H_2O_2: a plasmid constitutively expressing catalase is sufficient to totally (Δ*atf1*) or partially (Δ*pap1*) complement the oxidative stress sensitivity of several mutants of the two main oxidative stress responding pathways. It is intriguing to us, however, why the presence of Tpx1 is still required upon high doses of peroxides: over-expression of catalase is sufficient to sustain aerobic growth in cells lacking Tpx1, but not to avoid the sensitivity to extracellular H_2O_2 (Fig. 2D, upper right panel). We suspect that this is due to the role of Tpx1 in Pap1 activation. Thus some Pap1-dependent genes in addition to *ctt1* may still be critical to survive a H_2O_2 threat. Another possibility comes from the fact that Prxs have been described to switch from a peroxidase to a chaperone role (Jang *et al.*, 2004), but we have no indications to believe that cells lacking Tpx1 display sensitivity to heat shock (data not shown).

In bacteria, similar studies have provided genetic and biochemical evidences to demonstrate that putative H_2O_2 scavengers really perform such a role, and whether they have overlapping functions (for a review, see (Mishra and Imlay, 2012). In particular, the main peroxiredoxin of *Escherichia coli,* AhpC, was demonstrated to act as the primary scavenger of peroxides generated endogenously during aerobic growth, while catalase may specifically act when the peroxiredoxin becomes saturated (Seaver and Imlay, 2001). Then, why are *E. coli* cells lacking AhpC able to grow under aerobic conditions on solid plates? Interestingly enough, both in *S. pombe* and in *E. coli* catalase seems to have a backup role in cells lacking the peroxiredoxin Tpx1 or AhpC, respectively. In fact, expression of catalase in *E. coli* is dependent of the main sensor of peroxides and transcription factor OxyR. Thus, cells lacking AhpC display higher levels of intracellular peroxides, and OxyR-dependent over-expression of catalase, which sustains aerobic growth (Seaver and Imlay, 2001). In *S. pombe*, since *ctt1* activation at low doses of peroxides is Pap1-dependent and Pap1 activation is Tpx1-dependent, cells lacking Tpx1 cannot activate *ctt1* transcription and therefore cannot grow under aerobic conditions. The fact that fission yeast uses Tpx1 as both a H_2O_2 scavenger and as a sensor and transducer of the oxidative signal hampers the over-expression of catalase in a Δ*tpx1* background. In fact, we have confirmed by Western blot analysis that catalase protein levels are not increased in a

Δ*tpx1* strain, and conversely Tpx1 levels are not enhanced in cells lacking catalase (data not shown).

It is surprising to observe that over-expression of catalase is sufficient to rescue the sensitivity to peroxides of Δ*atf1* or Δ*pap1* cells, even though these two pathways are able to trigger many different genes in response to peroxides. Regarding the Sty1-Atf1 pathway (Fig. 5A), it is worth mentioning that several other types of life-threatening environmental stresses, such as osmotic stress, heat shock or nutrient deprivation, are also able to activate this MAP kinase signalling pathway, which could explain why hundreds of genes are up-regulated while over-expression of only catalase is sufficient to counteract the H_2O_2–mediated toxicity of Δ*atf1* cells.

It may sound like a good strategy to increase the levels of catalase to become more tolerant to oxidative threats, even prior to H_2O_2 imposition. However, excessive amounts of catalase may be prejudicial for growth, either by decreasing the steady-state levels of H_2O_2 and minimizing signalling events, or by activating other signalling cascades. Indeed, we have determined that constitutive over-expression of catalase upon H_2O_2 stress induces the iron starvation response, probably through sequestering available iron during reconstitution of the heme group (data not shown). Constitutive activation of the iron starvation response can halt cell growth, by repressing the transcription of many genes coding for essential iron-containing proteins (for a review, see (Labbe *et al.*, 2007).

EXPERIMENTAL PROCEDURES

Yeast strains and growth conditions.

Cells were grown in rich medium (YE, yeast extract) or in synthetic minimal medium (MM) at 30°C as described previously (Alfa *et al.*, 1993). Anaerobic liquid cultures were grown in flasks filled to the top with medium at 30°C without shaking. When indicated, 0.02 mg/ml thiamine was added to MM cultures to block gene expression from the *nmt* (no message in thiamine) promoter (see below, plasmids section). The origins and genotypes of strains used in this study are outlined in Table 1, and most of them were constructed by standard genetic methods. Strain EP302 (Δ*tpx1* Δ*trr1* Δ*ctt1 pnmt::ctt1*), carrying the episomal plasmid p418.41x to allow survival of cells carrying three synthetically lethal gene deletions, was constructed from strain SG156 carrying plasmid p418.41x (Δ*tpx1* Δ*trr1* + *pnmt::ctt1*), and further deletion of the *ctt1* gene using a linear *ctt1::ura4* DNA fragment and selection on MM plates without uracil.

Plasmids

The *ctt1* coding sequence was PCR-amplified from an *S. pombe* cDNA library using primers specific for the *ctt1*-coding gene. *ctt1* was cloned into the *nmt* (no message in thiamine)-driven expression vector pREP.41x (Maundrell, 1993) to yield plasmid p418.41x (*pnmt::ctt1*). Plasmid p418.41x was digested with *PstI/XhoI* to release the *nmt* promoter, which was replaced with a *PstI/XhoI* digested PCR amplified *sty1* promoter (including 797 bp from its ATG). The resulting episomal plasmid, p419 (p*sty1'::ctt1*) allowed constitutive expression of *ctt1*. Integrative plasmid AY025 (Sanso *et al.*, 2008) was digested with *PstI/SacI* to release the *nmt* promoter, multiple cloning site, and terminator which was replaced with the *sty1* promoter, the *ctt1* ORF and the terminator from p419 digested with *PstI/SacI,* yielding p422' (*psty1'::ctt1'*). p151.41x (*pHA-atf1.41x*) was previously described (Sanso *et al.*, 2008). The *gpx1, BCP/SPBC1773.02c* and *pmp20 genes* were PCR-amplified from an *S. pombe* cDNA library using specifics primers. *gpx1* ORF flanked with *BamHI* and *SmaI* restriction sites was cloned into p123.41x (Vivancos *et al.*, 2005) yielding plasmid p440.41x (*pnmt::gpx1*). *BCP* ORF was digested with *XhoI* and *SalI* and cloned into pREP.41x yielding plasmid p431.41x (*pnmt::BCP*). *pmp20* ORF flanked with

BamHI and *SmaI* sites was cloned into pREP.41x containing a MBP (maltose binding protein) tag cloned *SalI* and *BamHI*, yielding plasmid p217.41x (*pnmt::pmp20*).

Oxygen, caffeine and H_2O_2 sensitivity assays

For survival on solid plates, *S. pombe* strains were grown, diluted and spotted in YE or MM agar plates, with the indicated concentrations of H_2O_2 or 15 mM caffeine, and plates were incubated at 30ºC under aerobic or anaerobic conditions. To grow cells in solid media in an anaerobic environment, we incubated the plates at 30°C in a tightly sealed plastic bag containing a water-activated Anaerocult A sachet (Merck, Darmstadt, Germany) (Jara *et al.*, 2007), or in a nitrogen-filled anaerobic chamber (Forma Anaerobic System, Thermo Electron Corp.). When indicated 0.2 mg/ml thiamine was added to solid MM plates.

Preparation of *S. pombe* trichloroacetic acid extracts and immunoblot analysis.

To analyze the *in vivo* redox state of Pap1, trichloroacetic acid extracts were prepared as described elsewhere (Vivancos *et al.*, 2005). Pap1 was immuno-detected using polyclonal anti-Pap1 antibodies (Vivancos *et al.*, 2004).

Chromatin immuno-precipitation

To test the *in vivo* binding of Pap1 to stress promoters, the indicated strains were grown in MM, and cultures were treated or not with 0.2 mM H_2O_2 for 5 min. Chromatin isolation and immuno-precipitation was performed as previously described (Calvo *et al.*, 2012), using polyclonal antibodies against Pap1 (Vivancos *et al.*, 2004).

RNA analysis.

Total RNA from *S. pombe* MM cultures was obtained, processed and transferred to membranes as described previously (Castillo *et al.*, 2002). Membranes were hybridized with [α-32P] dCTP-labelled *caf5*, *obr1*, *SPCC663.08c*, *trr1*, *srx1, tpx1* or *ctt1* probes, containing the complete ORFs. We used ribosomal RNA or *act1* as loading controls.

Growth curves in liquid

To measure cellular growth, we used an assay based on automatic measurements of optical densities for small (100 µl) cell cultures. Cells were grown to an OD_{600} of 0.5 under continuous shaking in Erlenmeyer flasks and then diluted to an OD_{600} of 0.1. 100 µl of the diluted cultures (treated or not with the indicated amounts of stressors) were placed into a 96-well non-coated polystyrene microplate covered with an adhesive plate seal. A Power Wave microplate scanning spectrophotometer (Bio-Tek) was used to obtain the growth curves. The OD_{600} was automatically recorded using Gen5 software. The software was set as follows: OD was measured at 600 nm, incubation temperature was kept at 30ºC, the microplates were subjected to continuous shaking and the readings were done every 10 min during 48 hours.

H_2O_2 scavenging by whole cells

10 ml of exponentially growing cell cultures (OD_{600} 0.5) in MM were washed twice with equal volumes of PBS (phosphate buffer saline; 50 mM phosphate buffer containing 0.9% sodium chloride, pH 6.8) and were resuspended with 10 ml into PBS pH 6.8 containing 0.75% of glucose. After 30 min of incubation, cells were treated with different H_2O_2 concentrations. At the indicated times, an aliquot of 1 ml of cell culture was taken from the culture and centrifuged (1 min at 13,200 rpm) to separate cells from the media, and supernatants were stored on ice until sampling was over. The remaining H_2O_2 in the supernatants was measured with the Amplex red/horseradish peroxidase method, as described (Seaver and Imlay, 2001) with some minor modifications. Briefly, 50 µl of the supernatants, diluted in PBS when necessary, were mixed with 25 µl of 0.02 mg/ml horseradish peroxidise (HRP) (Sigma) and 25 µl of 200 µM Amplex Red (AR, Molecular Probes) in a 96-well black flat bottom plate (stock solutions of AR and HRP were prepared as follows: 200 µM AR in 50 mM buffer phosphate, pH 7.8, from 10 mM AR in DMSO; and 0.02 mg/ml HRP in 50 mM buffer phosphate, pH 7.8, from 1.7 mg/ml HRP in water). Fluorescence was immediately measured at λ_{ex} 530 nm and λ_{em} 590 nm in an Infinite 200 multimode reader (Tecan Group Ltd.). A blank was obtained with untreated samples, and this value was subtracted from the rest of measured fluorescence values. Relative fluorescent values were calculated using the maximum fluorescence value for each condition. At the low concentrations of H_2O_2 (10 µM) used in this assays, a decay of peroxides in the absence of

cells was detected during the course of the experiments, and subtracted from the scavenging values obtained with whole cells.

H_2O_2 scavenging by cell extracts

Cell cultures were grown to OD_{600}~0.5. Cells were pelleted, and pellets were washed twice with PBS buffer and were resuspended in 250 µl of NET-N buffer [20 mM Tris-HCl pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM phenylmethyl sulphonyl fluoride, 5 µg/ml aprotinin, protease inhibitor cocktail (Sigma)]. Cells were broken with glass beads in a BioSpec Mini-BeadBeater 16 Ring Rack. Lysates were then centrifuged to remove cell debris. The protein concentration was determined using the Bradford protein assay (Bio-Rad). 50 µl of protein extracts at a concentration of 0.25 or 1 µg/µl, as indicated, were then incubated with 2 µM H_2O_2. To inhibit the activity of catalase by azide as previously reported (Beers and Sizer, 1956), extracts were pre-treated with 1 mM azide during 5 min at room temperature prior to the addition of H_2O_2. To promote Tpx1 peroxide scavenging, we added 20 ng of recombinant Trx1 with or without 10 ng of recombinant Trr1 (Jara *et al.*, 2007), and 0.25 mM NADPH (Sigma), as indicated. To inhibit thioredoxin reductase from protein extracts and therefore block Tpx1 recycling, we treated the extracts with 30 µM DNCB [1-Chloro-2,4-dinitrobenzene (Sigma)], as reported (Arner *et al.*, 1995), during 10 min at room temperature prior to H_2O_2 addition. At the indicated times, the remaining H_2O_2 concentrations were measured with the AR/HPR method, exactly as described above.

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Table 1. Strains used in this work

Strain	Phenotype	Origin
972	*h⁻*	(Leupold, 1970)
HM123	*h⁻ leu1-32*	(Vivancos *et al.*, 2005)
PN513	*h⁻ ura4-D18 leu1-32*	Nurse lab stock
AD5	*h⁻ pap1::ura4+ ura4-D18 leu1-32 sty1'::ctt1::leu1⁺*	This work
AD6	*h⁻ leu1-32 sty1'::ctt1::leu1⁺*	This work
AD7	*h⁺ tpx1::natMX6 leu1-32 sty1'::ctt1::leu1⁺*	This work
AV25	*h⁻ pap1::kanMX6*	(Jara *et al.*, 2008)
CN513	*h- ctt1::ura4⁺ ura4-D18 ade6-M216 leu1-32*	(Nakagawa *et al.*, 1998)
EA37	*h⁻ gpx1::kanMX6 leu1-32*	(Vivancos *et al.*, 2005)
EA49	*h⁻ pmp20::kanMX6 leu1-32*	(Vivancos *et al.*, 2005)
EP193	*h⁻ atf1::natMX6 leu1-32*	This work
EP197	*h⁻ ctt1::natMX6 ura4-D18 leu1-32*	This work
EP198	*h⁺ ctt1::natMX6*	This work
EP231	*h⁻ ctt1::natMX6 ura4-D18 leu1-32 sty1'::ctt1::leu1⁺*	This work
EP232	*h⁻ atf1::natMX6 leu1-32 sty1'::ctt1::leu1⁺*	This work
EP302	*h⁺ tpx1::natMX6 trr1::kanMX6 ctt1::ura4⁺ nmt41x::ctt1::leu1⁺ ura4-D18 ade6-M210 leu1-32*	This work
IC1	*h⁻ pap1::ura4⁺ ura4-D18 leu1-32*	(Calvo *et al.*, 2012)
MJ11	*h⁻ tpx1::kanMX6*	This work
PG15	*h⁻ pap1::ura4⁺ atf1::natMX6 leu1-32 ura4-D18*	This work
SG5	*h⁺ tpx1::natMX6 leu1-32*	This work
SG156	*h⁺ tpx1::natMX6 trr1::kanMX6 ura4-D18 ade6-M210 leu1-32*	This work
SG164	*h⁻ tpx1::natMX6 trr1::kanMX6*	This work
SG167	*h⁺ trr1::natMX6*	(Garcia-Santamarina *et al.*, 2012)
SG224	*h⁺ pap1::ura4+ tpx1::natMX6 trr1::kanMX6 ura4-D18 ade6-M210 leu1-32*	This work
SG232	*h⁺ tpx1::natMX6 gpx1::kanMX6*	This work
SG253	*h⁻ SPBC1773.02c/BCP::hphMX6*	This work
SG255	*h⁻ pmp20::kanMX6 tpx1::natMX6 gpx1::kanMX6*	This work
SG256	*h⁺ pmp20::kanMX6 tpx1::natMX6*	This work
SG258	*h⁻ SPBC1773.02c/BCP::hphMX6 tpx1::natMX6*	This work
SG259	*h⁺ SPBC1773.02c/BCP::hphMX6 tpx1::natMX6 pmp20::kanMX6 gpx1::kanMX6*	This work
SG267	*h⁺ ctt1::ura4⁺ tpx1::kanMX6 ura4-D18*	This work

FIGURE LEGENDS

Fig. 1. Deletion of *trr1* rescues the aerobic defects of *Δtpx1* cells in a Pap1-dependent manner.

A. Deletion of *trr1* suppresses the aerobic growth defects of cells lacking Tpx1. Strains 972 (WT), MJ11 (Δ*tpx1*), SG167 (Δ*trr1*), SG164 (Δ*tpx1* Δ*trr1*) and AV25 (Δ*pap1*) were grown in liquid YE media, and the indicated number of cells were spotted onto plates with or without 1 mM H_2O_2 and 15 mM caffeine under aerobic (+O_2) or anaerobic (-O_2) conditions.

B, C and D. Pap1 is constitutively active in *Δtrr1* cells in a Tpx1-independent manner.

B. *In vivo* oxidation of Pap1. Cultures of strains as in A were treated or not with 0.2 mM H_2O_2 for 5 min. Trichloroacetic acid extracts were analyzed by non-reducing electrophoresis followed by Western blot analysis using polyclonal anti-Pap1 antibody. Reduced/inactive (red.) and oxidized/active (ox.) Pap1 forms are indicated with arrows.

C. Oxidized/nuclear Pap1 is constitutively recruited to all Pap1-dependent promoters in a Δ*tpx1* Δ*trr1* background. MM cultures of strains 972 (WT), MJ11 (Δ*tpx1*) and SG164 (Δ*tpx1* Δ*trr1*) were treated with 0.2 mM H_2O_2 for 5 min. Chromatin immuno-precipitation experiments using anti-Pap1 antibody were performed using primers covering promoter regions of *trr1, srx1, ctt1, caf5, obr1* and *SPCC663.08c* genes. Primers of an intergenic region were used as a negative control (control). Error bars (SD) were calculated from biological triplicates.

D. Constitutive activation of Pap1 dependent genes in a Δ*tpx1 Δtrr1* background. Cultures of strains as in C were treated or not with 0.2 mM H_2O_2 for the indicated times in min. Total RNA was obtained and analysed by Northern blot with probes for *caf5, obr1, SPCC663.08c*, *trr1, srx1,* and *ctt1*. Ribosomal RNA (rRNAs) and *act1* are shown as loading controls.

E. Survival under anaerobic and aerobic conditions in YE media plates of strains 972 (WT), MJ11 (Δ*tpx1*), SG164 (Δ*tpx1* Δ*trr1*) and SG224 (Δ*tpx1* Δ*trr1* Δ*pap1*) was analyzed as in A.

Fig. 2. Over-expression of catalase suppresses the aerobic defects of a Δ*tpx1* strain.

A. Expression of *ctt1* from the conditional Δ*tpx1* Δ*trr1* Δ*ctt1 pnmt::ctt1* strain. Cultures of strains 972 (WT), SG156 (Δ*tpx1* Δ*trr1*), SG156 carrying plasmid p418.41x (Δ*tpx1* Δ*trr1* + *pnmt::ctt1*) or EP302 (Δ*tpx1* Δ*trr1* Δ*ctt1* carrying plasmid p418.41x / *pnmt::ctt1*) were treated or not with 0.2 mM H_2O_2 for 15 min (H_2O_2 +), or with thiamine to block expression of *ctt1* from the thiamine

repressible plasmid p418.41x (thiamine +). Total RNA was obtained and analysed by Northern blot with probes for *trr1, tpx1,* and *ctt1* [a normal and a high exposure (o/exp.) are shown for *ctt1*]. Ribosomal RNA (rRNAs) and *act1* are shown as loading controls.

B. Survival to grow in MM plates (supplemented with adenine) in the presence or absence of thiamine of strains 972 (WT), SG156 carrying plasmid p418.41x (Δ*tpx1* Δ*trr1* + *pnmt::ctt1*) and EP302 (Δ*tpx1* Δ*trr1* Δ*ctt1* carrying plasmid p418.41x / *pnmt::ctt1*), was analyzed as described in Figure 1A, in conditions allowing (- thiamine) or not (+ thiamine) *ctt1* expression.

C. Over-expression of Ctt1 from a thiamine-repressible *nmt* promoter. Strains 972 (WT), SG167 (Δ*trr1*), SG164 (Δ*tpx1* Δ*trr1*), and of HM123 (WT) and SG5 (Δ*tpx1*) transformed with pREP.41x (empty epi.) or p418.41x (*pnmt::ctt1*), were grown with (thiamine +) or without thiamine to allow *ctt1* expression, were treated (H_2O_2 +) or not with 0.2 mM H_2O_2 for 15 min, and their RNA was analysed as described in Figure 1D.

D. Survival of strains HM123 (WT) and SG5 (Δ*tpx1*) transformed with pREP.41x (empty epi.) or p418.41x (*pnmt::ctt1*) was analyzed by sequential spotting as described in B, in the presence or not of 2 mM H_2O_2.

Fig. 3. The peroxiredoxin Tpx1 and catalase perform important roles in H_2O_2 detoxification.

A. Schematic representation of all *S. pombe* H_2O_2 scavengers. The positions of all cysteine residues are indicated.

B. Survival to aerobic conditions and to H_2O_2 of strains 972 (WT), AV25 (*Δpap1*), MJ11 (Δ*tpx1*), CN513 (Δ*ctt1),* EA37 *(*Δ*gpx1),* EA49 *(*Δ*pmp20)* and SG253 *(*Δ*BCP)* was analyzed as described in Figure 1A

C. The double mutant Δ*tpx1* Δ*ctt1* is even sicker than the single *⊿tpx1* strain. Strains 972 (WT), MJ11 (*⊿tpx1*), SG267 *(*Δ*tpx1* Δ*ctt1*) and SG259 (Δ*tpx1* Δ*gpx1* Δ*pmp20* Δ*BCP)* were streaked on YE plates and allowed to grow in the presence ($+O_2$) or absence ($-O_2$) of oxygen.

D. The phenotype of *⊿tpx1* strain is severely impaired by additional mutation of *ctt1*, but not by deletion of the other putative H_2O_2 scavengers. Survival of strains 972 (WT), MJ11 (Δ*tpx1*), SG267 (Δ*tpx1* Δ*ctt1),* SG232 (Δ*tpx1* Δ*gpx1),* SG256 (Δ*tpx1* Δ*pmp20),* SG258 (Δ*tpx1* Δ*BCP)*, SG255 (Δ*tpx1* Δ*gpx1* Δ*pmp20) and* SG259 (Δ*tpx1* Δ*gpx1* Δ*pmp20* Δ*BCP)* was analyzed as described in Figure 1A.

E. Growth curves of cultures of strains as in C. Log-phase cultures at an OD_{600} of 0.1 were grown into microculture wells. Growth was monitored by measuring OD_{600} every 10 min at 30º for 48 h.

Fig. 4. Scavenging of H_2O_2 both by whole cells and by cells extracts.

A. H_2O_2 scavenging by whole cells. Cell cultures from strains 972 (WT), MJ11 (Δ*tpx1*), EP198 (Δ*ctt1*) and SG267 *(*Δ*tpx1* Δ*ctt1*)* were treated with 1 mM (left panel) or 10 µM (right panel) H_2O_2. At various time points after addition of H_2O_2, fluorescence (equivalent to H_2O_2 concentration) was measured as described in Experimental Procedures. Error bars (SD) were calculated from biological triplicates.

B and C. H_2O_2 scavenging by protein extracts. Native protein extracts obtained from strains 972 (WT), MJ11 (Δ*tpx1*), EP198 (Δ*ctt1*) and SG267 *(*Δ*tpx1* Δ*ctt1*)*, at the concentrations indicated in the figure (0.25 or 1 µg/µl), were incubated with 2 µM H_2O_2. At various time points after addition of H_2O_2, fluorescence (equivalent to H_2O_2 concentration) was measured as described in Experimental Procedures. Inhibition of catalase was accomplished by the addition of azide. When indicated, recombinant Trx1 and Trx1, and NADPH, were added to promote Tpx1 activity. The peroxiredoxin Tpx1 was inhibited by the addition of DNCB. Error bars (SD) were calculated from biological triplicates.

Fig. 5. Over-expression of catalase is the main cellular strategy for the adaptation to H_2O_2 stress.

A. Scheme showing the activation of the two *S. pombe* pathways in response to oxidative stress. High doses of H_2O_2 trigger a cascade of phosphorylations ending up in the activation of the MAP kinase Sty1 and its transcription factor Atf1/Pcr1 (left). Lower doses of H_2O_2 activate the Tpx1-Pap1 pathway (right). *ctt1* transcription depends on any of the two pathways.

B. Relative mRNA levels of Δ*ctt1* transformed with different plasmids carrying the *ctt1* gene. RNA from cultures of strains PN513 transformed with pREP.41x (WT empty epi.), or EP197 (*Δctt1*) transformed with pREP.41x (empty epi.), p418.41x (*pnmt::ctt1*), p419 (*pctt1* epi.) or p422' (*pctt1* int.), either untreated (-) or treated (15) with 1 mM H_2O_2 during 15 minutes, was

obtained and analyzed by Northern blot with a probe for *ctt1*. Ribosomal RNA (*rRNA*) is shown as a loading control.

C. Over-expression of Ctt1 fully complements the H_2O_2 defects of a *Δctt1* strain. Survival to H_2O_2 of strains PN513 transformed with pREP.41x (WT empty epi.), or EP197 transformed with pREP.41x (*Δctt1* empty epi.), EP197 transformed with p419 (*Δctt1 pctt1* epi.), and EP231 (*Δctt1 pctt1* int.) was analyzed as described in Figure 1A.

D. Over-expression of Ctt1 partially complements the H_2O_2 defects of a *Δpap1* strain. Survival to H_2O_2 of strains HM123 transformed with pREP.41x (WT empty epi.), AD6 (WT *pctt1* int.), PN513 transformed with p419 (WT *pctt1* epi.), IC1 transformed with pREP.41x (*Δpap1* empty epi.), AD5 (*Δpap1 pctt1* int.), and IC1 transformed with p419 (*Δpap1 pctt1* epi.) was analyzed as described in Figure 1A.

E. Over-expression of Ctt1 fully complements the H_2O_2 defects of a *Δatf1* strain. Survival to H_2O_2 of strains PN513 transformed with pREP.41x (WT empty epi.), PN513 transformed with p419 (WT *pctt1* epi.), EP193 transformed with pREP.41x (*Δatf1* empty epi.), EP193 transformed with p151.41x (*Δatf1 patf1* epi.), and EP193 transformed with p419 (*Δatf1 pctt1* epi.) were spotted on MM plates containing uracil (MM + U) and analyzed as described in Figure 1A.

F. YE media cultures of strains HM123 transformed with pREP.41x (WT empty epi.), AD6 (WT *pctt1* int.), IC1 transformed with pREP.41x (*Δpap1* empty epi.), AD5 (*Δpap1 pctt1* int.), EP193 transformed with pREP.41x (*Δatf1* empty epi.) and EP232 (*Δatf1 pctt1* int.) were left untreated or were treated with 25 mM H_2O_2 (arrow) for the indicated times. After treatment cells were washed three times with YE media and then serially diluted and spotted on YE media plates to determine cell survival.