Hydrogen Peroxide-sensitive Cysteines in the Sty1 MAPK Regulate the Transcriptional Response to Oxidative Stress*  
Alison M. Day and Elizabeth A. Veal

MAPK are activated by and orchestrate responses to multiple, diverse stimuli. Although these responses involve the increased phosphorylation of substrate effector proteins, e.g. transcription factors, the mechanisms by which responses are tailored to particular stimuli are unclear. In the fission yeast Schizosaccharomyces pombe, the Sty1 MAPK is crucial for changes in gene expression that allow adaptation to many forms of environmental stress. Here, we have identified two cysteine residues in Sty1, Cys-153 and Cys-158, that are important for hydrogen peroxide-induced gene expression and oxidative stress resistance but not for other functions of Sty1. Many Sty1-dependent changes in gene expression are mediated by the Atf1 transcription factor. In response to stress, Sty1 increases Atf1 levels by (i) promoting increases in atf1 mRNA and by (ii) directly phosphorylating and stabilizing Atf1 protein. Although dispensable for phosphorylation and stabilization of Atf1 protein, we find that both Cys-153 and Cys-158 are required for increases in atf1 mRNA levels and Atf1-dependent gene expression in response to hydrogen peroxide but not osmotic stress. Indeed, our data indicate that oxidation of Sty1, by formation of a disulfide bond between Cys-153 and Cys-158, is important for maintaining atf1 mRNA stability at high concentrations of hydrogen peroxide. Together, these data reveal that redox regulation of cysteine thiols in Sty1 is involved in a stress-specific mechanism regulating transcriptional responses to oxidative stress. Intriguingly, the conservation of these cysteine residues in other MAPK raises the possibility that similar mechanisms may ensure appropriate responses to hydrogen peroxide in other eukaryotes.

In eukaryotes, mitogen-activated protein kinases (MAPK) play vital roles in orchestrating appropriate responses to a vast array of stimuli, including growth factors, cytokines, and environmental stress. For a recent review, see Ref. 1.) MAPK activation by these stimuli is important for changes in gene expression that promote cell growth, apoptosis, or survival/adaptation. Consequently, MAPK play important roles in regulating many fundamental biological processes (1). For instance, in metazoans, p38 and JNK MAPK are important for innate immunity and inflammation (2, 3). Conversely, MAPK in pathogenic fungi trigger adaptive changes that aid survival/evasion of the host immune system and are important for virulence (4, 5). The association of MAPK activity with various diseases, including cancer, has generated great medical interest in targeting drugs to regulate MAPK activity (6). However, as MAPK are activated by multiple stimuli and important for a large number of processes, drugs that target the kinase activity have inevitable side effects (7). The identification/understanding of mechanisms by which MAPK tailor responses to particular stimuli will be an important step toward developing drug treatments that target specific aspects of MAPK function.

The amenability to genetic manipulation and presence of fewer functionally redundant MAPK genes have allowed studies in the model eukaryote, the fission yeast Schizosaccharomyces pombe, to yield important insight into MAPK function/regulation (8). For instance, the S. pombe ortholog of the p38 and JNK families of MAPK, Sty1 (Spe1, Pkh1), is important for cell cycle progression and also essential for cell survival and adaptation in response to environmental change (9–12). Significantly, like its mammalian counterparts, p38 and JNK, Sty1 is activated in response to a variety of stresses including heat, oxidative stress, osmotic stress, nutrient limitation, and UV (9–12). In response to these stimuli, signaling mechanisms are initiated that lead to phosphorylation of these MAPK and consequent activation of their kinase activity (9–15). Among the key substrates that are phosphorylated by p38, JNK and Sty1 MAPK are ATF transcription factors (16–19). For instance, Sty1 phosphorylates Atf1 (18, 19). As a heterodimer with a second bZip transcription factor, Pcr1, Atf1 regulates the expression of many genes. Indeed, global analysis of mRNA has identified a Sty1/Atf1-dependent core environmental stress response that is activated by a variety of stress stimuli and important for protection against different stress conditions, including osmotic and oxidative stress (20). Stress-induced activation of the Sty1 kinase increases the phosphorylation of Atf1, inhibiting its degradation and thus leading to increased Atf1 levels and increased transcription of Atf1 target genes (21). Under oxidative stress conditions, in addition to increasing Atf1 protein stability, Sty1 is also required for atf1 mRNA stability (22).
In addition to the core environmental stress response, sub-
sets of Sty1-regulated genes are only induced in response to
certain specific stresses (20). Indeed, different sets of genes
have been shown to be induced in response to different levels of the
same stress. For instance, the transcriptional response to low
levels of hydrogen peroxide (0.07 mm H2O2) is significantly dif-
ferent from the response to higher levels (0.5 and 6 mm H2O2)
(23, 24). Although the mechanisms by which Sty1 tailors
responses to specific stimuli are poorly understood, this dif-
ferential response to different levels of hydrogen peroxide is par-
tially orchestrated through the use of different transcription factors.
The hydrogen peroxide-specific transcriptional response to
low levels of hydrogen peroxide is mediated by the AP-1 (acti-
vator protein 1)-like transcription factor, Pap1, with Atf1 more
important for the response to higher levels of hydrogen perox-
ide (23, 24).

The hydrogen peroxide-induced activation of Pap1 involves
reversible oxidation of cysteine thiols in Pap1 (25). Indeed, the
susceptibility of particular cysteine residues to hydrogen per-
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| Yeast Strains | Source or reference |
|--------------|---------------------|
| Laboratory stock | Laboratory stock |
| This study | This study |
| This study | This study |
| This study | This study |
| This study | This study |
| This study | This study |
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| Ref. 18 | Gift† |
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† Gift of C. Wilkinson/N. Jones.

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ity of an increasing number of proteins (26, 27). Notably, cys-
teine thiol oxidation has also been implicated in stress-sensing
mechanisms leading to activation of MAPK. For instance, oxida-
tion of cysteine thiols in the MAPKK (mitogen-activated
protein kinase kinase kinase), Ask1, and MEKK1 is involved in
the regulation of downstream MAPK, JNK, and p38 (28, 29).
Moreover, previous work in our laboratory revealed that hydro-
gen peroxide-induced activation of Sty1 is associated with the
formation of mixed disulfide bonds between Sty1 and the typi-
cally 2-Cys peroxiredoxin Tpx1 (30).

Here, we have explored the possibility that redox regulation of
cysteines in Sty1 might also be involved in tailoring down-
stream signaling events to generate hydrogen peroxide-specific
responses in S. pombe. Indeed, we have identified two redox-
sensitive cysteine residues in Sty1 that are required specifically
for the response to hydrogen peroxide. We show that these two
cysteines are required for the hydrogen peroxide-induced
increase in Atf1 levels and hence for the hydrogen peroxide-
induced expression of Atf1-dependent genes and oxidative
stress resistance. Our data support a role for reversible oxida-
tion of these cysteine residues in preventing hydrogen perox-
ide-induced destabilization of atf1 mRNA.

**TABLE 1**

**S. pombe** strains used in this study

| Yeast Strains | Source or reference |
|---------------|---------------------|
| Laboratory stock | Laboratory stock |
| This study | This study |
| This study | This study |
| This study | This study |
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| Gift† | Gift† |
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Strains were constructed containing alleles expressing wild-type Sty1 (sty1+) or mutant forms of Sty1 with serine residues substituted for specified cysteine residue(s) from the normal chromosomal locus. Plasmids pRip2AD1-Sty1, pRip2AD1-Sty1C13S, pRip2AD1-Sty1C35S, pRip2AD1-Sty1C153S, pRip2AD1-Sty1C202S, pRip2AD1-Sty1C242S, and pRip2AD1-Sty1C153SC158S were linearized by digestion with NheI and introduced into AD22 (sty1::his7) to generate AD38 (sty1+,WT), AD39 (sty1C13S), AD40 (sty1C35S), AD41 (sty1C153S), AD42 (sty1C153S), AD67 (sty1C202S), AD43 (sty1C242S), and AD44 (sty1C153SC158S), respectively. URA + transformants were screened by PCR for appropriate genomic integration of the wild-type Sty1 or Sty1 cysteine mutant expressing fragment. The sty1 open reading frame in each mutant was fully sequenced to confirm the presence of the appropriate mutation and the absence of any additional mutations. All oligonucleotide sequences are available upon request.

AD68 (sty1+,WT) and AD52 (sty1C153SC158S) were obtained by crossing AD38 (sty1+,WT) and AD44 (sty1C153SC158S) with NT4. Strains AD69 and AD59 were obtained from crosses between KS1479 and AD68 or AD52, respectively. Strains AD65 and AD66 were obtained from crosses between NJ260 and AD38 or AD44, respectively. Strain AD70 was obtained from a cross between AD44 and NJ543.

**Analysis of Proteins by Western Blotting**—Protein concentrations were estimated using the bicinchoninic acid protein assay (Perbio) for denatured samples or the Coomassie Blue protein assay (Perbio) for non-denatured samples. Equal amounts of protein were separated by SDS-PAGE on 10% gels and electroblotted to nitrocellulose membrane (Schleicher & Schuell). Nonspecific interactions were blocked with 10% bovine serum albumin in TBST (20 mM Tris-HCl, pH 7.6, 140 mM NaCl, 0.1% Tween 20) before incubation with primary antibodies diluted 1 to 1000 with 5% bovine serum albumin in TBST. Following TBST washes, horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibodies were utilized in conjunction with ECL (Amersham Biosciences) and x-ray film (Fujiﬁlm). For determination of Sty1 phosphorylation, 50 mM NaF and 2 mM sodium vanadate were included as phosphatase inhibitors in all blotting and antibody incubation steps. TAT1 anti-tubulin antibodies (Cancer Research UK) were used as a loading control.

**Analysis of the Redox State of Cysteines in Sty1**—Cell lysates were prepared from 7.5 × 10⁶ cells using the trichloroacetic acid method essentially as described previously (36) but without phosphatase treatment. Proteins were resuspended in either 200 mM Tris-HCl, pH 8.0, 1% SDS, 1 mM EDTA or 200 mM Tris-HCl, pH 8.0, 1% SDS, 1 mM EDTA containing 25 mM 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS, Molecular Probes) at 25 °C for 25 min followed by 37 °C for 5 min. Thiol-modified and unmodiﬁed forms of Sty1 were separated by SDS-PAGE and then analyzed by Western blotting with rabbit polyclonal anti-Sty1 antibodies raised against the peptides CSEVLSFHMDNEQSL and CNTLR-FVQSLPQKEKV (Eurogentec) whose speciﬁcity for Sty1 was conﬁrmed (supplemental Fig. S1A).

**Analysis of Sty1 Phosphorylation**—Cell lysates were prepared using lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% (v/v) Nonidet P-40, 10 mM imidazole with 2 μg/ml pepstatin A, 2 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride, and 1% (v/v) aprotinin) containing 10 mM β-mercaptoethanol as described previously (37). Proteins were separated by SDS-PAGE on 10% polyacrylamide gels, and then phosphorylated Sty1 was examined by Western blotting using anti-phospho-p38 antibody (New England Biolabs). Sty1 levels were determined with polyclonal anti-Sty1 antibodies.

**Analysis of Atf1**—7.5 × 10⁶ cells were harvested, and lysates were prepared by trichloroacetic acid lysis essentially as described previously (36) but without phosphatase treatment. Proteins were resuspended in 200 mM Tris-HCl, pH 8.0, 1% SDS, 1 mM EDTA. Alternatively, 2.5 × 10⁶ cells were harvested, and lysates were prepared using lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% (v/v) Nonidet P-40, 10 mM imidazole with 2 μg/ml pepstatin A, 2 μg/ml leupeptin, 100 μg/ml phenylmethylsulfonyl fluoride, and 1% (v/v) aprotinin) containing 10 mM β-mercaptoethanol (37). Atf1 tagged with six histidines and the hemagglutinin (HA) epitope (Atf1-HA-His₆) was partially puriﬁed from 0.75 mg of protein using Ni²⁺-nitrotetracetic acid agarose (Qiagen) and dephosphorylated (as indicated) with λ-phosphatase (New England Biolabs). Atf1-HA-His₆ in acid-lysed or nickel-puriﬁed samples was analyzed by Western blotting using monoclonal anti-HA antibodies (Sigma).

**RNA Analysis**—2.5 × 10⁶ cells grown in YE5S were harvested and RNA-extracted, denatured, and separated on glyoxal gels (38). Northern blotting was performed using [32P]dCTP gene-speciﬁc cDNA probes in QuikHyb (Stratagene).

**Sensitivity Tests**—For spot tests, equal numbers of exponentially growing cells (~1 × 10⁶) were subjected to 10-fold serial dilutions and then spotted onto YE5S agar containing the indicated concentrations of stress-inducing agents using a 96-well replicating tool. Plates were incubated at 30 °C for up to 4 days. To assay recovery/survival following exposure to H₂O₂, similar numbers of exponentially growing cells in liquid culture were treated with 1.0 or 25 mM H₂O₂. Duplicate samples containing the appropriate numbers of cells were plated onto YE5S agar at the indicated time points to give between 30 and 300 colonies/plate. Plates were incubated at 30 °C for up to 4 days, after which time colonies were counted. Experiments were repeated, and two-factor analysis of variance conﬁrmed that differences between curves were statistically signiﬁcant (p < 0.0001).

**RESULTS**

**High Concentrations of Hydrogen Peroxide Induce Reversible Oxidation of Cysteines in Sty1**—We have previously shown that during hydrogen peroxide-induced activation of the Sty1 MAPK, a small proportion of Sty1 becomes oxidized through
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**Figure 1.** Sty1 is susceptible to reversible hydrogen peroxide-induced cysteine thiol oxidation. A and B, proteins extracted from wild-type cells (CHP429) treated for 10 min with the indicated concentrations of hydrogen peroxide were reacted (as indicated by +) with the thiol-active agent AMS and then analyzed by Western blotting with anti-Sty1 antibodies. A, following AMS treatment, the mobility of Sty1 prepared from untreated cells was decreased (Sty1-red), consistent with cysteines in Sty1 being in the reduced thiol state in untreated cells (Sty1-red). Following exposure to increasing concentrations of hydrogen peroxide, a more mobile form of AMS-modified Sty1 was detected (Sty1ox), consistent with an increasing proportion of Sty1 becoming oxidized and hence less reactive with AMS. B, electrophoretic separation of Sty1 from hydrogen peroxide-treated cells into two forms is dependent on treatment of protein samples with AMS (as indicated by +), suggesting that the more mobile hydrogen peroxide-induced form of Sty1 is an oxidized, AMS-resistant form of Sty1 (Sty1ox). C, the absence of oxidized Sty1 (Sty1ox) when proteins extracted from wild-type cells (CHP429) exposed to 25 mM hydrogen peroxide for 10 min were treated with DTT (as indicated by +) prior to AMS treatment suggests that hydrogen peroxide-induced thiol oxidation of Sty1 is reversible. D and E, proteins extracted from wild-type cells (CHP429) treated, as indicated, for 10 min with 25 mM (D) or 6.0 mM (E) hydrogen peroxide were treated sequentially (as depicted in supplemental Fig. S2A) with (i) the thiol-modifying agent IAA, (ii) DTT (as indicated by +), and then (iii) AMS (as indicated by +). Analysis of these proteins by Western blotting with anti-Sty1 antibodies revealed that following DTT treatment, a proportion of IAA-modified Sty1 (Sty1-IAA) from hydrogen peroxide-treated cells was AMS-modified (Sty1-IAA+AMS), decreasing its mobility. Experiments were repeated at least twice, and representative experiments are shown.

It is possible that this oxidized form of Sty1 (Fig. 1A) is produced by irreversible oxidation of one or more cysteine thiols in Sty1. However, when proteins from hydrogen peroxide-treated cells were pretreated with the reducing agent DTT prior to reaction with AMS, only reduced forms of Sty1 were detected (Fig. 1C). The DTT reversibility of this oxidation suggests that the oxidized form of Sty1 is produced by formation of one or more disulfide bonds between cysteines in Sty1 rather than by the irreversible oxidation of cysteine thiols to sulfinic or sulfonic acid derivatives. To examine the possibility that the oxidized form of Sty1 contains one or more disulfide bonds, protein extracts prepared from wild-type cells, before or following treatment with 25 mM hydrogen peroxide, were treated stepwise with (i) the thiol-modifying agent iodoacetamide to alkylate any reduced cysteine residues, (ii) DTT to reduce any disulfide bond(s), and (iii) AMS to bind to any cysteine residues reduced by DTT (supplemental Fig. S2A).

Again, following exposure to 25 mM hydrogen peroxide, lower levels of Sty1 were detected, reflecting the increase in SDS-insoluble Sty1 in hydrogen peroxide-treated cells following acid lysis (Fig. 1D). Consistent with a stress-induced increase in Sty1 phosphorylation, we noted that Sty1 mobility was slightly decreased in cells exposed to hydrogen peroxide (Fig. 1, D and E). Prior reduction with DTT of samples from hydrogen peroxide-treated cells containing oxidized Sty1 increased the amount of Sty1 detected and, importantly, also allowed AMS modification of Sty1 (Fig. 1D). A similar AMS-dependent band was also detected following DTT treatment of Sty1 extracted from cells treated with 6.0 mM hydrogen peroxide (Fig. 1E). These data are consistent with Sty1 in which one or more cysteines is oxidized, reducing its capacity to bind AMS. As the levels of hydrogen peroxide increased, the proportion of oxidized to reduced protein also increased such that the greatest proportion of oxidized to reduced protein was observed at the highest concentration of hydrogen peroxide tested (25 mM) (Fig. 1A). At this highest concentration of hydrogen peroxide (25 mM), Sty1 protein levels were also apparently reduced. However, when proteins were extracted from cells treated with 25 mM hydrogen peroxide under non-denaturing conditions at pH 7.5, comparable levels of Sty1 were detected by Western analysis (supplemental Fig. S1B).

This suggests that the reduced levels of Sty1 detected following acid lysis of 25 mM hydrogen peroxide-treated cells reflects a hydrogen peroxide-induced increase in the formation of acid-precipitated, SDS-resistant insoluble forms of Sty1 rather than a decrease in total Sty1 protein levels.

formation of a mixed disulfide with the peroxiredoxin, Tpx1 (30). In Saccharomyces cerevisiae, hydrogen peroxide-induced activation of the Yap1 transcription factor involves formation of a mixed disulfide with the peroxiredoxin, Gpx3, as an essential but transient step preceding the formation of multiple intramolecular disulfide bonds in Yap1 (39). Hence, we examined the possibility that other oxidation events might occur at cysteines in Sty1 in response to hydrogen peroxide. First, we examined whether the oxidation state of cysteines in Sty1 changed when cells were exposed to hydrogen peroxide. Proteins were extracted from wild-type cells under acidic conditions to preserve the redox state of cysteine thiols, allowing electrophoretic separation of forms containing different numbers of modified cysteines. In unstress cells, AMS treatment produces an ~3.6-kDa shift in the electrophoretic mobility of Sty1, consistent with all six cysteine residues being reduced (Fig. 1A). Similarly, when cells were exposed to low levels of hydrogen peroxide, there was no decrease in the ability of Sty1 to bind AMS, indicating that all the cysteines in Sty1 remain reduced. However, following exposure of cells to concentrations of hydrogen peroxide above 1.0 mM, a form of Sty1 with an increased electrophoretic mobility was detected (Fig. 1A). This lower molecular weight form of Sty1 was only detected when samples were treated with AMS (Fig. 1B), strongly suggesting that it represents a form of Sty1 in which one or more cysteines is oxidized, reducing its capacity to bind AMS. As the levels of hydrogen peroxide increased, the proportion of oxidized to reduced protein also increased such that the greatest proportion of oxidized to reduced protein was observed at the highest concentration of hydrogen peroxide tested (25 mM) (Fig. 1A). At this highest concentration of hydrogen peroxide (25 mM), Sty1 protein levels were also apparently reduced. However, when proteins were extracted from cells treated with 25 mM hydrogen peroxide under non-denaturing conditions at pH 7.5, comparable levels of Sty1 were detected by Western analysis (supplemental Fig. S1B). This suggests that the reduced levels of Sty1 detected following acid lysis of 25 mM hydrogen peroxide-treated cells reflects a hydrogen peroxide-induced increase in the formation of acid precipitated, SDS-resistant insoluble forms of Sty1 rather than a decrease in total Sty1 protein levels.
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FIGURE 2. Cysteines 153 and 158 are required for hydrogen peroxide-induced oxidation of Sty1 and resistance to oxidative stress. A, Sty1 contains six cysteine residues. The positions of these cysteines (black) in the primary sequence of Sty1 are indicated relative to the protein kinase domain (pale gray) and its key features, the ATP binding site (dark gray) and MAPKK phosphorylation sites (Thr-171 and Tyr-173). N, N terminus; C, C terminus. B–D, Western blot analysis of proteins extracted from WT (AD38) and sty1ΔC153S (AD41) cells (B), Δsty1 cells (AD22) containing pRep41sty1 (WT) or pRep41sty1ΔC158S (C) wild-type (AD38) and sty1ΔC153S (AD44) cells (D) treated for 10 min with 25 mM hydrogen peroxide. Proteins were sequentially (i) IAA-modified and (ii) DTT-treated (as indicated by +) followed by (iii) AMS modification of DTT-reduced cysteine thiols before SDS-PAGE and Western blotting with anti-Sty1 antibodies. E, equal numbers of exponential phase WT (AD38), Δsty1 (AD22), and sty1ΔC153S (AD44) cells were serially diluted and spotted onto plates containing, as indicated, 1% KCl, 250 mM CaCl2, 1.0% H2O2, or Cd2+ (0.1 mM CdSO4). F, the growth and survival of exponential phase WT (AD38), Δsty1 (AD22), and sty1ΔC153S (AD44) cells (grown in rich media) following treatment with 1.0 or 25 mM hydrogen peroxide for the indicated length of time. Error bars indicate the S.D. Experiments were repeated three times, and representative experiments are shown.

consistent with the reversible hydrogen peroxide-induced oxidation of Sty1 involving formation of one or more intramolecular disulfide bonds.

The genetic amenability of S. pombe allowed us to investigate which of the six cysteine residues in Sty1 are required for the formation of hydrogen peroxide-induced disulfide bond(s) in Sty1 (Fig. 2A). First, a series of S. pombe mutant strains was generated, each expressing, in place of wild-type Sty1, a Sty1 mutant protein in which a single cysteine residue was substituted with serine. We then examined the ability of each of these Sty1 mutant proteins to form a disulfide bond in response to hydrogen peroxide. Previous work in our laboratory has shown that cysteine 35 of Sty1 is able to form a hydrogen peroxide-induced disulfide bond with Tpx1 (30). However, serine substitution of cysteine 35 did not prevent the AMS modification of DTT-treated Sty1ΔC153S extracted from hydrogen peroxide-treated cells (supplemental Fig. S2B). This indicates that cysteine 35 is not involved in the hydrogen peroxide-induced formation of intramolecular disulfide bond(s) in Sty1. Similarly, Sty1 mutant proteins in which cysteine 13, cysteine 202, or cysteine 242 was replaced with serine were also able to form Sty1 intramolecular disulfide bonds (supplemental Fig. S2, B and C). In contrast, the reduced effect of AMS on the mobility of DTT-treated oxidized Sty1C153S suggests that cysteine 153 in Sty1 is required for normal Sty1 oxidation (Fig. 2B and supplemental Fig. S2D). However, pretreatment with DTT still increased AMS binding to IAA-modified Sty1C153S, suggesting that an additional cysteine is reversibly oxidized even in the absence of cysteine 153 (Fig. 2B and supplemental Fig. S2D). We examined the possibility that this cysteine might be cysteine 158. Interestingly, although serine substitution of the other cysteines in Sty1 had a negligible effect upon the levels of Sty1 protein (Fig. 2B and supplemental Fig. S2, B, C, and D), the levels of Sty1C158S protein produced were far higher than wild type (supplemental Fig. S2D) as a result of increased sty1C158S mRNA levels. Furthermore, when protein levels were adjusted for this (by loading 10-fold less protein onto the gel), we found that DTT treatment did not allow Sty1C158S extracted from hydrogen peroxide-treated cells and modified with IAA to bind AMS (supplemental Fig. S2D, lower panel). This indicates that as well as its role in inhibiting

sty1 gene expression, this cysteine was also required for hydrogen peroxide-induced oxidation of Sty1. It was possible that the insensitivity of Sty1C158S to hydrogen peroxide-induced oxidation might be due to the increased amount of Sty1C158S protein present. To eliminate this possibility we examined whether Sty1C158S protein was DTT reversibly oxidized in cells expressing Sty1 and Sty1C158S protein from another promoter (nmt) and thus containing equivalent amounts of Sty1 protein (32) (Fig. 2C). Consistent with cells expressing Sty1 from its normal promoter (Fig. 1 and supplemental Fig. S2, B–D), DTT treatment of IAA-modified Sty1 and Sty1C158S extracted from hydrogen peroxide-treated cells expressing Sty1 and Sty1C158S from the Rep41 nmt promoter allowed wild-type Sty1, but not Sty1C158S protein, to be modified by AMS (Fig. 2C). Thus, our data suggest that the hydrogen peroxide-induced oxidation of Sty1 may involve reversible oxidation of cysteine 158.

3 A. M. Day and E. A. Veal, unpublished results.
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As substitution of cysteine 153 with serine also reduced the AMS reactivity of Sty1 (Fig. 2B), this suggested that cysteine 153 might participate in formation of a disulfide with cysteine 158. However, the DTT-induced AMS reactivity of Sty1<sup>C153SC158S</sup> suggests that in the absence of cysteine 153, cysteine 158 still becomes oxidized, possibly through formation of a disulfide with an alternative cysteine. The absence of any AMS-reactive form following DTT treatment of IAA-modified Sty1<sup>C153SC158S</sup>, in which both cysteine 153 and cysteine 158 are replaced with serine, is consistent with this possibility (Fig. 2D).

Taken together, these data suggest that in response to hydrogen peroxide, cysteine 158 in Sty1 becomes reversibly oxidized. Moreover, our data suggest that in wild-type Sty1, this reversible oxidation is likely to involve formation of a disulfide bond between cysteines 153 and 158. Intriguingly, not only did Sty<sup>1<sup>C153SC158S</sup></sup> not undergo oxidation, but it was also expressed at normal levels, suggesting that cysteine 153 is required for the increased Sty1 protein levels in the <i>sty1<sup>C153S</sup></i> mutant.

**Cysteines 153 and 158 of Sty1 Are Required for Resistance to Oxidative Stress**—Sty1 is important for cell cycle progression and for resistance to a variety of stress conditions, including osmotic and oxidative stress. Having determined that cysteines 153 and 158 play important roles in regulating Sty1 levels and redox sensitivity, we examined whether substitution of cysteines 153 and 158 with serine affected the ability of cells to grow under a variety of different stress conditions that inhibit the growth of Δsty1 mutant cells (10, 11). Although Sty1<sup>C158S</sup> is insensitive to thiol oxidation, to eliminate the possibility that the increased levels of Sty1<sup>C158S</sup> might be responsible for any phenotypes associated with the single mutation, these investigations were undertaken with Sty1<sup>C153SC158S</sup>. Importantly, <i>sty1<sup>C153SC158S</sup></i> cells were of a similar size and grew at a similar rate to wild-type (WT) cells, suggesting that neither cysteine is important for Sty1 function in controlling cell cycle progression (9) (supplemental Fig. S3 and Fig. 2F). Indeed, the similar growth of wild-type and <i>sty1<sup>C153SC158S</sup></i> mutant cells on plates containing 1 M potassium chloride or 250 mM calcium chloride (Fig. 2E) suggests that these cysteines are also dispensable for Sty1 function in resistance to osmotic stress and calcium. However, although much less sensitive than the Δsty1 mutant, the reduced growth of <i>sty1<sup>C153SC158S</sup></i> cells when compared with the WT control strain, on plates containing hydrogen peroxide or the heavy metal cadmium (Fig. 2E) suggested that these cysteines might be important for resistance to oxidative stress. Indeed, this was also evident in the substantially reduced capacity of the <i>sty1<sup>C153SC158S</sup></i> cells to recover and grow following transient exposure to either a level of hydrogen peroxide (1.0 mM) that is sublethal to wild-type cells or an acutely toxic level (25 mM) (Fig. 2F). Together, these data suggest that cysteines 153 and 158 are specifically important for Sty1 function under oxidative stress conditions.

**Cysteines 153 and 158 of Sty1 Are Not Required for Hydrogen Peroxide-induced Phosphorylation of Sty1 or Its Substrate, Atf1**—Activation of the kinase activity of Sty1 by dual phosphorylation of threonine 171 and tyrosine 173 by the MAPKK, Ws1, is essential for Sty1-dependent phosphorylation of downstream substrates in response to a variety of stress conditions, including hydrogen peroxide (11). Hence, it was possible that the increased oxidative stress sensitivity of cells expressing Sty1<sup>C153SC158S</sup> was due to reduced hydrogen peroxide-induced phosphorylation of Sty1<sup>C153SC158S</sup>. However, we found that Sty1<sup>C153SC158S</sup> was inducibly phosphorylated to an even greater extent than wild-type Sty1 in response to 1.0 mM H<sub>2</sub>O<sub>2</sub> (Fig. 3A). Thus, Cys-153 and Cys-158 are not required for hydrogen peroxide-induced phosphorylation of Sty1.

Although dispensable for Sty1 phosphorylation, it was possible that serine substitution of Cys-153 and Cys-158 reduced the kinase activity of Sty1, thus reducing the phosphorylation of substrate proteins such as the bZip transcription factor Atf1 (18, 19). We therefore examined whether cysteines 153 and 158 of Sty1 were required for hydrogen peroxide-induced phosphorylation of Atf1. As expected, the mobility of HA epitope-tagged Atf1 (Atf1-HA) extracted from WT cells treated with hydrogen peroxide was significantly decreased, consistent with hydrogen peroxide-induced Atf1-HA phosphorylation (Fig. 3B) (21). The similar decrease in Atf1-HA mobility observed when cells expressing Sty1<sup>C153SC158S</sup> were treated with hydrogen peroxide suggests that hydrogen peroxide-induced phosphorylation of Atf1 is unimpaired by serine substitution of these cysteines (Fig. 3B). From this we conclude that cysteines 153 and 158 are not required for Sty1-dependent, hydrogen peroxide-induced phosphorylation of Atf1.

**Cysteines 153 and 158 Are Required for Hydrogen Peroxide-induced Increases in Atf1 Levels**—The stress-induced phosphorylation of Atf1 by Sty1 inhibits degradation of Atf1, thus leading to an increase in Atf1 protein levels (21). However, despite apparently normal hydrogen peroxide-induced phosphorylation, we noticed that hydrogen peroxide appeared to induce a smaller increase in Atf1-HA levels in <i>sty1<sup>C153SC158S</sup></i> mutant than in WT cells (Fig. 3B). To examine possible effects on Atf1 protein levels more closely, we examined the levels of Atf1-HA in phosphatase-treated samples prepared from wild-type and Sty1<sup>C153SC158S</sup> mutant cells before and after exposure to 1.0 mM H<sub>2</sub>O<sub>2</sub>. As expected, in wild-type cells, there was a significant increase in the levels of Atf1 following exposure to hydrogen peroxide. However, a much smaller increase in Atf1 levels was detected in cells expressing Sty1<sup>C153SC158S</sup> (Fig. 3C). This suggests that although not necessary for H<sub>2</sub>O<sub>2</sub>-induced phosphorylation of Atf1, cysteines 153 and 158 of Sty1 are required to ensure increased levels of Atf1 protein in response to peroxide stress.

**Cysteines 153 and 158 of Sty1 Are Required for Hydrogen Peroxide-induced Increases in atf1 mRNA**—In addition to stabilizing Atf1 by phosphorylation, Sty1 also promotes oxidative stress-induced increases in the levels of <i>atf1</i> mRNA (21, 22). To examine the possibility that the failure to effectively increase Atf1 protein levels in <i>sty1<sup>C153SC158S</sup></i> mutant cells (Fig. 4, B and C) was due to a failure to increase <i>atf1</i> mRNA levels in response to hydrogen peroxide, we compared the levels of <i>atf1</i> mRNA in wild-type and <i>sty1<sup>C153SC158S</sup></i> cells before and after treatment with hydrogen peroxide. As expected, <i>atf1</i> mRNA levels were increased following exposure of wild-type cells to hydrogen peroxide or osmotic stress (0.6 M KCl) (Fig. 3D). However, the hydrogen peroxide-induced increase in <i>atf1</i> mRNA levels was significantly (p = 0.03) lower in <i>sty1<sup>C153SC158S</sup></i> cells (Fig. 3D). This implies that the reduced levels of Atf1 protein following
hydrogen peroxide treatment of \textit{sty}^{C153SC158S} cells may be due, at least in part, to a failure to effectively increase \textit{atf1} mRNA levels. There was no significant difference between the levels of \textit{atf1} mRNA in untreated \textit{sty}^{C153SC158S} and wild-type cells (Fig. 3D). Moreover, osmotic stress induced similar increases in \textit{atf1} mRNA levels in both wild-type cells and \textit{sty}^{C153SC158S} mutant cells (Fig. 3D). Together, these data are consistent with cysteines 153 and 158 being specifically required for hydrogen peroxide-induced increases in \textit{atf1} mRNA levels.

Previous studies have suggested that Sty1 influences \textit{atf1} mRNA levels by affecting mRNA stability (22). However, it was possible that the reduced levels of \textit{atf1} mRNA in \textit{sty}^{C153SC158S} cells could reflect either reduced transcription or reduced \textit{atf1} mRNA stability. To distinguish between these two possibilities, we examined the effect of hydrogen peroxide on \textit{atf1} mRNA levels in wild-type and \textit{sty}^{C153SC158S} mutant cells treated with the transcriptional inhibitor 1,10-phenanthroline (40). As expected, treatment with 1,10-phenanthroline prevented any increase in \textit{atf1} mRNA levels in either wild-type or \textit{sty}^{C153SC158S} cells following \textit{H}_{2}\text{O}_{2} treatment, indicating that transcription had been effectively blocked (Fig. 3E). However, although in wild-type cells \textit{atf1} mRNA levels remained relatively constant up to 60 min after exposure to hydrogen peroxide, in \textit{sty}^{C153SC158S} cells treated with 1,10-phenanthroline, \textit{atf1} mRNA levels decreased (Fig. 3E). This suggests that \textit{atf1} mRNA was less stable in these cells than in wild-type cells. Indeed, although the stability of \textit{atf1} mRNA was similar in untreated and osmotically stressed \textit{sty}^{C153SC158S} and wild-type cells (Fig. 3E and data not shown), following treatment
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FIGURE 4. Cysteines 153 and 158 in Sty1 are required to limit the hydrogen peroxide-induced destabilization of atf1 mRNA at increasing concentrations of hydrogen peroxide (A, B), allowing Atf1-dependent gene expression (C). A, Northern blot analysis of atf1 mRNA levels in WT (AD38) and sty1Δ153Δ158Δ (AD44) cells before and after exposure to 1.0, 6.0, and 25 mM H2O2 reveals that the induction of atf1 mRNA decreases as the concentration of hydrogen peroxide increases in both wild-type cells and sty1Δ153Δ158Δ cells. The graph shows the mean ± fold induction of atf1 mRNA levels relative to leu1 mRNA (loading control) determined from PhosphorImager analysis of data obtained in at least four independent experiments. Error bars indicate the S.E. B, Northern blot analysis of atf1 mRNA levels in WT (AD38) and sty1Δ153Δ158Δ (AD44) cells treated with 300 μg/ml L,10-phenanthroline and for 0, 10, 20, or 40 min with 1.0, 6.0, or 25 mM H2O2 reveals that the half-life of atf1 mRNA is lower in sty1Δ153Δ158Δ (AD44) than in WT (AD38) cells following treatment with 1.0, 6.0, or 25 mM H2O2. The half-life of atf1 mRNA in each strain and under each condition was calculated from the decay curves (log 2 atf1 mRNA/25 S rRNA against time), and mean values determined from at least four experiments are shown. Error bars indicate the S.E. C, Northern blot analysis of RNA from WT (AD38) and sty1Δ153Δ158Δ (AD44) cells treated for 0, 20, 40, 60, or 80 min with 1.0 mM H2O2 revealed a significantly smaller H2O2-induced increase in gpx1 mRNA compared with WT. Graphs show mean ± fold induction of each mRNA at 0, 20, and 40 min calculated from data obtained in at least six independent experiments. (Two-way analysis of variance analysis: atf1 and sty1Δ153Δ158Δ, p = 0.0237, n = 6).

Cysteines 153 and 158 of Sty1 Are Required for Hydrogen Peroxide-induced Oxidation of Sty1 Is Associated with Maintenance of atf1 mRNA Stability—Having found that cysteines 153 and 158 in Sty1 (i) are required for atf1 mRNA stability following exposure to hydrogen peroxide (Fig. 3E) and (ii) become oxidized in response to hydrogen peroxide (Figs. 1 and 2), we examined whether oxidation of these cysteines was important for hydrogen peroxide-induced increases in atf1 mRNA levels. When we compared the levels of atf1 mRNA in wild-type cells treated with 1.0, 6.0, and 25 mM H2O2, we found that treatment with 1.0 mM H2O2, at which oxidation of Sty1 is minimal (Fig. 1A), produced a much bigger increase in atf1 mRNA levels than with 6.0 or 25 mM (Fig. 4A), at which an increasing proportion of Sty1 is oxidized (Fig. 1A). This smaller increase in atf1 mRNA as H2O2 concentrations increase suggested that oxidation of cysteines 153 and 158 might prevent Sty1 from maintaining atf1 mRNA stability. However, if either serine substitution or oxidation of cysteines 153 and 158 in Sty1 prevents Sty1-dependent stabilization of atf1 mRNA, then it would be expected that the induction of atf1 mRNA in sty1Δ153Δ158Δ cells would be reduced to a similar extent at both low and high concentrations of H2O2. In contrast, we found that as H2O2 concentrations increase, there was an even smaller increase in atf1 mRNA levels in sty1Δ153Δ158Δ cells than in WT cells such that atf1 mRNA levels actually decreased following exposure of sty1Δ153Δ158Δ cells to 25 mM H2O2 (Fig. 4A). This suggested that oxidation of Sty1 at these concentrations of H2O2 might be important for maintaining atf1 mRNA stability. Consistent with this, although exposure to increasing levels of hydrogen peroxide reduced the half-life of atf1 mRNA in wild-type cells, it produced a much greater decrease in atf1 mRNA stability in sty1Δ153Δ158Δ cells (Fig. 4B). Indeed, we found that the half-life of atf1 mRNA was still significantly reduced in sty1Δ153Δ158Δ cells exposed to 25 mM hydrogen peroxide when compared with wild-type cells similarly treated (at 25 mM hydrogen peroxide, half-lives of atf1 mRNA are: wild-type, 8.00 ± 0.95 min; sty1Δ153Δ158Δ, 4.60 ± 0.41 min; Student’s t test p = 0.00938) (Fig. 4B). Thus, our data indicate that the oxidation of cysteines 153 and 158 in Sty1 that occurs at these concentrations of hydrogen peroxide (Fig. 1A) is important for maintenance of atf1 mRNA stability following oxidative stress.

Cysteines 153 and 158 of Sty1 Are Required for Hydrogen Peroxide-induced Atf1-dependent Gene Expression—Our data suggest that the smaller hydrogen peroxide-induced increase in

with 1.0 mM hydrogen peroxide, the half-life of atf1 mRNA was substantially decreased in sty1Δ153Δ158Δ cells when compared with wild-type cells. These data suggest that cysteines 153 and 158 in Sty1 are required for atf1 mRNA stability following exposure to 1.0 mM H2O2.
Atf1 levels in \textit{sty1}^{C153SC158S} cells when compared with wild-type (Fig. 3C) is due to the reduced capacity of \textit{sty1}^{C153SC158S} to maintain \textit{atf1} mRNA stability under these conditions (Figs. 3E and 4B). We next examined whether the reduction in Atf1 levels in hydrogen peroxide-treated \textit{sty1}^{C153SC158S} mutant cells was sufficient to reduce the hydrogen peroxide-induced expression of Atf1-dependent genes, encoding the glutathione peroxidase, \textit{gpx1}, and the protein tyrosine phosphatase \textit{pyp2}. As expected, levels of both \textit{gpx1} and \textit{pyp2} mRNA increased in wild-type cells following exposure to 1.0 mM H$_2$O$_2$ (Fig. 4C). \textit{gpx1} and \textit{pyp2} mRNA levels also increased following exposure of \textit{sty1}^{C153SC158S} mutant cells to 1.0 mM H$_2$O$_2$. However, the increases in each mRNA were markedly reduced when compared with wild-type cells ($p < 0.025$) (Fig. 4C). This suggests that the role of cysteines 153 and 158 of Sty1 in \textit{atf1} mRNA stability is important for the \textit{Sty1}-dependent transcriptional response to hydrogen peroxide and may be responsible for the reduced oxidative stress resistance of \textit{sty1}^{C153SC158S} mutant cells (Fig. 2, E and F). Indeed, as negative feedback regulation of Sty1 involves dephosphorylation by Pyp2 (10, 11), it is possible that the reduced levels of \textit{pyp2} expression may also be responsible for the slight increase in hydrogen peroxide-induced \textit{Sty1} phosphorylation detected in \textit{sty1}^{C153SC158S} mutant cells (Fig. 3A).

The Role of Cysteines 153 and 158 in Oxidative Stress Resistance Is Atf1-dependent—It was possible that the reduced H$_2$O$_2$-induced expression of Atf1-dependent genes might account for the increased hydrogen peroxide sensitivity of \textit{sty1}^{C153SC158S} mutant cells, or, alternatively, that these cysteines have additional roles that contribute to their essential role in oxidative stress resistance. To examine these possibilities, we compared the sensitivity of \textit{sty1}^{C153SC158S}, \textit{Δatf1}, and \textit{Δatf1} \textit{sty1}^{C153SC158S} cells to hydrogen peroxide. The sensitivity of \textit{Δatf1} \textit{sty1}^{C153SC158S} cells was similar to \textit{Δatf1} cells, both on plates containing low levels of hydrogen peroxide (Fig. 5A) and following exposure to high levels of hydrogen peroxide in liquid culture (Fig. 5B). This indicates that loss of cysteines 153 and 158 of Sty1 does not further reduce the oxidative stress resistance of cells lacking Atf1. The Atf1 dependence of the oxidative stress-protective role of cysteines 153 and 158 is consistent with their function in oxidative stress resistance being predominantly through their role in maintaining \textit{atf1} mRNA stability. Indeed, the reduced ability of \textit{sty1}^{C153SC158S} cells to survive transient exposure to 25 mM hydrogen peroxide was completely rescued by ectopic expression of Atf1 (pRep41 atf1), suggesting that increased expression of Atf1 is sufficient to restore wild-type levels of hydrogen peroxide resistance to \textit{sty1}^{C153SC158S} cells (Fig. 5C). Together, these data also indicate that the \textit{Sty1}-dependent maintenance of \textit{atf1} mRNA stability, for which Cys-153 and Cys-158 are required, makes an important contribution to the role of \textit{Sty1} in oxidative stress resistance.

\textit{Sty1} Regulates \textit{atf1} mRNA Stability and Atf1 Phosphorylation by Independent Mechanisms to Increase Atf1 Protein Levels and Oxidative Stress Resistance—Stress-induced phosphorylation of Atf1 by Sty1 increases the stability of Atf1 protein such that a mutant in which 11 consensus MAPK phosphorylation sites have been substituted (Atf1-11M) is no longer phosphorylated or stabilized by \textit{Sty1} (21). Our data suggest that cysteines 153 and cysteine 158 in \textit{Sty1} act to increase Atf1 protein levels independently from \textit{Sty1}-dependent phosphorylation of Atf1 (Fig. 3). A prediction of this model is that serine substitution of these cysteines in \textit{Sty1} in cells containing the Atf1-11M mutant form of Atf1 will prevent stress-induced stabilization of \textit{atf1} mRNA and thus further reduce the levels of Atf1 protein. To test this hypothesis, we constructed a strain containing both the \textit{atf1-11M-\textit{HA}} and \textit{atf1-11M} alleles and compared the levels of Atf1-\textit{HA} protein in these cells with those in the \textit{atf1-11M-\textit{HA}} and \textit{sty1}^{C153SC158S} parent strains before and after treatment with hydrogen peroxide. This Western blot analysis revealed the hydrogen peroxide-induced increase in Atf1-\textit{HA}} levels.
observed in wild-type cells is reduced to a similar extent by Ser substitution of Cys-153 and Cys-158 in Sty1 as by mutation of all the consensus MAPK phosphorylation sites on Atf1 (Atf1-11M) (Fig. 6A). Furthermore, in atf1-11M-HA sty1\(^{C153SC158S}\) cells, the hydrogen peroxide-induced increase in Atf1 levels was completely abolished, indicating that Sty1-dependent phosphorylation of Atf1 and stabilization of atf1 mRNA act independently and are together vital for the hydrogen peroxide-induced increase in Atf1 levels (Fig. 6A). This was also reflected in the heightened oxidative stress sensitivity of atf1-11M-HA sty1\(^{C153SC158S}\) mutant cells, in which both Sty1-dependent phosphorylation of Atf1 and Sty1-dependent stabilization of atf1 mRNA are defective when compared with atf1-11M-HA and sty1\(^{C153SC158S}\) cells (Fig. 6B).

Thus, we propose that S. pombe have evolved to use the Sty1 MAPK in two independent mechanisms to increase Atf1 levels in response to hydrogen peroxide. Firstly, formation of a disulfide between cysteines 153 and 158 in Sty1 is required to maintain atf1 mRNA stability. Secondly, Sty1-mediated direct phosphorylation of Atf1 increases Atf1 protein stability (21) (Fig. 7). The evolution of multiple mechanisms to increase Atf1 levels in response to oxidative stress indicates the critical role that Atf1 plays in survival and adaptation to oxidative stress conditions.

**DISCUSSION**

Activation of MAPK by phosphorylation is a key step in the initiation of biological responses to a multitude of stimuli (1). ATF transcription factors are important mediators of MAPK-dependent transcriptional responses in mammals and yeast (16, 23, 24). Phosphorylation by MAPK increases ATF activity by multiple mechanisms, including the inhibition of ATF protein degradation (21, 41). In S. pombe, the Sty1 MAPK regulates Atf1 to elicit both core stress responses common to different stimuli and also responses specific to particular stress conditions (20). However, it is unclear how different stimuli, which produce similar increases in phosphorylation of Sty1 and Atf1, produce different responses appropriate for adaptation to a particular stimulus. Here, we have identified two cysteines in Sty1 that are specifically required to increase Atf1 levels and Sty1-dependent transcription in response to hydrogen peroxide. Under oxidative stress conditions, in addition to phosphorylating and stabilizing Atf1 protein, Sty1 is also required to maintain atf1 mRNA stability (22). Indeed, we find that cysteines 153 and 158 in Sty1 are dispensable for phosphorylation of Atf1 but instead required to inhibit the hydrogen peroxide-induced destabilization of atf1 mRNA (Figs. 3E, 4B, and 7).

Previous studies have suggested that phosphorylation by Sty1 and heterodimerization with Pcr1 together act to increase the protein stability of Atf1 (21). In addition, at least two Sty1-dependent mechanisms are important for maintaining atf1 mRNA stability following oxidative stress. One of these mechanisms involves the RNA-binding proteins Csx1 and Upf1, which act in the same pathway to increase atf1 mRNA stability (22, 42). However, Sty1 is also required for atf1 mRNA stability, even in the absence of Csx1 (22). Indeed, we find that cysteines 153 and 158 in Sty1 regulate atf1 mRNA stability independently from Csx1 (data not shown). Thus, Sty1 is involved in multiple
mechanisms for increasing Atf1 levels in response to stress. The evolution of several mechanisms for increasing Atf1 levels following stress reflects the important role of this transcription factor in stress survival/adaptation (18, 19). In addition, it suggests that constitutively high levels of Atf1 do not benefit, or indeed may adversely affect, cells under non-stress conditions. Strikingly, the two cysteines identified here are important for hydrogen peroxide-induced increases in atf1 mRNA and oxidative stress resistance but not for osmotic stress-induced increases in atf1 mRNA or osmotic stress resistance (Figs. 2 and 3). This raises the possibility that the regulation of Atf1 by different mechanisms in response to different stresses allows S. pombe to tailor the levels of Atf1 appropriately to initiate distinct transcriptional responses to a particular stress. Indeed, cells containing an Atf1 mutant protein that is not phosphorylated by Sty1 in response to stress are also sensitive to oxidative stress but have normal resistance to osmotic stress (21). Together, these data suggest that Sty1-dependent increases in Atf1 levels are particularly important for oxidative stress resistance.

Intriguingly, we have also found that Sty1 is sensitive to hydrogen peroxide-induced oxidation. The DTT reversibility of this oxidation and its rapid reversal following removal from hydrogen peroxide (data not shown) suggest that it involves the formation of disulfide bond(s) between cysteine thiols in Sty1. Indeed, our analysis of this oxidation in Sty1 mutants, in which each cysteine is substituted with serine, suggest that cysteines 153 and 158 form an intramolecular disulfide in response to high levels of hydrogen peroxide. Comparisons with the crystal structures of other MAPK suggest that both Cys-153 and Cys-158 are likely to be on the surface of Sty1, where they are accessible to hydrogen peroxide, and moreover that it is structurally feasible for a disulfide bond to form between them. Given the important role of these cysteines in the response of cells to hydrogen peroxide, it was possible that this oxidation might be important for the function of Sty1 in increasing atf1 mRNA stability. Indeed, as hydrogen peroxide concentrations increase, the greater decline in atf1 mRNA stability in sty1C153SC158S than in wild-type cells (Fig. 4) suggests that the increased oxidation of these cysteines in wild-type Sty1 is important for maintenance of atf1 mRNA stability under these conditions (Fig. 4). Although the mechanism by which formation of a disulfide bond between cysteines 153 and 158 in Sty1 could prevent hydrogen peroxide-induced destabilization of atf1 mRNA is not clear, this suggests that redox regulation of these cysteines allows S. pombe to modulate the response to high levels of hydrogen peroxide.

Previous transcriptional profiling studies have suggested that Atf1-dependent gene expression is reduced at higher concentrations of hydrogen peroxide (24). Here, we show that this could reflect smaller increases in atf1 mRNA levels as hydrogen peroxide concentrations increase (Fig. 4A). It is possible that the reduced “protective” transcriptional response at these higher levels may allow commitment to an alternative cellular response, e.g. apoptosis rather than initiation of mechanisms to ensure survival. Thus, under these conditions, regulation of atf1 mRNA stability may be critical in determining whether or not cells survive. In support of this, the survival of sty1C153SC158S cells is greatly reduced following exposure to hydrogen peroxide levels that substantially reduce atf1 mRNA stability in these cells (Figs. 2, 3E, 4B, and 5). Furthermore, the ability of ectopic expression of atf1 to restore normal oxidative stress resistance to sty1C153SC158S cells suggests that reduced Atf1 levels are responsible for the sensitivity of these cells to hydrogen peroxide (Fig. 5C).

The critical role that SAPK (stress-activated protein kinase) and bZip transcription factors also play in coordinating responses to stress in other eukaryotes suggests that this regulatory mechanism might be conserved. Indeed, Cys-153 is conserved in all MAPK, and Cys-158 is also conserved in fungal orthologs of Sty1, such as Hog1, which is important for the virulence of opportunistic human pathogens, Candida albicans and Cryptococcus neoformans (supplemental Fig. S4) (4, 43). As hydrogen peroxide is generated by mammalian immune cells as a potent weapon against pathogens, the ability to activate responses to hydrogen peroxide is likely to be important for the virulence of these fungi. Hence, these cysteines, particularly Cys-158, which is not conserved in mammalian p38/JNK MAPK, may represent a potential target for the treatment of fungal infections.

Although cysteine 158 is not conserved in mammalian p38 and JNK MAPK, it is conserved in the ERK family of MAPK (supplemental Fig. S4). This raises the intriguing possibility that ERK may have a role in regulating mRNA stability. ERK is activated in response to growth factors, many of which have been shown to lead to the generation of hydrogen peroxide as a second messenger. Hydrogen peroxide promotes signal transduction by causing the reversible inactivation of catalytic cysteine in protein tyrosine phosphatases (44). If cysteines 153 and 158 are important for ERK1/2 function, then it is an intriguing possibility that hydrogen peroxide may also regulate signal transduction by reversible oxidation of these cysteine thiols.

In summary, together with previous studies (21, 22), our data suggest that Sty1 is involved in multiple mechanisms to ensure that adequate levels of Atf1 protein are produced and maintained to initiate appropriate levels of antioxidant gene expression in response to hydrogen peroxide. Moreover, our studies reveal that the function of the MAPK activity of Sty1 in increasing Atf1 protein stability (21) can be genetically separated from the role of Sty1 in prevention of hydrogen peroxide-induced destabilization of atf1 mRNA (22). Significantly, both activities are important for adaptation and survival of S. pombe cells following exposure to oxidative stress. These data imply that distinct dose- and stimului-specific responses can be initiated by MAPK by regulating the levels of a single transcription factor by multiple mechanisms. It is not clear whether the stability of atf mRNA is stress-regulated in other eukaryotes. However, the conserved role of bZip transcription factors as effectors of MAPK signaling pathways raises the intriguing possibility that similar mechanisms may be employed to ensure appropriate patterns of gene expression in response to different stimuli in mammals.

4 M. Wouters, personal communication.
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REFERENCES

1. Wagner, E. F., and Nebreda, A. R. (2009) Nat. Rev. Cancer 9, 537–549
2. Dong, C., Davis, R. J., and Flavell, R. A. (2002) Curr. Opin. Cell Biol. 14, 389–393
3. Livingstone, C., Patel, G., and Jones, N. (1995) EMBO J. 14, 1785–1797
4. Shiozaki, K., and Russell, P. (1996) Genes Dev. 10, 2276–2288
5. Wilkinson, M. G., Samuels, M., Takeda, T., Toone, W. M., Shieh, J. C., Toda, T., Millar, J. B., and Jones, N. (1996) Genes Dev. 10, 2289–2301
6. Chen, D., Toone, W. M., Mata, J., Lyne, R., Burns, G., Kivinen, K., Brazma, A., Jones, N., and Bähler, J. (2003) Mol. Biol. Cell 14, 214–229
7. Lawrence, C. L., Maekawa, H., Worthington, J. L., Reiter, W., Wilkinson, C. R., and Jones, N. (2007) J. Biol. Chem. 282, 5160–5170
8. Rodriguez-Gabriel, M. A., Burns, G., McDonald, W. H., Martin, V., Yates, J. R., 3rd, Bähler, J., and Russell, P. (2003) EMBO J. 22, 6256–6266
9. Quinn, J., Findlay, V. J., Dawson, K., Millar, J. B., Jones, N., Morgan, B. A., and Toone, W. M. (2002) Mol. Cell. Biol. 13, 805–816
10. Chen, D., Wilkinson, C. R., Watt, S., Penkett, C. J., Toone, W. M., Jones, N., and Bähler, J. (2008) Mol. Biol. Cell 19, 308–317
11. Degols, G., Shiozaki, K., and Russell, P. (1996) Mol. Cell. Biol. 16, 2870–2877
12. Nguyen, A. N., and Shiozaki, K. (1999) Mol. Cell. Biol. 19, 1169–1181
13. Nguyen, A. N., and Shiozaki, K. (1999) Genes Dev. 13, 1653–1663
14. Buck, V., Quinn, J., Soto Pino, T., Martin, H., Saldanha, J., Makino, K., Morgan, B. A., and Millar, J. B. (2001) Mol. Biol. Cell 12, 407–419
15. Nadeau, P. J., Charette, S. J., Toledano, M. B., and Morgan, B. A. (2004) Mol. Cell 15, 129–139
16. Moreno, S., Klar, A., and Nurse, P. (1991) Methods Enzymol. 194, 795–823
17. Coulthard, L. R., White, D. E., Jones, D. L., McDermott, M. F., and Burchill, S. A. (2001) Trends Mol. Med. 7, 537–549
18. Cohen, P. (2009) Gene 123, 127–130
19. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene 77, 51–59
20. Apolinaro, E., Nocero, M., Jin, M., and Hoffman, C. S. (1993) Curr. Genet 24, 491–495
21. Lawrence, C. L., Maekawa, H., Worthington, J. L., Reiter, W., Wilkinson, C. R., and Jones, N. (2007) J. Biol. Chem. 282, 5160–5170
22. Rodriguez-Gabriel, M. A., Burns, G., McDonald, W. H., Martin, V., Yates, J. R., 3rd, Bähler, J., and Russell, P. (2003) EMBO J. 22, 6256–6266
23. Quinn, J., Findlay, V. J., Dawson, K., Millar, J. B., Jones, N., Morgan, B. A., and Toone, W. M. (2002) Mol. Cell. Biol. 13, 805–816
24. Chen, D., Wilkinson, C. R., Watt, S., Penkett, C. J., Toone, W. M., Jones, N., and Bähler, J. (2008) Mol. Biol. Cell 19, 308–317
25. Vivancos, A. P., Castillo, E. A., Jones, N., Ayte, J., and Hidalgo, E. (2004) Mol. Microbiol. 52, 1427–1435
26. Barford, D. (2004) Curr. Opin. Struct. Biol. 14, 679–686
27. Veal, E. A., Day, A. M., and Morgan, B. A. (2007) Mol. Cell 26, 1–14
28. Cross, J. V., and Templeton, D. J. (2004) Biochem. J. 381, 675–683
29. Nadeau, P. J., Charette, S. J., Toledano, M. B., and Landry, J. (2007) Mol. Biol. Cell 18, 3903–3913
30. Veal, E. A., Findlay, V. J., Day, A. M., Bozonen, S. M., Evans, J. M., Quinn, J., and Morgan, B. A. (2004) Mol. Cell 15, 129–139
31. Maundrell, K. (1993) Mol. Cell. Biol. 13, 2285–2300
32. Bahn, Y. S., Kojima, K., Cox, G. M., and Heitman, J. (2005) J. Biol. Chem. 280, 6256–6264
33. Quinn, J., Findlay, V. J., Dawson, K., Millar, J. B., Jones, N., Morgan, B. A., and Toone, W. M. (2002) Mol. Cell. Biol. 13, 805–816
34. Apolinaro, E., Nocero, M., Jin, M., and Hoffman, C. S. (1993) Curr. Genet 24, 491–495
35. Delaunay, A., Isnard, A. D., and Toledano, M. B. (2000) EMBO J. 19, 5157–5166
36. Veal, E. A., Toone, W. M., Jones, N., and Morgan, B. A. (2002) J. Biol. Chem. 277, 35523–35531
37. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
38. Delaunay, A., Isnard, A. D., and Toledano, M. B. (2000) EMBO J. 19, 5157–5166
39. Parker, R., Herrick, D., Peltz, S. W., and Jacobson, A. (1991) J. Biol. Chem. 266, 6347–6356
40. Bahn, Y. S., Kojima, K., Cox, G. M., and Heitman, J. (2005) Mol. Biol. Cell 16, 2285–2300
41. Tonks, N. K. (2005) Cell 121, 667–670