Reversible thiol oxidation in the H_2O_2-dependent activation of the transcription factor Pap1

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Accepted 4 March 2013
Journal of Cell Science 126, 2279–2284
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doi: 10.1242/jcs.124370

Summary

Reversible thiol oxidation is both a mark of hydrogen peroxide (H_2O_2) toxicity and an initiator of signalling events. H_2O_2 sensors contain exposed and reactive cysteine residues, which become transiently oxidized as an activation mechanism. In fission yeast, the Pap1 (pombe AP-1) transcription factor is normally cytosolic, and upon H_2O_2 stress it undergoes post-translational modifications impairing its nuclear export; genetic evidences suggested the formation of a disulphide bond in Pap1 as a triggering activation event. Nuclear Pap1 is then recruited to about 50–80 promoters and induces an adaptation response. We have now dissected the role of all seven cysteine residues in Pap1 using genetic and proteomic techniques, and we show that four of them are required for Pap1 to be activated by H_2O_2 stress. Thus, mutants lacking each one of these cysteine residues display sensitivity to peroxides. Furthermore, these mutant proteins do not become oxidized by H_2O_2 and cannot bind to promoters or trigger the Pap1-dependent gene expression program. We also demonstrate, by proteomic analysis of reduced and oxidized Pap1, that these four cysteine residues are reversibly oxidized upon H_2O_2 stress. Our study suggests that not just one but probably two disulphide bonds are required to promote the important conformational changes that trigger Pap1 activation and nuclear accumulation.

Key words: H_2O_2 sensor, Pap1, Disulphide bond, Redox cascade, Fission yeast

Introduction

Unbalances in the generation and scavenging of reactive oxygen species give rise to the so-called oxidative stress. The elevated steady state levels of superoxide, hydrogen peroxide (H_2O_2) or hydroxyl radical can damage all biomolecules, inducing their loss-of-function and/or degradation. H_2O_2 has reactivity towards many amino acids in proteins, and cysteine (Cys) residues are classical targets or peroxide reactivity. Thus, the thiol group is susceptible to oxidation towards sulfenic acid (SOH), which is then prone to react with a nearby thiol group forming a disulphide bond, or to over-oxidation to either sulfinic (SO_2H) and sulfonic acid (SO_3H) forms, both of which are considered in general irreversible modifications. Only SO_2H formation in peroxiredoxins can be enzymatically reduced by sulfiredoxins (Biteau et al., 2003).

H_2O_2 sensors can also use thiol oxidation as a gain-of-function modification leading to the activation of a signalling pathway. This was initially proposed for the bacterial sensor OxyR (Zheng et al., 1998), and this regulatory mechanism has been extended to unicellular eukaryotes and animal cells. OxyR, the *Escherichia coli* sensor of H_2O_2, undergoes thiol oxidation as a result of a mild H_2O_2 stress, which transforms it into a potent transcriptional activator of an antioxidant response. The modification proposed by Storz and colleagues is an intramolecular disulphide bond, even though it has also been postulated that oxidation of the thiol to SOH or to S-nitrosothiol (SNO) (when treated with nitric oxid) also activates OxyR as a transcription factor (Kim et al., 2002).

Yap1 (yeast AP-1 homolog) is the more extensively studied H_2O_2-responding transcription factor in eukaryotes. It contains two clusters of Cys residues in the centre and the C-terminal region of the polypeptide, with three Cys present in each domain. Yap1 becomes oxidized and activated by H_2O_2 through the formation of an internal disulphide bond, which can be detected *in vitro* by a change in electrophoretic mobility in non-reducing gels (Delaunay et al., 2000). Genetic analysis demonstrated that two Cys residues, one of each domain, are essential for the H_2O_2-dependent oxidation and activation of Yap1, since substitution of these residues led to a constitutively reduced and inactive form, and cells expressing these mutant forms displayed sensitivity to peroxides. However, mutations of the other two Cys residues in this transcription factor also yielded mild phenotypes involving sensitivity to peroxides and activation of the antioxidant gene response (Delaunay et al., 2000). Later work also showed that several disulphides do occur in Yap1 upon H_2O_2 exposure, some preceding the others, and leading to different activation kinetics (regarding both levels of activity and duration of the signal) of the transcription factor (Okazaki et al., 2007). It is worth pointing out that the actual sensor of H_2O_2 in this redox system is the glutathione peroxidase Gpx3/Orp1, which seems to receive the input directly from H_2O_2, the thiol group of the peroxidatic Cys becoming oxidized to SOH, which would then react with a Cys of Yap1 forming a transient mixed disulphide, which is resolved with the formation of an intra-molecular disulphide in Yap1 (Delaunay et al., 2002).

The *Schizosaccharomyces pombe* Pap1 transcription factor is the homolog of budding yeast Yap1 (Kudo et al., 1999; Toda et al., 1991; Toone et al., 1998). It also responds to moderate levels of H_2O_2 by rapidly adopting a conformation that blocks its

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nuclear export and allows its transient accumulation at the nucleus (Vivancos et al., 2004). Pap1 contains seven Cys residues clustered in two domains, one at the centre of the polypeptide called N-terminal Cys rich domain (nCRD) and the second at the carboxyl-terminal domain (cCRD). Upon H_2O_2 stress, the transcription factor accumulates at the nucleus, and again a disulphide bond has been hypothesized to contribute to the dissociation between Pap1 and the Hba1-Crm1 nuclear export machinery (Bozonet et al., 2005; Castillo et al., 2002; Castillo et al., 2003; Vivancos et al., 2005; Vivancos et al., 2004). Two Cys substitutions, Cys-278-Ala and Cys-501-Ala, fully prevent the H_2O_2-dependent nuclear accumulation of GFP-fused Pap1 (Castillo et al., 2002). However, the exact role of the other five Cys residues is unknown. Furthermore, in previous studies the mutant proteins were overexpressed from a regulated heterologous promoter, and their effect on peroxide sensitivity could not be determined since high levels of Pap1 can induce the antioxidant response in a H_2O_2-independent manner.

In an attempt to decipher the exact mechanism of activation by H_2O_2 of a eukaryotic transcription factor, we decided to mutagenize all seven Cys residues of Pap1 and substitute the endogenous *pap1* locus with the mutant open reading frames, so that peroxide sensitivity could be determined. Our results indicate that Cys278, Cys285 and Cys532 are essential to trigger the conformational change observed with non-reducing electrophoresis, for nuclear accumulation and for wild-type tolerance to peroxides. Cys501 is also required for Pap1 oxidation and for nuclear accumulation; however, cells expressing this mutant form still display partial, although severely impaired, transcriptional response to peroxides and an intermediate sensitivity to peroxides. We have confirmed by proteomic analysis the reversible oxidation of these four Cys residues. The identification of all Cys residues involved in the response to peroxides opens new perspectives regarding the dissection of the molecular events that define a redox cascade.

Results

Cys278 and Cys285 of the nCRD of Pap1 are essential for the activation of Pap1

We decided to mutagenize all Cys residues of Pap1 to test their role in the H_2O_2-responding redox cascade. We generated two sets of mutagenized strains. On one hand, the mutated *GFP*-tagged genes were integrated downstream of an inducible promoter to determine the subcellular localization of the mutant proteins by fluorescent microscopy. On the other hand, the untagged mutagenized genes were integrated at the chromosomal *pap1* locus to yield wild-type levels of the transcription factor, so that the otherwise different protein levels could not interfere with the phenotypic characterization of strains harbouring the mutations. The nCRD of Pap1 contains four Cys residues (Fig. 1A), and we performed several individual substitutions to serine or alanine, which are summarized in Fig. 1B. Fluorescent microscopy analysis of the GFP-tagged mutant proteins clearly demonstrated that not only Cys278 but

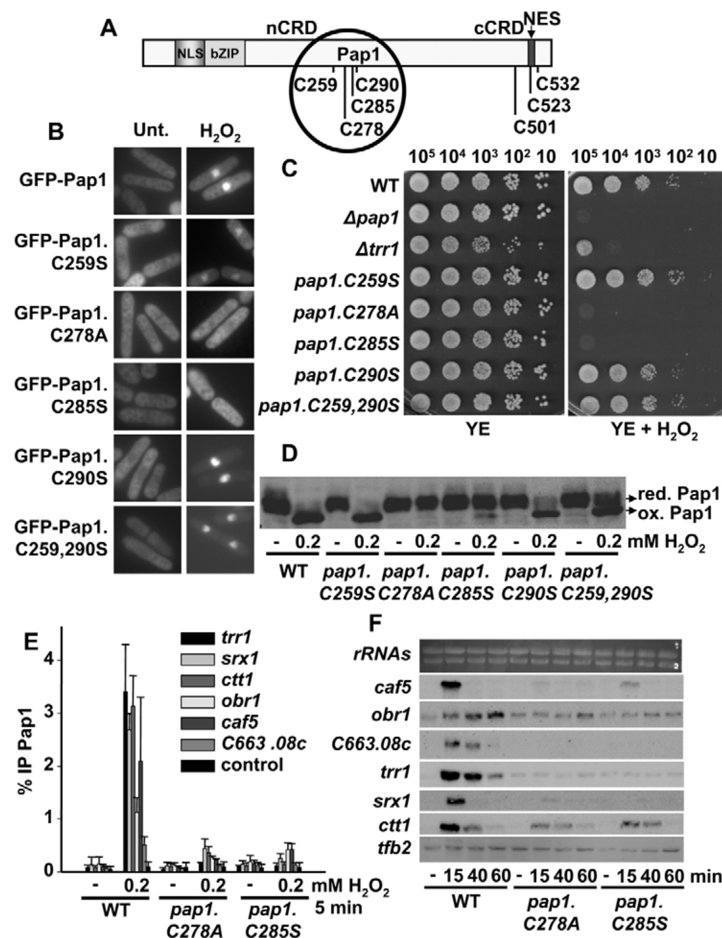


Fig. 1. Characterization of the role of the four Cys residues located at the nCRD of Pap1: Cys278 and Cys285 are required for H_2O_2 sensing. (**A**) Schematic representation of Pap1. The amino (nCRD) and carboxyl (cCRD) Cys-rich domains, and the relative positions of the seven Cys residues, are indicated. (**B**) The cellular distribution of GFP–Pap1 in wild-type (EHH14) and derived mutant strains either untreated (Unt.) or treated with 0.2 mM H_2O_2 for 5 minutes. (**C**) Sensitivity to H_2O_2 exposure. Strains IC2 (WT), IC1 (Δ*pap1*), NG25 (Δ*trr1*), IC2.C259S (*pap1.C259S*), IC2.C278A, IC2.C285S, IC2.C290S and IC2.C259,290S were grown in liquid YE medium, and the indicated number of cells were spotted onto plates with or without 2 mM H_2O_2. (**D**) *In vivo* oxidation of Pap1. Cultures of strains used in C were either untreated (−) or treated with 0.2 mM H_2O_2 for 5 minutes. Reduced/inactive (red.) and oxidized/active (ox.) Pap1 forms are indicated with arrows. (**E**) Pap1 is not recruited to promoters in some *pap1* mutants. Strains IC2 (WT), IC2.C278A and IC2.C285S were either untreated (−) or treated with 0.2 mM H_2O_2 for 5 minutes. ChIP experiments using anti-Pap1 antibody, coupled to quantification by real-time PCR, were performed as described in the Materials and Methods. (**F**) Stress-dependent transcriptional analysis of wild-type and mutant strains. Cultures of strains used in E were either untreated (−) or treated with 0.2 mM H_2O_2 for the indicated times. Total RNA was obtained and analysed by northern blotting with the indicated Pap1-dependent probes. Ribosomal RNAs (rRNAs) and *tfb2* are shown as loading controls.

also Cys285 are fully required for the H_2O_2-dependent nuclear accumulation of Pap1, whereas Cys259 and Cys290 are fully dispensable (Fig. 1B). We confirmed this last observation by generating a double Cys259Ser Cys290Ser mutant, which also showed to be wild-type regarding subcellular localization (Fig. 1B). Importantly enough, cells expressing Pap1.C259,290S display wild-type tolerance to peroxides, while mutant cells expressing Pap1.C278A or Pap1.C285S are as sensitive to peroxides as *Δpap1* cells (Fig. 1C).

We obtained extracts from cells expressing each one of the mutant forms of Pap1, before and after stress imposition. As shown in Fig. 1D, wild-type Pap1 became oxidized upon H_2O_2 stress, as reflected by its faster mobility in non-reducing electrophoresis. Pap1.C259,290S behaved as wild-type Pap1 regarding this conformational change, whereas Pap1.C278A or Pap1.C285S did not show any change in electrophoretic mobility upon stress. Consistent with the above characterization, while wild-type Pap1 was recruited to stress promoters in a H_2O_2-dependent manner, mutant Pap1.C278A or Pap1.C285S proteins were not detected at these chromosomal loci, as determined by chromatin immunoprecipitation (Fig. 1E). Furthermore, cells expressing the mutant Pap1.C278A or Pap1.C285S were unable to engage the Pap1-dependent antioxidant response, as shown by northern blot analysis (Fig. 1F). The conclusion from these experiments is that two Cys residues at the nCRD, Cys278 and Cys285, are absolutely required to sense and transduce the H_2O_2 signal.

Cys532 is essential and Cys501 partially required for Pap1 activation

We performed the same analysis for the Cys residues of the cCRD of Pap1. This domain surrounds the nuclear export signal (NES) of Pap1, and some specific substitutions alter the interaction with the export machinery even under untreated conditions (Castillo et al., 2002). This is the case of Pap1.C523D, which displays constitutive nuclear localization (Fig. 2B) (Castillo et al., 2002), high sensitivity to peroxides (Fig. 2C) and constitutively reduced conformation (Fig. 2D); this may be due to the exclusion of the upstream sensor Tpx1 from the nucleus (Calvo et al., 2012). When we mutated Cys523 to alanine, we concluded that Cys523 does not seem to have any role on the H_2O_2-dependent redox relay: GFP–Pap1.C523A accumulates at the nucleus upon stress (Fig. 2B), and cells expressing Pap1.C523A display wild-type sensitivity to peroxides (Fig. 2C), and the mutant protein displays a shift in mobility under non-reducing electrophoresis, as wild-type Pap1 does (Fig. 2D).

Pap1 carrying a Cys-532-Thr mutation behaves very similar to the above characterized mutants Cys278A and Cys285S: it does not respond to peroxides (Fig. 2). Similarly, a Cys-501-Ala mutation prevented the nuclear accumulation of Pap1 at the nucleus upon stress (Fig. 2B), and Pap1.C501A does not display a mobility shift upon exposure to H_2O_2 (Fig. 2D). However, while the sensitivity to peroxides of cells expressing Pap1.C532T is similar to that of *Δpap1* cells, *pap1.C501A* cells only display

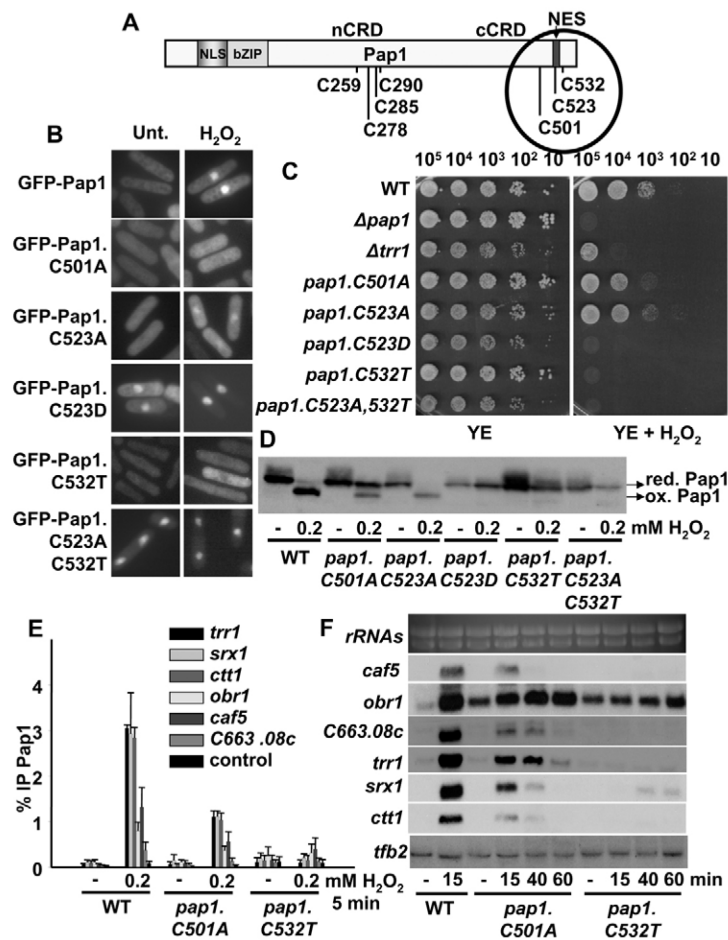


Fig. 2. Characterization of the role of the three Cys residues located at the cCRD of Pap1: Cys501 and Cys532 are required for H_2O_2 sensing. (**A**) Schematic representation of Pap1 (details as in Fig. 1A). (**B**) The cellular distribution of GFP–Pap1 in WT and mutant strains either untreated (Unt.) or treated with 0.2 mM H_2O_2 for 5 minutes. (**C**) Sensitivity to H_2O_2 exposure. Strains IC2 (WT), IC1 (*Δpap1*), NG25 (*Δtrr1*), IC2.C501A (*pap1.C501A*), IC2.C523A, IC2.C523D, IC2.C532T and IC2.C523A,532T were analysed as described in Fig. 1C. (**D**) *In vivo* oxidation of Pap1. Cultures of strains used in C were either untreated (−) or treated as described in Fig. 1D. (**E**) Pap1 recruitment to promoters in wild-type and *pap1* mutants. ChIP analysis of strains IC2 (WT), IC2.C501A and IC2.C532T was performed as described in Fig. 1E. (**F**) Stress-dependent transcriptional analysis of wild-type and mutant strains. Cultures of strains used in E were analysed as described in Fig. 1F.

moderate sensitivity to peroxides (Fig. 2C). Concomitantly, Pap1.C532T is not recruited to stress promoters upon H_2O_2 nor triggers an antioxidant transcriptional response (Fig. 2E,F), whereas cells expressing Pap1.C501A are partially able to accumulate mutant Pap1 at promoters and to induce gene activation. We conclude that while Cys532 is also indispensable for the H_2O_2-dependent signal transduction, Cys501 has a secondary role which may be redundant to the other Cys residue left at the cCRD, Cys523.

Proteomic identification of reduced and reversibly oxidized thiols in Pap1 before and after stress

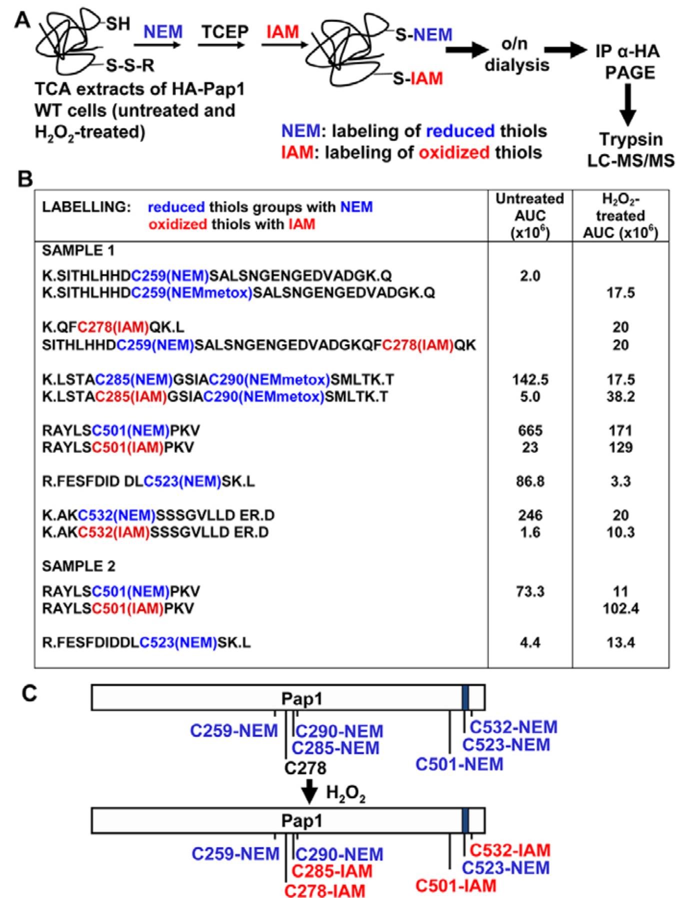
To confirm the relevant role of Cys residues 278, 285, 501 and 532 of Pap1 in redox transduction, we purified wild-type GFP–HA–Pap1 from fission yeast cells exposed or not to H_2O_2, and analysed the redox state of all seven Cys residues. To do so, we obtained acidic cell extracts to freeze the redox state of Cys residues, and blocked all free thiols with *N*-ethylmaleimide. Then, after sequential reduction of reversibly oxidized thiols and further alkylation with iodoacetamide of the newly exposed groups, we dialyzed the extracts to solubilize denatured proteins, and then immunoprecipitated GPF–HA–Pap1 with anti-HA antibodies. We loaded the immunoprecipitates onto SDS-PAGE gels, and gel slices from the position of the molecular mass GFP–HA–Pap1 were trypsinized and peptides were analysed by LC/MS-MS (Fig. 3A). The results of this analysis are depicted in Fig. 3B,C. Although all seven Cys residues in Pap1 appear as reduced under untreated conditions, four of them were alkylated with iodoacetamide (and therefore were reversibly oxidized) after exposure to H_2O_2: Cys278, 285, 501 and 532. These experiments confirm the essential role of four Cys residues in Pap1 activation.

Discussion

Cys residues are targets of H_2O_2-dependent oxidation, and such post-translational modification is both a mark of oxidative damage and a mechanism of activation of signalling cascades. However, the rate constants for thiol-to-sulfenic acid formation upon moderate doses of H_2O_2 are in general very low and only very reactive and exposed Cys residues will initiate signalling events. It has been proposed that only Cys residues from glutathione peroxidases and peroxiredoxins have the capacity to initiate these processes (Bozonet et al., 2005; Delaunay et al., 2002; Vivancos et al., 2005; Winterbourn, 2008; Winterbourn and Hampton, 2008). In the case of Pap1, a transcription factor that indirectly responds to H_2O_2, the peroxiredoxin Tpx1 is the upstream sensor of H_2O_2 (Bozonet et al., 2005; Vivancos et al., 2005). We have here dissected the role of all seven Cys residues of Pap1 in the response to H_2O_2, and demonstrated that three of them are fully dispensable, three are essential to respond to the H_2O_2–Tpx1 redox relay and one Cys has a clear but intermediate role in this redox response.

The proteomic analysis we have performed clearly indicates that Pap1 contains four reversibly oxidized thiols in its active form. We propose the presence of two intramolecular disulphide bonds in activated Pap1, both of which are probably required to maintain Pap1 on its active conformation, since individual mutation of each cysteine residue almost fully abrogates Pap1 function. Mass spectrometry experiments to isolate disulphide-linked mixed peptides are on their way.

The role of Cys501 in this redox cascade, however, is unclear. Pap1.C501A and the other three mutant proteins (Pap1.C278A, Pap1.C285S and Pap1.C532T) clearly block Pap1 activation, as



LABELLING: reduced thiols groups with NEM; oxidized thiols with IAM	Untreated AUC (x10⁶)	H2O2-treated AUC (x10⁶)
SAMPLE 1		
K.SITHLHHDC259(NEM)SALSNGENGEDVADGK.Q	2.0	
K.SITHLHHDC259(NEMmetox)SALSNGENGEDVADGK.Q		17.5
K.QFC278(IAM)QK.L		20
SITHLHHDC259(NEM)SALSNGENGEDVADGKQFC278(IAM)QK		20
K.LSTAC285(NEM)GSIAC290(NEMmetox)SMLTK.T	142.5	17.5
K.LSTAC285(IAM)GSIAC290(NEMmetox)SMLTK.T	5.0	38.2
RAYLSC501(NEM)PKV	665	171
RAYLSC501(IAM)PKV	23	129
R.FESFDID DLC523(NEM)SK.L	86.8	3.3
K.AKC532(NEM)SSSGVLLD ER.D	246	20
K.AKC532(IAM)SSSGVLLD ER.D	1.6	10.3
SAMPLE 2		
RAYLSC501(NEM)PKV	73.3	11
RAYLSC501(IAM)PKV		102.4
R.FESFDIDDLC523(NEM)SK.L	4.4	13.4

Fig. 3. Proteomic analysis of H_2O_2-dependent reversible thiol oxidation in Pap1. (**A**) Scheme for the *in vivo* labelling of reduced and oxidized thiols in Pap1. TCA protein extracts from strain EHH14, either untreated or treated with H_2O_2, were obtained and thiols (SH) were initially alkylated with NEM in each sample. Upon reduction of oxidized thiols (S-S-R) with TCEP, resulting thiols were biotin-labelled with IAM. After renaturing the protein extracts by dialysis, GFP–HA–Pap1 was immunoprecipitated with anti-HA antibody (previously cross-linked to Sepharose beads) and further enriched by non-reducing PAGE. Gel slices corresponding to tagged Pap1 were trypsinized and analysed by LC-MS/MS. (**B**) Table of the Cys-containing peptides identified by LC-MS/MS, and their relative abundance. The sequence of the peptides identified, and the areas under the curve (AUC) were represented in millions of arbitrary units, which correspond to the identified peptides alkylated with NEM (peptides containing reduced thiols) or IAM (peptides containing reversibly oxidized thiols) in extracts from untreated or H_2O_2-treated cultures (0.2 mM H_2O_2 for 5 minutes). (**C**) Scheme depicting the final outcomes of the analysis of the redox state of Pap1 Cys residues by LC-MS/MS, as described in B.

determined by its nuclear accumulation and by detection of a conformational change by non-reducing electrophoresis. However, cells expressing Pap1.C501A display intermediate phenotypes regarding sensitivity to peroxides, recruitment to promoters and gene activation. We can only hypothesize that in this particular mutant the other two Cys at the cCRD (Cys523 and Cys532) can substitute the role of Cys501. Unfortunately, accumulation of several mutations at the cCRD alters the structure of the NES, disrupts the interaction of the transcription factor with the export machinery and leads to constitutively nuclear Pap1 mutants (Castillo et al., 2002).

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The study we have performed here is essential for understanding the molecular events that rule a redox signalling cascade. Thus, while it is likely that only a peroxiredoxin such as Tpx1 can initiate the process and sense fluctuations in H_2O_2, it remains unclear how the transduction of the signal towards Pap1 takes place. We suspect that an internal disulphide in Tpx1 could oxidize Pap1 by thiol disulphide exchange, since both Cys residues in the peroxiredoxin are required for Pap1 activation, and therefore it is unlikely that an SOH in Tpx1 could transfer the H_2O_2 signal downstream (Bozonet et al., 2005; Vivancos et al., 2005). Thiol disulphide exchange takes place by the transient formation of a mixed disulphide between the oxidative and reducing substrates, which could be trapped with a particular mutation in the resolving Cys of the electron donor of this redox couple exchange. Therefore, we expect that in extracts from cells expressing one of the four Cys mutants (Pap1.C278A, Pap1.C285S, Pap1.C501A or Pap1.C532T) the mixed disulphide with Tpx1 could be exacerbated. The corresponding experiments are on their way.

Materials and Methods

Yeast strains and growth conditions

The origins and genotypes of strains used in this study are outlined in supplementary material Table S1. Cells were grown in rich medium (YE; yeast extract) or in synthetic minimal medium as described previously (Alfa et al., 1993).

Plasmids

The plasmid p85.41x′ and its mutant derivatives (Castillo et al., 2002), containing the *GFP-pap1* coding region at the *leu1* locus, were used. In order to construct new *pap1* mutant alleles, p85.41x′ was used as a template for PCR reactions using pairs of mutagenic and complementary primers containing the codon change of interest (Castillo et al., 2002), yielding plasmids p85.41x′.C259S, p85.41x′.C285S and p85.41x′.C290S. Double and triple Cys substitutions were generated using the single-codon mutants as templates of second or third mutagenesis reactions. All the mutations were confirmed by sequencing. The p85.41x′ plasmid derivatives were used to generate chromosomal insertions of GFP-tagged *pap1* genes into the *leu1* locus of strain EHH108, as described before (Castillo et al., 2002).

Fluorescence microscopy

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

H_2O_2 sensitivity assay

For survival on solid plates, *S. pombe* strains were grown, diluted and spotted in YE5S agar plates, containing 2 mM H_2O_2 or 15 mM caffeine, as described previously (Calvo et al., 2009).

Preparation of *S. pombe* TCA extracts and immunoblot analysis

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

RNA analysis

Total RNA from *S. pombe* minimal medium cultures was obtained, processed and transferred to membranes as described previously (Castillo et al., 2002). Membranes were hybridized with [α-^{32}P]dCTP-labelled *caf5*, *obr1*, *SPCC663.08c*, *trr1*, *srx1* or *ctt1* probes, containing the complete open reading frames. We used rRNA or *tfb2* as loading controls.

Chromatin immunoprecipitation

The *in vivo* binding of Pap1 to stress promoters was analysed as described previously (Calvo et al., 2012), using polyclonal antibodies against Pap1 (Vivancos et al., 2004).

TCA extracts to detect Cys modifications in Pap1 by MS analysis

Each 300 ml-culture were pelleted, washed with 20% TCA and lysed with glass beads in a BioSpec Minibeadbeater with 4.5 ml of 12.5% TCA. Cell lysates were then pelleted, washed twice in cold acetone and dried. Free thiols were blocked by resuspending each pellet in 4.5 ml of alkylating buffer (100 mM Tris-HCl pH 8, 1 mM EDTA and 1% SDS) containing 100 mM *N*-ethylmaleimide (NEM; Sigma-Aldrich, St. Louis, MO, USA), and incubated at 30℃ for 60 minutes in the dark. Aggregates were spun down by centrifugation and proteins were precipitated by addition of 4.5 ml of 20% TCA and incubated at −20℃ for 10 minutes. A TCA protein pellet was obtained, washed twice in cold acetone and dried. Reversibly oxidized Cys were then reduced by resuspending each pellet in 3 ml of a solution containing 10 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP; Sigma-Aldrich, St. Louis, MO, USA) in alkylating buffer followed by incubation at 30℃ for 30 minutes. TCA protein precipitation and acetone washing removed excess TCEP. To label the newly reduced thiols, the resulting pellet was resuspended in 3 ml of alkylating buffer containing 100 mM iodoacetamide (IAM; Sigma-Aldrich, St. Louis, MO, USA) and incubated at 25℃ in the dark for 1 hour. TCA protein precipitation and acetone washing removed unbound IAM. After resuspending protein pellets in 3 ml of alkylating buffer, protein concentration was determined by Bradford protein assay (Bio-Rad, Hercules, CA, USA). In order to renature our protein extracts, we dialyzed our samples using Slide-A-Lyzer G2 dialysis cassettes of 3 ml capacity (Thermo Scientific, Rockford, IL, USA) in dialysis buffer (20 mM Tris-HCl pH 8, 100 mM NaCl and 1 mM EDTA), with three cycles of one hour, one hour and overnight at 4℃. Renatured protein extracts (3 mg) were immunoprecipitated with monoclonal anti-HA antiserum (12CA5); the antibodies were previously cross-linked to protein-G–Sepharose. After 2 hours of incubation, immunoprecipitates were washed three times with the same dialysis buffer and eluted from beads with PAGE sample buffer and electrophoretically separated by SDS-PAGE. After Coomassie Blue colloidal staining, gel slices corresponding to GFP–HA–Pap1 were analysed by mass spectrometry (see below). To verify the migration of the fusion protein on gels, 10 µg of total extracts before and after dialysis and 100 µg of the immunoprecipitates were also resolved on SDS-PAGE, but transferred to nitrocellulose membranes and Pap1 was immunodetected using polyclonal anti-Pap1 antibody (Vivancos et al., 2004).

Mass spectrometry analysis

Gel slices of GFP–HA–Pap1 (see above) were in-gel digested with trypsin (Promega, CA, USA). After digestion, peptides were separated in a reverse phase column, 100 µm×150 mm (Nikkyo Technos Co., Ltd, Tokyo, Japan) with a gradient of 12–36% acetonitrile with 0.1% formic acid in 58 minutes at a flow of 0.5 µl/minutes. Samples were then analysed by LC-MS/MS on an LTQ-Orbitrap Velos (Thermo Scientific, Rockford, IL, USA) fitted with a nanospray source (Thermo Scientific, Rockford, IL, USA) previous nanoLC separation in an EasyLC system (Proxeon, Odense, Denmark). The Orbitrap Velos was operated in positive ion mode with nanospray voltage set at 2.1 kV and source temperature at 250℃. An internal calibration was performed using the background polysiloxane ion signal at *m/z* 445.120025 as the calibrant. The instrument was operated in DDA mode. In all experiments, full mass spectrometer scans were acquired over a mass range of *m/z* 350–2000 with detection in the Orbitrap mass analyzer at a resolution setting of 30,000. For each mass spectrometer scan, the twenty most intense ions with multiple charged ions above a threshold ion count of 5000 were selected for fragmentation at normalized collision energy of 35% in the LTQ linear ion trap. All data were acquired with Xcalibur 2.1 software (Thermo Scientific, Rockford, IL, USA). Data was searched against Gene_DB_SPombe database using an internal version of the search algorithm Mascot v.2.2 (http://www.matrixscience.com/). Peptides were filtered using a Mascot Ion Score of 20. Area under the curve (AUC) in for each cysteine-containing peptide was calculated using Proteome Discoverer 1.3 (Thermo Scientific, Rockford, IL, USA) and were represented as number of millions of arbitrary units.

Acknowledgements

We thank Mercè Carmona for technical assistance. We are grateful to the joint CRG-UPF Proteomic Facility (Barcelona, Spain), where the mass spectrometry experiments were performed, and especially thank Cristina Chiva and Guadalupe Espadas.

Author contributions

I.A.C. performed the experiments, I.A.C., J.A. and E.H. analyzed the data and E.H. wrote the manuscript.

Funding

This work was supported by the Spanish Ministry of Science and Innovation, PLAN E and FEDER [grant numbers BFU2009-06933, BFU2012-32045 to E.H.]; the Spanish program Consolider-Ingenio 2010 [grant number CSD 2007-0020 to E.H.]; the Generalitat de Catalunya (Spain) [grant number SGR2009-196 to E.H.]; and Institució Catalana de Recerca i Estudis Avançats Academia Awards (Generalitat de Catalunya) to E.H. and J.A.

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.124370/-/DC1

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