

Cip1 and Cip2 Are Novel RNA-Recognition-Motif Proteins That Counteract Csx1 Function during Oxidative Stress

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Eukaryotic cells reprogram their global patterns of gene expression in response to stress. Recent studies in *Schizosaccharomyces pombe* showed that the RNA-binding protein Csx1 plays a central role in controlling gene expression during oxidative stress. It does so by stabilizing *atf1*⁺ mRNA, which encodes a subunit of a bZIP transcription factor required for gene expression during oxidative stress. Here, we describe two related proteins, Cip1 and Cip2, that were identified by multidimensional protein identification technology (MudPIT) as proteins that coprecipitate with Csx1. Cip1 and Cip2 are cytoplasmic proteins that have RNA recognition motifs (RRMs). Neither protein is essential for viability, but a *cip1Δ cip2Δ* strain grows poorly and has altered cellular morphology. Genetic epistasis studies and whole genome expression profiling show that Cip1 and Cip2 exert posttranscriptional control of gene expression in a manner that is counteracted by Csx1. Notably, the sensitivity of *csx1Δ* cells to oxidative stress and their inability to induce expression of Atf1-dependent genes are partially rescued by *cip1Δ* and *cip2Δ* mutations. This study emphasizes the importance of a modulated mRNA stability in the eukaryotic stress response pathways and adds new information to the role of RNA-binding proteins in the oxidative stress response.

INTRODUCTION

Reactive oxygen species (ROS) are found in all aerobically growing cells. Changes in the intracellular redox state actively regulate several signaling pathways that control essential biological processes (Finkel, 2003; Torres, 2003). However, cell survival requires tight control of the cellular redox state. When ROS increase beyond homeostatic concentrations, they react with a multitude of molecules (such as lipids, proteins, and nucleic acids), resulting in impaired cellular functions and formation of toxic species. In fact, oxidative stress is thought to be an important factor in aging, in the progression of cancer, and in many common neurodegenerative diseases such as amyotrophic lateral sclerosis, Alzheimer's disease, Huntington's disease, and Parkinson's disease (Migliore and Coppede, 2002; Barnham *et al.*, 2004). Antioxidant proteins such as catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase are part of the defense mechanisms that help the cells to deal with oxidative stress.

The evolutionary conserved mitogen-activated protein kinase (MAPK) pathways control the expression of many genes in response to oxidative stress (reviewed in Martindale and Holbrook, 2002). In *Schizosaccharomyces pombe*, the Spc1 (Sty1, Phh1) MAPK pathway is essential for the cellular response to different forms of stress, including oxidative

stress, hyperosmotic stress, heat, UV light, and nutrient limitation (Millar *et al.*, 1995; Shiozaki and Russell, 1995; Degols *et al.*, 1996; Degols and Russell, 1997). Spc1 is activated through phosphorylation by the MAPK kinase (MAPKK) Wis1, which in turn is activated through phosphorylation by two MAPKK kinases (MAPKKK), Wis4 and Win1 (Samejima *et al.*, 1997; Shieh *et al.*, 1997, 1998; Shiozaki *et al.*, 1997; Samejima *et al.*, 1998; Quinn *et al.*, 2002). In addition to Wis1, the tyrosine phosphatases Pyp1 and Pyp2 participate in the negative regulation of Spc1 (Millar *et al.*, 1995; Shiozaki and Russell, 1995; Samejima *et al.*, 1997; Shieh *et al.*, 1997, 1998; Shiozaki *et al.*, 1997, 1998). Spc1 regulates stress-dependent transcription through the Atf1–Pcr1 heterodimeric bZip transcription factor complex (Toda *et al.*, 1991; Takeda *et al.*, 1995; Kumada *et al.*, 1996; Wilkinson *et al.*, 1996; Toone *et al.*, 1998; Yamada *et al.*, 1999; Nguyen *et al.*, 2000; Quinn *et al.*, 2002). Pap1, a bZip transcription factor whose activity seems to be indirectly influenced by Spc1, also participates in transcriptional regulation during oxidative stress (Vivancos *et al.*, 2004, 2005). Pap1 is a homologue of c-Jun and activates target genes in response to low levels of H₂O₂, whereas the transcriptional response to higher concentrations of H₂O₂ and other kinds of stress is mediated by Atf1–Pcr1 (Toone *et al.*, 1998; Quinn *et al.*, 2002).

Components of the Spc1 MAPK cascade are functionally and structurally homologous to members of the HOG MAPK pathway in *Saccharomyces cerevisiae* and to the mammalian and *Drosophila* c-Jun NH₂-terminal kinase (JNK) and p38 stress-activated protein kinase cascades (reviewed in Toone and Jones, 1998). In contrast to the HOG pathway, which almost exclusively senses and responds to osmotic stress, the Spc1, JNK and p38 pathways are activated by a wide range of stress stimuli. However, depending on the stimulus, different patterns of gene expression result.

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The recent discovery of the RNA binding protein Csx1, which regulates global gene expression during oxidative stress in *S. pombe*, has helped to elucidate how cells tailor specific gene expression responses to each kind of stress. The cytoplasmic protein Csx1 contains three RNA recognition motifs (RRMs) and binds to *atf1*⁺ mRNA, stabilizing *atf1*⁺ mRNA and allowing cells to maintain normal levels of Atf1 protein under conditions of oxidative stress (Rodriguez-Gabriel *et al.*, 2003). The sensitivity of Csx1-deficient cells to H₂O₂ is partly explained by a deregulated expression of Atf1-dependent genes. However, Csx1 also possesses Atf1-independent functions in the response to oxidative stress, because *csx1Δ* mutants are more sensitive to H₂O₂ than *atf1Δ* cells. Both Csx1 and Spc1 are necessary to maintain normal levels of *atf1*⁺ mRNA. Csx1 and Spc1 coordinately regulate expression of many genes. However, microarray analyses of cells subjected to oxidative stress show that many genes whose expression is Spc1-dependent are Csx1-independent and vice versa, indicating that Csx1 and Spc1 have certain nonoverlapping functions (Rodriguez-Gabriel *et al.*, 2003).

A more thorough understanding of the precise mechanisms that participate in the posttranscriptional control of gene expression in H₂O₂-treated *S. pombe* cells relies on the identification and biochemical characterization of the specific molecules involved in this pathway. Here, we describe two novel RRM proteins of *S. pombe* that interact with Csx1 and participate in the control of gene expression under conditions of oxidative stress.

MATERIALS AND METHODS

Yeast Strains, Media, and General Methods

Standard procedures and growth media for *S. pombe* genetics have been described previously (Moreno *et al.*, 1991). *Cip1* and *Cip2* were deleted by replacement of the entire open reading frame of each gene with the KanMx6 module as described previously (Bahler *et al.*, 1998). Epitope-tagged *Cip1* and *Cip2* were also generated as described previously (Bahler *et al.*, 1998), placing a FLAG, green fluorescent protein (GFP), or cyan fluorescent protein (CFP) epitope at the C terminus of each protein and marking the allele with the kanamycin resistance gene. The epitope-tagged alleles seemed to be functional because they did not rescue *csx1Δ*.

All strains used in these studies were *ura4-D18 leu1-32*. Their genotypes are PR 109, wild-type; VM3770, *cip1*-CFP-KanMx6; VM3771, *cip1*::KanMx6; VM2772, *cip2*::KanMx6; VM3773, *cip2*-GFP-KanMx6; VM3774, *cip1*::KanMx6 *cip2*::KanMx6; VM3775, *cip2*::KanMx6 *sx1*::KanMx6; VM3776, *cip1*::KanMx6 *csx1*::KanMx6; VM3777, *cip1*::KanMx6 *cip2*::KanMx6 *csx1*::KanMx6; VM3778, *cip1*-FLAG-KanMx6; VM3779, *cip2*-FLAG-KanMx6; VM3780, *kanMx6-ntm1*-*cip2*⁺; VM3781, *cip1*::KanMx6 *atf1*::*ura4*; VM3782, *cip2*::KanMx6 *atf1*::*ura4*; VM3783, *spc1*::*ura4* *cip1*-FLAG-kanMx6; VM3784, *spc1*::*ura4* *cip2*-FLAG; VM3785, *csx1*::*kanMx6* *cip1*-FLAG-kanMx6; VM3786, *csx1*::*kanMx6* *cip2*-FLAG-kanMx6; MR3213 *csx1*::*kanMx6*; KS1497, *atf1*::*ura4*, and KS1605, *spc1*::*ura4* (this strain is *leu1*⁺).

For plate survival assays, serial dilutions of yeast culture were plated in media containing 0.6 or 0.8 mM H₂O₂. For cell survival assays, cells were grown in the presence of H₂O₂ for different times, plated in rich media, and colonies were counted after 4 d at 30°C.

RNA and Microarray Methods

RNA for Northern blots and microarray analysis was obtained as described in http://www.sanger.ac.uk/PostGenomics/S_pombe/protocols/. Sample labeling, microarray hybridization and data acquisition were performed as described previously (Lyne *et al.*, 2003).

Mass Spectrometry and Protein Methods

Csx1-TAP protein was purified from fission yeast cells treated with 1 mM H₂O₂ using a previously described method (Saitoh *et al.*, 2002). The resulting peptide mixture was analyzed by multidimensional protein identification technology (MudPIT) (MacCoss *et al.*, 2002). MudPIT combines multidimensional chromatography with mass spectrometry, obviating the need for visualization and excision of protein bands from gels for peptide identification (Graumann *et al.*, 2004). This approach has been successfully used in our laboratory for the identification of partners of other proteins (Boddy *et al.*, 2001, 2003). For immunoblotting, the FLAG epitope was detected using

mouse monoclonal antibodies. Immunoprecipitated *Cip2*-FLAG was used as substrate for λ phosphatase treatment.

Microscopy

CFP and GFP fluorescence was visualized in mid-log phase live cells. Cells were photographed using a Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan) equipped with a Photometrics Quantix charge-coupled device camera (Photometrics, Tucson, AZ).

RESULTS

Identification of *Cip1* and *Cip2*

For a better understanding of how Csx1 mediates posttranscriptional regulation of gene expression in response to oxidative stress, we sought to identify interaction partners by using MudPIT (see *Materials and Methods* for details). To this end, the *csx1*⁺ endogenous locus was engineered to encode a protein with a C-terminal TAP tag (Rodriguez-Gabriel *et al.*, 2003).

Cells were then treated for 15 min with 1 mM H₂O₂ and subsequently processed as described previously (Saitoh *et al.*, 2002). Before the MudPIT analysis, we confirmed by immunoblotting that the Csx1-TAP protein had been efficiently precipitated (our unpublished data). The percentage of coverage of the primary sequence and the number of peptides obtained for each of the proteins recovered was used as an indication of the relative abundance of each protein in the sample. We have performed many TAP purifications of nuclear and cytosolic proteins, and this allowed us to generate a list of the highly abundant proteins that commonly contaminate TAP purifications (e.g., metabolic enzymes, actin, and ribosomal proteins). The list of proteins identified in the Csx1-TAP purification was compared with this list to exclude the proteins that were nonspecifically purified.

As expected, mass spectrometric analysis of the affinity-purified Csx1-TAP sample revealed extensive peptide coverage of Csx1 (86.9%; Figure 1A). We found two novel proteins that coprecipitated with Csx1-TAP, which we termed *Cip1* and *Cip2*. *Cip1* ("Csx1-interacting protein 1") was identified by peptides covering 18.4% of its 490 amino acid primary sequence (Figure 1A). Peptides covering 11.6% of another novel protein, *Cip2*, were also obtained (Figure 1A). *Cip2* is a 576-amino acid protein that showed significant homology to *Cip1* (Figure 1, B and C). Both *Cip1* and *Cip2* were identified with greater than 98% confidence.

The sequence of both *Cip1* and *Cip2* contained an RNA recognition motif (RRM) (Figure 1, B and C). RRM domains are typically present in proteins involved in RNA processing, a relevant example being Csx1 (Rodriguez-Gabriel *et al.*, 2003). *Cip2* also harbored an R3H motif (Figure 1B). R3H motifs are thought to function in sequence-specific binding to single-stranded nucleic acids (Grishin, 1998). As observed in the sequence of *Cip2*, R3H motifs usually occur in association with other DNA- or RNA-binding domains (Letunic *et al.*, 2002; Bateman *et al.*, 2004).

Performing a BLAST search, most *Cip1* and *Cip2* homologues showed sequence homology only across the RRM domain. In addition, Rna15—a subunit of the cleavage and polyadenylation factor I complex in *S. cerevisiae* (Gross and Moore, 2001)—also shared common residues outside the RNA recognition motif with *Cip1* and *Cip2*. Rna15, *Cip1*, and *Cip2* seemed to be direct evolutionary counterparts because all of them were included in the same cluster of orthologous group (COG) (Figure 1D) (Marchler-Bauer *et al.*, 2005). SPAC644.16, which is the closest homologue of Rna15 in fission yeast, was not present in the group of proteins coprecipitating with Csx1-TAP. The only *S. pombe* ortho-

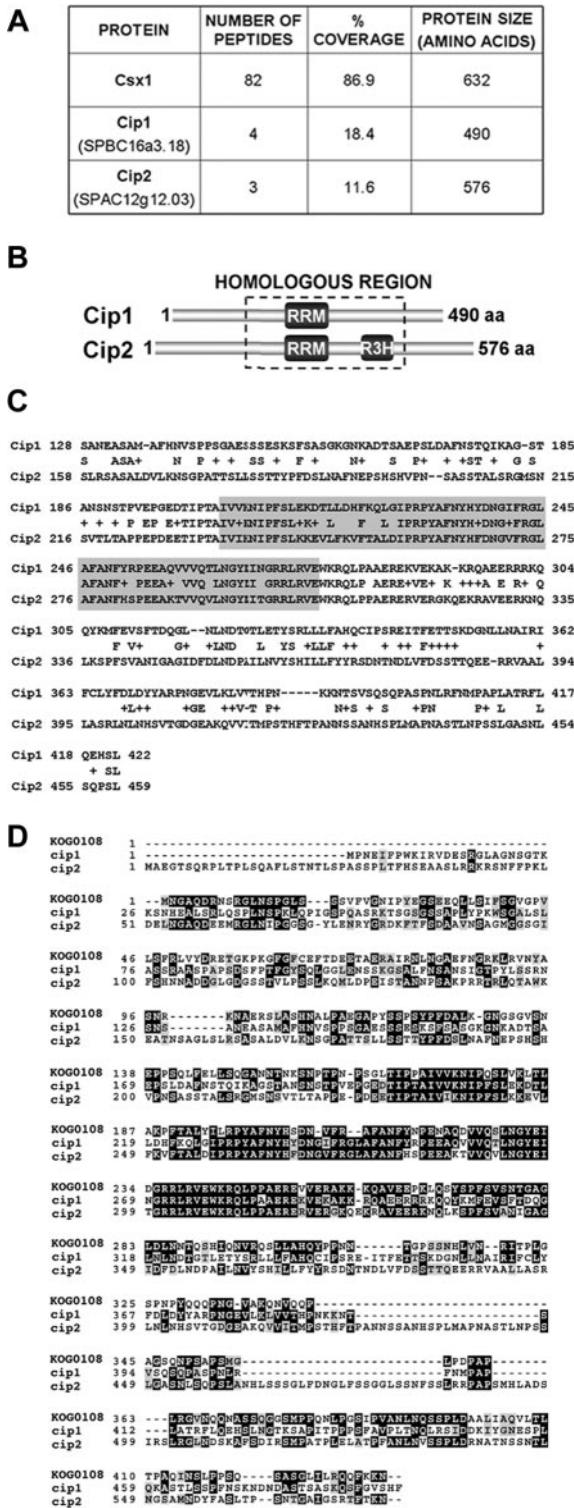


Figure 1. Identification of Cip1 and Cip2. (A) Number of independent peptides and percent of primary sequence coverage obtained by mass spectrometry for each of the indicated proteins. The TAP purification was performed after treating the cells for 15 min with 1 mM H₂O₂. (B) RNA-binding domain distribution in Cip1 (SPBC16A3.18) and Cip2 (SPAC12G12.03). The region with the highest sequence identity between the two proteins is boxed. (C) Alignment of the homologous regions of Cip1 and Cip2. Shaded residues represent the RRM. The percentage of homology is 58%, with 41% of the amino acid residues being identical in both proteins.

logue of the budding yeast cleavage and polyadenylation factor I complex identified in our TAP purification was the poly (A) binding protein Pabp, with six peptides covering 15% of its sequence.

Phenotypes of cip1Δ and cip2Δ Cells

Csx1 is a cytoplasmic protein (Rodriguez-Gabriel *et al.*, 2003). We predicted that to be able to interact with Csx1, Cip1 and Cip2 should also localize to the cytoplasm. To determine the localization of Cip1 and Cip2, we tagged each protein at its genomic locus with a C-terminal epitope, creating the fusion proteins Cip1-CFP and Cip2-GFP. Both fusion proteins were expressed from their endogenous promoters. Consistent with a possible role in the control of mRNA stabilization or degradation, both proteins were predominantly cytoplasmic at all stages of the cell cycle, and in the presence or absence of H₂O₂ (Figure 2A).

To gain insight into the function of Cip1 and Cip2, we replaced the entire open reading frame of both genes with the kanMx6 cassette (Bahler *et al.*, 1998). Precise replacement of the chromosomal copies of *cip1*⁺ and *cip2*⁺ by the kanamycin marker was confirmed by Southern blot and PCR analyses (our unpublished data). *cip1Δ* and *cip2Δ* cells were viable and grew at the same rate as wild-type cells. Microscopic analysis showed no significant difference between wild-type cells and *cip1Δ* (Figure 2B). In contrast, the morphology of *cip2Δ* cells was noticeably different from wild type, with many of the *cip2Δ* cells seeming to be swollen and shorter (Figure 2B). This phenotype was even more profound in *cip1Δ cip2Δ* double mutants. Conversely, overexpression of Cip2, by using the *nmf1* promoter, caused an elongation of the cells (Figure 2C). Although deletion of *cip2*⁺ did not alter the cells' sensitivity to H₂O₂, overexpression of *cip2*⁺ led to a slightly increased H₂O₂ sensitivity. As expected, *csx1Δ*, *atf1Δ* and *spc1Δ* mutants were more susceptible to H₂O₂ (Figure 2C).

cip1Δ cip2Δ double mutant strains grew slower than wild-type cells (Figure 3A) and showed an enhanced version of the *cip2Δ* morphology phenotype (Figure 2B). The sensitivity of *cip1Δ cip2Δ* strains to oxidative stress, osmotic stress, UV light, γ irradiation, and hydroxyurea was comparable to the single mutants and to wild-type cells (Figure 3A; our unpublished data).

Cip1 and Cip2 Participate in the Response to Oxidative Stress

Cells deficient in Csx1 are sensitive to oxidative stress (Rodriguez-Gabriel *et al.*, 2003; Figure 2C). We investigated whether the absence of Cip1 and/or Cip2 in a *csx1Δ* background changed sensitivity to oxidative stress. As expected, strains lacking Csx1 were incapable of growing in the presence of H₂O₂. However, *cip1Δ csx1Δ* and *cip2Δ csx1Δ* strains were less sensitive to oxidative stress treatments, both chronic and acute, compared with *csx1Δ* strains (Figure 3, A and B). This effect was specific for oxidative stress, because it was not possible to detect any other difference between the double mutants and the single mutant *csx1Δ* in response to other forms of stress (our unpublished data). The rescue of the H₂O₂ sensitivity of *csx1Δ* mutants by elimination of Cip1 or Cip2 was stronger at higher H₂O₂ concentrations (Figure 3A). The differences in the

(D) Sequence comparison between Cip1 and Cip2 and the eukaryotic KOG0108 domain, corresponding to the mRNA cleavage and polyadenylation factor I complex, subunit 15. Each COG includes proteins that are inferred to be orthologues and represents an ancient conserved domain.

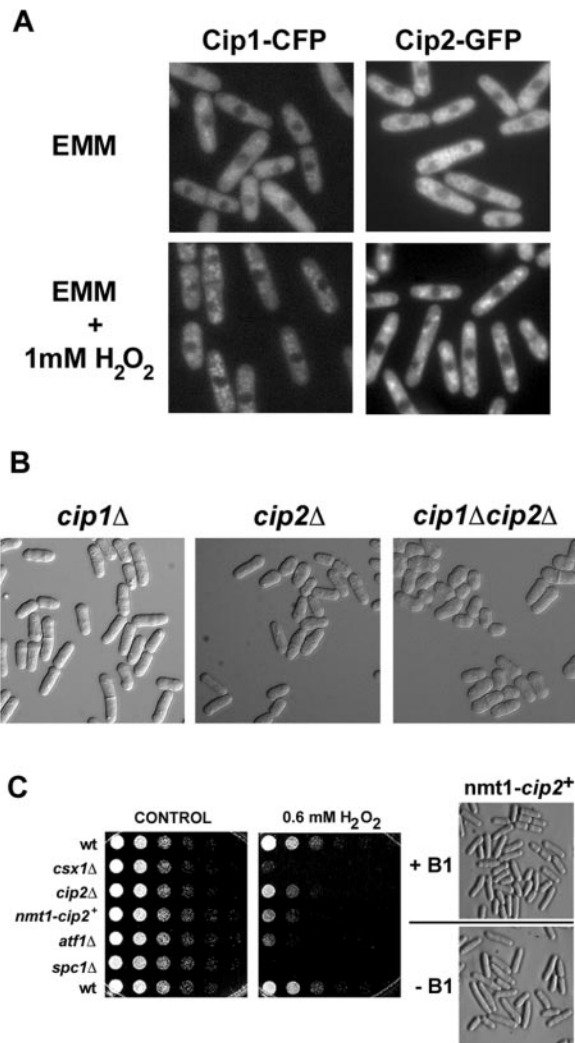


Figure 2. Phenotype of *cip1Δ* and *cip2Δ* mutant strains. (A) Strains carrying Cip1 or Cip2 tagged at their genomic loci with CFP or GFP, respectively, were used to determine the localization of each protein. Cip1 and Cip2 show cytoplasmic localization in cells growing in minimal medium (EMM), without (top) or during treatment with 1 mM H₂O₂ for 15 min (bottom). (B) *cip1Δ*, *cip2Δ* and *cip1Δcip2Δ* strains were grown to mid-log phase at 30°C and analyzed under the microscope. (C) Left, spot assays of wild-type and mutant strains. Fourfold serial dilutions were plated on EMM plates (CONTROL) or EMM plates supplemented with 0.6 mM H₂O₂. No thiamine was added to the media to induce overexpression of Cip2. Pictures were taken after incubating the plates for 5 d at 30°C. Right, morphology of Cip2-overexpressing cells. Wild-type cells containing the endogenous copy of *cip2⁺* under the control of the *nmt1* promoter were grown in liquid EMM media in the absence (-B1) or presence (+B1) of thiamine. Pictures were taken after growing cells to mid-log phase for 21 h in the indicated media at 30°C.

subset of genes and transcription factors that are activated in response to low and high H₂O₂ levels could explain the variation of the sensitivity of *cip1Δ csx1Δ* and *cip2Δ csx1Δ* strains to different concentrations of H₂O₂.

Triple deletions *cip1Δ cip2Δ csx1Δ* were also generated. The simultaneous absence of Cip1 and Cip2 caused a rescue level of *csx1Δ* H₂O₂ sensitivity identical to the level obtained by the elimination of any of the proteins independently (our unpublished data). The partial rescue of the H₂O₂ sensitivity of *csx1Δ* strains points to a specific role of Cip1 and Cip2 in

tolerance to oxidative stress in *S. pombe* and is consistent with the idea that Csx1, Cip1, and Cip2 have related functions in the response to oxidative stress.

The sensitivity of *atf1Δ* cells to H₂O₂ was not much improved by deletion of either Cip1 or Cip2 (Figure 3C), implying that Cip1 and Cip2 required Atf1 for their function. However, Cip1 and Cip2 must also have targets other than Atf1, because these *cip1Δ atf1Δ* and *cip2Δ atf1Δ* double mutants were slightly less sensitive to H₂O₂ compared with *atf1Δ* single mutant cells.

Whole genome expression profiling has shown that the abundance of *cip1⁺* mRNA increases by about twofold in response to H₂O₂ treatment, whereas the amount of *cip2⁺* mRNA is unaffected (Chen *et al.*, 2003). We monitored protein abundance by immunoblotting with an anti-FLAG antibody. Immunoblot analyses detected multiple electrophoretic mobility species of Cip1 and Cip2, but no significant increase in protein levels after treatment with H₂O₂ (Figure 3, D and E). It is possible that increased expression of *cip1⁺* mRNA during oxidative stress is required to compensate for reduced mRNA translation or accelerated turnover of Cip1 protein. Interestingly, oxidative stress caused Cip1 and Cip2 proteins to have reduced electrophoretic mobility (Figure 3D), indicating that Cip1 and Cip2 might become phosphorylated in the presence of H₂O₂. We therefore treated a Cip2 immunoprecipitate with λ phosphatase and analyzed the samples using SDS-PAGE conditions that accentuated changes in the electrophoretic mobility of proteins. This analysis showed that the Cip2 mobility shift induced by oxidative stress was caused by phosphorylation (Figure 3E). It has been previously shown that phosphorylation of Csx1 depends on the Spc1 MAPK (Rodriguez-Gabriel *et al.*, 2003). In a similar way, deletion of *spc1⁺* abolished the change in Cip1 and Cip2 electrophoretic mobility (Figure 3F). In contrast to Cip2, Cip1 phosphorylation was also dependent on Csx1 (Figure 3F), suggesting that Cip1 and Cip2 might be regulated differently during the oxidative stress response. Based on these results, Spc1 seems to be controlling directly or indirectly the phosphorylation status of these three RRM-containing proteins (Csx1, Cip1, and Cip2). Further studies will be required to determine the functional significance of this phosphorylation.

Role of Cip1 in the Global Transcriptional Response to Oxidative Stress

The fact that *cip1Δ csx1Δ* and *cip2Δ csx1Δ* mutants were more resistant to H₂O₂ treatment than the *csx1Δ* mutant suggested that Cip1 and Cip2 might participate in the control of gene expression during oxidative stress. Therefore, we decided to analyze whether Cip1 modulated the transcriptional response to oxidative stress. We focused our studies on Cip1 because the *cip1Δ* mutation suppressed the oxidative stress phenotype of *csx1Δ* cells without altering cell morphology (see above).

We isolated total RNA from wild-type, *csx1Δ*, *cip1Δ*, and *csx1Δ cip1Δ* strains without stress or 15 or 60 min after treatment with 1 mM H₂O₂. *atf1⁺* mRNA accumulated after H₂O₂ treatment in wild-type and *cip1Δ* cells (Figure 4A), consistent with the observation that *cip1Δ* mutants were not sensitive to oxidative stress (Figure 3, A and B). The H₂O₂-sensitive *csx1Δ* cells, however, failed to accumulate *atf1⁺* mRNA after oxidative stress (Figure 4A; Rodriguez-Gabriel *et al.*, 2003). Interestingly, *csx1Δ cip1Δ* double mutants were able to induce expression of *atf1⁺* during the oxidative stress response, although the increase in *atf1⁺* mRNA levels was not as strong as the one detected in wild-type or *cip1Δ* cells (Figure 4A). This latter finding correlates with the H₂O₂ sensitivity of *csx1Δ cip1Δ* cells, which was intermediate between the one of wild-type and *csx1Δ* cells (Figure 3, A and B).

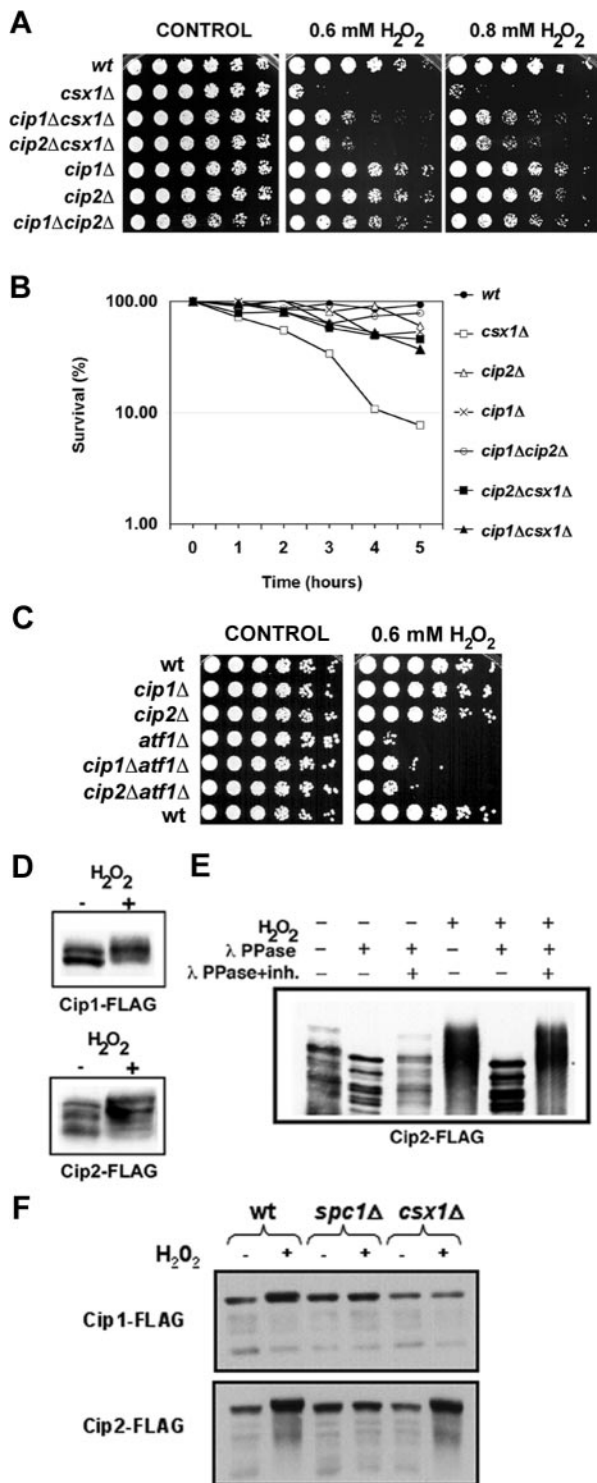


Figure 3. Cip1 and Cip2 participate in the oxidative stress response. (A) Serial dilutions of wild-type and mutant strains were plated in rich YES (yeast extract, glucose, and supplements) medium (left) or YES media with 0.6 mM H₂O₂ (middle) or 0.8 mM H₂O₂ (right). Photographs were taken after incubating the plates for 4 d at 30°C. (B) Survival of wild-type and mutant strains after H₂O₂ treatment. Liquid cultures were incubated in the presence of 1 mM H₂O₂ for 0–5 h, and cells were then plated on YES plates. Colonies were counted after 4 d of incubation at 30°C. (C) Fourfold serial dilutions of wild-type and the indicated mutant strains were plated on YES plates (left) or YES plates supplemented with 0.6 mM H₂O₂ (right). Pictures were taken after incubating the plates for 4 d at

To analyze the global effect of Cip1 in the expression profile of the fission yeast genome, RNAs isolated from wild-type, *csx1Δ*, *cip1Δ*, and *csx1Δ cip1Δ* strains without treatment or 15 and 60 min after treatment with 1 mM H₂O₂ were labeled during reverse transcription. The resulting cDNA was hybridized onto DNA microarrays containing probes of all known and predicted fission yeast genes. After eliminating the genes that failed to give measurable data in all the samples, we were able to monitor the expression of ~3500 genes. We first compared the total number of genes whose expression was induced two- or fivefold in wild-type, *cip1Δ*, *cip1Δ csx1Δ*, and *csx1Δ* cells, 15 or 60 min after treatment with 1 mM H₂O₂. This analysis showed that the global pattern of induction of gene expression in wild-type and *cip1Δ* strains was very similar (Figure 4B). The magnitude of the transcriptional response to H₂O₂ was considerably weaker in *cip1Δ csx1Δ* and more so in *csx1Δ* mutants (Figure 4B).

We studied the expression of the 477 genes that were induced twofold or more in at least one time point in the wild-type strain. In *cip1Δ* mutants, 406 of these genes were induced as well, indicating that the oxidative stress response in *cip1Δ* and wild-type cells followed similar patterns. Interestingly, 362 of the genes induced in wild-type cells were not induced in *csx1Δ* mutants compared with 155 genes in *cip1Δ csx1Δ* double deficient mutants (Figure 4C). From these results, we conclude that elimination of Cip1 in a *csx1Δ* background partially restores the defect in gene expression induced by H₂O₂. The fact that 90 (43.5%) of the 207 genes whose induction is partially restored in *cip1Δ csx1Δ* mutants are Atf1-independent indicates that part of the effect of Cip1 in the oxidative stress response does not require Atf1.

We further evaluated whether the level of induction of the 207 genes “rescued” in *cip1Δ csx1Δ* mutants was similar to the one observed in wild-type cells. In at least one of the time points, 85% of these genes were induced stronger in wild-type cells than in *cip1Δ csx1Δ* cells (Figure 4D). This defective induction together with the lack of induction in the remaining 155 genes (Figure 4C) could explain why *cip1Δ csx1Δ* mutants show higher H₂O₂ sensitivity than wild-type cells. Consistent with this idea we found that just 59% of the genes belonging to this group showed reduced induction upon H₂O₂ treatment in *cip1Δ* mutants compared with wild-type cells (Figure 4E).

Similar comparisons were performed for the 110 genes that were induced after oxidative stress in all the strains, i.e., wild type, *cip1Δ*, *csx1Δ*, and *cip1Δ csx1Δ*. Almost all of them (>97%) were expressed less in *csx1Δ* mutants compared with wild-type cells (Figure 5A). The differences were much smaller when the comparison was done between *cip1Δ csx1Δ* mutants or *cip1Δ* mutants and wild-type cells (Figure 5, B and C). These results indicate that the *cip1Δ* mutation reverses some of the expression defects observed in *csx1Δ* cells

30°C. (D) Cip1 and Cip2 Western blots. Cip1-FLAG and Cip2-FLAG cultures were treated with 1 mM H₂O₂, and cells were collected after 60 min. The same amount (50 μg) of whole cell extract was loaded in each lane. Proteins were detected using anti-FLAG monoclonal antibodies. (E) Wild-type Cip2-FLAG cultures were treated with 1 mM H₂O₂ for 60 min, and whole cell extracts were prepared. After immunoprecipitation with anti-FLAG agarose, the pull-down was treated with λ phosphatase. Cip2-FLAG protein was detected by Western blotting with anti-FLAG monoclonal antibodies. (F) Cip1 and Cip2 Western blots. Wild-type, *spc1Δ*, and *csx1Δ* strains expressing Cip1-FLAG or Cip2-FLAG were treated with 1 mM H₂O₂, and cells were collected after 60 min. The same amount (50 μg) of whole cell extract was loaded in each lane. Proteins were detected using anti-FLAG monoclonal antibodies.

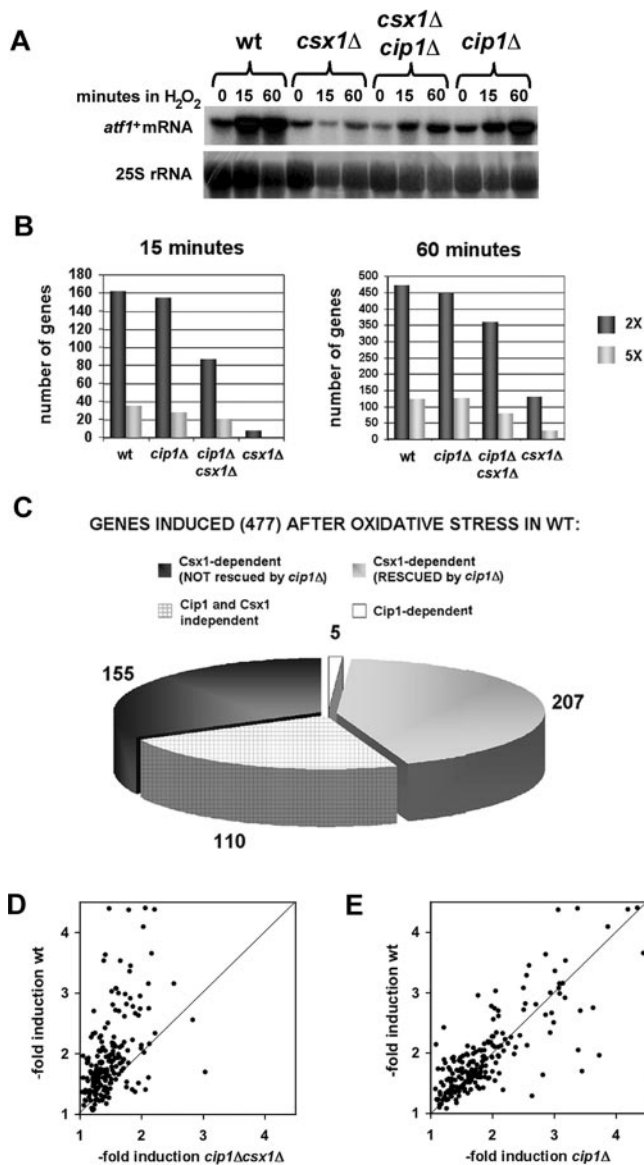


Figure 4. Influence of Cip1 on global gene expression after oxidative stress. (A) Northern blot of *atf1⁺* mRNA before (time 0) and after treatment of wild-type, *csx1Δ*, *cip1Δ*, and *cip1Δ csx1Δ* cells with 1 mM H₂O₂ for 15 or 60 min. 25S rRNA is shown as loading control. (B) Comparison of the total number of genes with two- or fivefold induction in wild-type, *cip1Δ*, *cip1Δ csx1Δ*, or *csx1Δ* cells 15 and 60 min after treatment with 1 mM H₂O₂. (C) Role of Cip1 and Csx1 in controlling the expression of genes induced at least twofold at one or both time-points (15 and/or 60 min) in wild-type cells. (D and E) Scatter chart analyses of the induction levels of the genes shown in Figure 4B as Csx1-dependent (RESCUED by *cip1Δ*). Comparisons were made between wild-type cells and *cip1Δ csx1Δ* mutants (D) or wild-type and *cip1Δ* cells (E).

after H₂O₂ treatment. Collectively, we conclude that Cip1 and Csx1 have counteractive roles in controlling gene expression in response to oxidative stress.

DISCUSSION

In this report, we have analyzed the roles of two novel proteins, Cip1 and Cip2, in the cellular response to oxidative

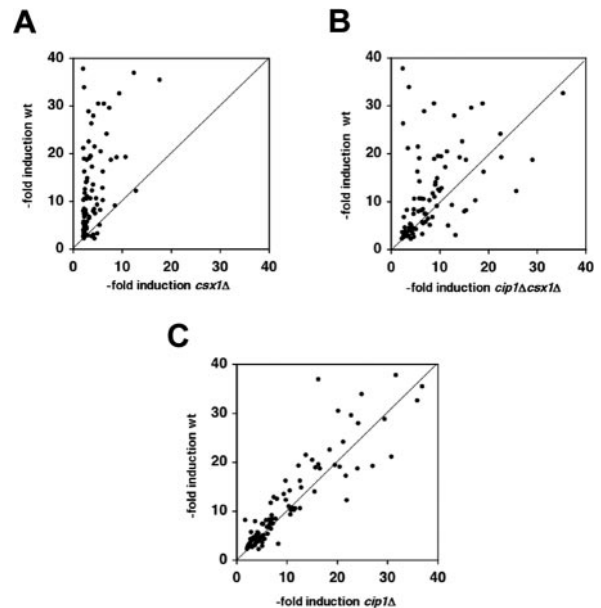


Figure 5. Importance of Cip1 in the control of the expression of oxidative-stress induced genes. Scatter chart analyses of the induction levels of the subset of genes (110) whose expression increased at least twofold (at 15 and/or 60 min) after oxidative stress wild-type, *cip1Δ*, *cip1Δ csx1Δ*, or *csx1Δ* cells. (A) Comparison of the induction levels between wild-type and *csx1Δ* strains. (B) Comparison of the induction levels between wild-type cells and *cip1Δ csx1Δ* double mutants. (C) Comparison of the levels of induction between wild-type and *cip1Δ* cells.

stress in *S. pombe*. Cip1 and Cip2 were discovered by MudPIT analysis of TAP-tagged Csx1. The physiological significance of this interaction is indicated by the analysis of *cip1⁺* and *cip2⁺* deletions. Notably, these mutations ameliorate the oxidative stress phenotype of *csx1Δ* cells, a specific and striking genetic interaction that could not have been predicted beforehand. Furthermore, analysis of global patterns of gene expression has shown *cip1Δ* to partially correct the defect in oxidative-stress induced gene expression in *csx1Δ* cells. This effect potentially explains the suppression of *csx1Δ* by *cip1Δ* and *cip2Δ* mutations.

Involvement of Cip1 and Cip2 in the Response to Oxidative Stress

We have identified Cip1 and Cip2 as two proteins that coprecipitate with Csx1-TAP analyzed by MudPIT. The detection of these related proteins is highly specific for Csx1-TAP because they have not been identified in >30 other TAP purifications done in our laboratory; however, it should be noted that they do not coprecipitate when analyzed by conventional immunoprecipitation analysis. This finding suggests that Csx1, Cip1, and Cip2 do not form a stable complex, and their interaction might be bridged through their association with mRNA.

Although *cip1Δ* and *cip2Δ* single and double mutants are not abnormally sensitive to H₂O₂, both mutations are able to partially rescue the oxidative stress-sensitive phenotype of the *csx1Δ* strain. Genetic analysis revealed that Csx1 functions in the pathway that connects Spc1 with Atf1 (Rodriguez-Gabriel *et al.*, 2003). We tested whether the sensitivity of *spc1Δ* mutants to different treatments, including H₂O₂ treatment, was rescued by independent or simultaneous deletion of *cip1⁺* and/or *cip2⁺*; however, we could not observe any rescue (our unpub-

lished data). In addition, *cip1Δ* and *cip2Δ* mutants were not sensitive to osmotic stress, UV light, γ irradiation, or hydroxyurea treatment, although they show a slight sensitivity to arsenite (our unpublished data), which has been related to the production of ROS (Harris and Shi, 2003). Thus, the function of these two putative RNA-binding proteins in survival to stress is only obvious in *csx1Δ* mutants treated with H₂O₂.

Interestingly, Cip1 and Cip2 seem to become heavily phosphorylated in response to oxidative stress but not in response to osmotic stress (our unpublished data). This phosphorylation is dependent on Spc1 MAPK (Figure 3F). The phosphorylation state of many RNA-binding proteins determines the fate of their associated transcripts and is controlled by MAPKs. For example, phosphorylation of the mRNA-destabilizing protein TPP increases the half-life of its target mRNA, whereas phosphorylation of the RNA stabilizing protein HuR helps to prevent degradation of interleukin-3 mRNA (reviewed in Bevilacqua *et al.*, 2003). In fission yeast, phosphorylation of the RNA-binding proteins Rnc1 and Csx1 has been shown to be dependent on the Pmk1 and Spc1 MAPKs, respectively (Rodríguez-Gabriel *et al.*, 2003; Sugiura *et al.*, 2003). Similar regulation could also control Cip1 and Cip2 activities.

Consequences of the Absence of Cip1 and Csx1 in the Global Transcription Response to Oxidative Stress

In *S. pombe*, the levels of proteins required for survival under stress conditions are fine-tuned by posttranscriptional mechanisms. *csx1Δ* mutants were unable to induce expression of most of the genes necessary for tolerance to H₂O₂ (Figure 4B). *cip1Δ* cells, on the contrary, were very well able to elaborate a response almost identical to wild-type strains. Accordingly, *cip1Δ* mutants were as resistant as wild-type cells to H₂O₂, whereas *csx1Δ* mutants were highly sensitive to such treatment. Interestingly, the induction level of the genes activated in *csx1Δ* when treated with H₂O₂ was always lower than the level found for those same genes in wild-type, *cip1Δ*, and *cip1Δ csx1Δ* cells. Therefore, the sensitivity of *csx1Δ* mutants to oxidative stress could derive from the combination of two factors: absence of induction of crucial genes, such as the transcription factor *atf1*⁺, and reduced levels of induction of other genes, such as the phosphatase *pyp2*⁺.

Microarray analyses of the *cip1Δ csx1Δ* cells were consistent with Csx1 and Cip1 (and possibly also its homologue Cip2) participating in opposite processes in RNA metabolism after H₂O₂ stress. The absence of Cip1 restored the induction of more than half of the genes, which were not induced in *csx1*⁺ null mutants, *atf1*⁺ among them. In many cases, however, the level of induction was still lower compared with wild-type cells, which could explain why the sensitivity to oxidative stress was only partially rescued in *cip1Δ csx1Δ* double mutants compared with wild-type cells. The genes with the most impaired induction in *csx1Δ* mutants (e.g., *pyp2*⁺) recover to greatest extent the normal induction levels when *cip1*⁺ is eliminated.

Possible Roles of Cip1 and Cip2 in the Control of mRNA Stability

RRMs occur in several proteins involved in all the steps of RNA processing: from nuclear events, such as transcriptional regulation, splicing, and 3' processing to nuclear export, localization in the cytoplasm, translation, and stability (reviewed in Dreyfuss *et al.*, 2002). The cytoplasmic localization of Cip1 and Cip2 and their interaction with Csx1 suggests that they participate in the control of mRNA stability. As mentioned, Cip1 and Cip2 share homology throughout

their sequences with Rna15, a protein that interacts with poly (A) sequences and one of the components of the multisubunit complex that forms the 3' ends of mRNAs in *S. cerevisiae* (Gross and Moore, 2001). In contrast, the human poly (A) ribonuclease DAN (Korner and Wahle, 1997; Korner *et al.*, 1998) and xPARN, a deadenylating nuclease from *Xenopus* (Copeland and Wormington, 2001), contain R3H domains in their sequences. Deadenylation is the first step of the major pathway of mRNA decay in yeast.

Recent studies indicate that in yeast and human cells, mRNA decay occurs in cytoplasmic processing bodies (Sheth and Parker, 2003; Cougot *et al.*, 2004). After oxidative stress, Cip1 and Cip2 localized to phase-dense cytoplasmic sites, which could link these proteins to the mRNA decay pathways. It is tempting to propose that oxidative stress could activate destabilizing factors or lead to mRNA degradation pathways, which are promoted by Cip1 and Cip2 and counteracted by Csx1. Alternatively, oxidants could directly affect the mRNA stability and Csx1 binding would protect mRNAs from degradation. It is known that after treatments causing stress, mammalian mRNAs are dynamically sorted into so-called "stress granules" in which RNA-binding proteins such as TIA-1, TIAR, and HuR control mRNA translation and stability (Kedersha and Anderson, 2002; Stoecklin *et al.*, 2004). Perhaps the function of Cip1 and Cip2 is related to the assembly of these structures or the regulation of the transition of mRNAs between the different cytoplasmic compartments after oxidative stress.

This work contributes to understanding the detailed mechanism of the oxidative stress response in eukaryotic cells. However, more studies are needed to address the question of how general this type of regulation is and which specific roles RNA-binding proteins play during the post-transcriptional control of gene expression after cell stress.

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