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Upf1, an RNA Helicase Required for Nonsense-Mediated mRNA Decay, Modulates the Transcriptional Response to Oxidative Stress in Fission Yeast

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In the fission yeast *Schizosaccharomyces pombe*, oxidative stress triggers the activation of the Spc1/Sty1 mitogen-activated protein kinase, which in turn phosphorylates the Atf1/Pcr1 heterodimeric transcription factor to effect global changes in the patterns of gene expression. This transcriptional response is also controlled by Csx1, an RNA-binding protein that directly associates with and stabilizes *atf1*$^+$ mRNA. Here we report the surprising observation that this response also requires Upf1, a component of the nonsense-mediated mRNA decay (NMD) system. Accordingly, *upf1*Δ and *csx1*Δ strains are similarly sensitive to oxidative stress, and the effects of the mutations are not additive, suggesting that Upf1 and Csx1 work in the same pathway to stabilize *atf1*$^+$ mRNA during oxidative stress. Consistent with these observations, whole-genome expression profiling studies have shown that Upf1 controls the expression of more than 100 genes that are transcriptionally induced in response to oxidative stress, the large majority of which are also controlled by Atf1 and Csx1. The unexpected connection between an NMD factor and the oxidative stress response in fission yeast may provide important new clues about the physiological function of NMD in other species.

Production of reactive oxygen species (ROS), such as hydroxyl radicals (OH^-), superoxide anions (O_2^-), and hydrogen peroxide (H_2O_2), is a normal by-product of aerobic metabolism in all eukaryotic organisms. Elevation of intracellular ROS can also arise through exposure to environmental toxicants, such as heavy metals/metalloids (e.g., arsenic) and some pesticides (10–12). Oxidative stress in the form of ROS can be highly toxic, causing damage to proteins, lipids, and nucleic acids. Indeed, the cumulative effects of exposure to ROS are thought to be a causative factor in many of the most widespread and debilitating human diseases, such as atherosclerosis, Alzheimer's and Parkinson's disease, and cancer, as well as the aging process itself (5, 19, 24, 35). As a consequence, all eukaryotic organisms have multiple cellular mechanisms to prevent the excessive accumulation of ROS and protect against their harmful effects. Antioxidant defense mechanisms include those that involve nonenzymatic molecules, such as glutathione and several vitamins, as well as ROS scavenger enzymes, such as superoxide dismutase and catalase and glutathione peroxidase (10, 11).

Transcriptional control of gene expression plays a central role in the oxidative stress response of eukaryotic organisms. One of the most well-studied model systems for this process is the fission yeast *Schizosaccharomyces pombe*. In this organism, accumulation of ROS triggers the activation of a mitogen-activated protein (MAP) kinase cascade that leads to the activation of Spc1 (Sty1/Phh1) (9, 42). Spc1 is a homolog of mammalian p38 (14) and the Hog1 protein kinase of the budding yeast *Saccharomyces cerevisiae* (6). Like its counterpart in humans, activated Spc1 phosphorylates and regulates a bZIP heterodimeric transcription factor, known as Atf1/Pcr1 in fission yeast (37, 42). Spc1 also appears to indirectly regulate Pap1, a transcription factor that is specifically involved in the response to oxidative stress (39). Atf1/Pcr1 controls gene expression in response to a broad variety of insults that activate Spc1, such as oxidative stress, exposure to heavy metals, and osmotic stress. Pap1 is especially important in the response to low levels of hydrogen peroxide and several other ROS-inducing agents (30, 40).

The Spc1 pathway in fission yeast and the p38 pathway in mammals respond to diverse forms of stress by generating gene expression patterns that are tailored to each type of stress. For example, osmotic and oxidative stress responses are both transmitted by Spc1 and yet lead to different patterns of gene expression (7). How a single pathway achieves this specificity is unknown. We made recent progress on this question through the discovery of Csx1, an RNA-binding protein that plays an important role in the response to oxidative stress (32). We found that cells deficient in Csx1 are specifically sensitive to oxidative stress. Csx1 associates with *atf1*$^+$ mRNA and is required for stabilization of this transcript during oxidative stress. Accordingly, *csx1*Δ cells are sensitive to oxidative stress. In more-recent studies, we found that Csx1 can be found in a protein complex with Cip1 and Cip2, two proteins with predicted RNA recognition motifs (23). Intriguingly, *cip1*Δ and *cip2*Δ mutations were found to partially rescue the oxidative stress sensitivity of *csx1*Δ cells (23), suggesting that Cip1 and Cip2 participate in a mRNA degradation pathway that is counteracted by Csx1.

Nonsense-mediated mRNA decay (NMD) is an RNA surveillance system that degrades mRNAs with premature translation termination (nonsense) codons (22). This response is thought to be a quality control mechanism that protects cells

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from the potential dominant-negative effects of truncated proteins. Recent studies have suggested that NMD factors also promote efficient translation of normal mRNAs in mammalian cells (41), raising the possibility that NMD factors do not function only in RNA surveillance. Transcripts that have mutations that insert an upstream open reading frame (uORF) in the 5′ untranslated region are degraded through NMD. However, several naturally occurring uORF-containing transcripts in *S. cerevisiae* are resistant to NMD. These mRNAs have a stabilizer element that prevents rapid NMD by interacting with the RNA-binding protein Pub1 (34). Interestingly, Pub1 is one of the closest relatives of Csx1.

While the mechanistic details of NMD have been steadily unveiled, the physiological role of NMD has remained a mystery. NMD mutants in *S. cerevisiae* have a partial impairment in respiratory growth that is enhanced at low temperature, whereas *Caenorhabditis elegans* mutants have defects in the male bursa and the hermaphrodite vulva (8). In neither case have these phenotypes been attributed to defects in the degradation of specific transcripts. In contrast to the situations in budding yeast and worms, NMD factors are essential for cell viability in *Drosophila* and mouse cells (25, 31), although the reasons for cell death are unknown.

The connection between Pub1 and NMD in *S. cerevisiae* and the sequence similarity of Pub1 and Csx1 prompted us to investigate whether NMD might be involved in the turnover of *atf1*$^+$ mRNA in response to oxidative stress in fission yeast. Quite unexpectedly, we have found that NMD factors are required for survival of oxidative stress in *S. pombe*. This phenotype can be traced to an unanticipated defect in mounting a global transcriptional response to oxidative stress. These findings unveil a new facet of NMD that may be relevant to understanding its function in other species.

MATERIALS AND METHODS

Yeast methods. The methods used for the handling of fission yeast have been described elsewhere (27). The disruption of *upf1*$^+$ (SPAC16C9.06c) and *upf2*$^+$ (SPAC19A8.08) was performed by substitution of the respective ORFs with the *kanMX6* gene (3). For plate survival assays, serial dilutions of yeast cultures were spotted in rich medium plates (yeast extract, glucose, and supplements [YES]) in the presence or absence of hydrogen peroxide. Plates were incubated for 2 or 3 days at 30°C, and pictures were taken. For survival to oxidative or osmotic stress in liquid, aliquots of each culture were treated with hydrogen peroxide or potassium chloride and plated in YES. For survival to UV irradiation, aliquots of each culture were plated in YES plates and irradiated. After incubation for 3 or 4 days at 30°C, colonies were counted, and relative survival was estimated by comparison with the untreated control. The strains used in these studies were PR109 (wild type), KS1366 (*spc1*::*URA4*) (36), MR3213 (*csx1*::*kanMx6*) (32), MR3567 (*upf1*::*kanMx6*), MR3568 (*csx1*::*kanMx6 upf1*::*kanMx6*), MR3569 (*upf2*::*kanMx6*), MR3570 (*upf1*::*kanMx6 upf2*::*kanMx6*), PS2917 (*ade6-M26*), MR3571 (*ade6-M26 upf1*::*kanMx6*), MR3572 (*ade6-M26 csx1*::*kanMx6*), MR4022 (*upf1*::*HA*::*kanMx6*), MR4023 (*upf1*::*HA*::*kanMx6 ade6-M26*), VM3771 (*cip1*::*kanMx6*), VM3772 (*cip2*::*kanMx6*), MR4024 (*cip1*::*kanMx6 upf1*::*kanMx6*), and MR4025 (*cip2*::*kanMx6 upf1*::*kanMx6*). All strains were h$^-$ *leu1-32 ura4-D18*.

RNA methods. RNA was obtained following the protocol described at http://www.sanger.ac.uk/PostGenomics/S_pombe/. For Northern analysis, 10 μg of total RNA was loaded in denaturing formaldehyde-agarose gels. After electrophoretic separation, RNA was transferred to Hybond-H+ membranes (Amersham), and RNA loading was monitored by methylene blue staining. RNAs were detected using probes amplified by PCR that were labeled with [α-^{32}P]dCTP using Prime-It II kit (Stratagene).

Microarray design, labeling, hybridization, and data processing were performed as described before (21). Two independent biological repeats were performed from all samples with dye swap (12 arrays in total). The twofold cutoff was from the average of two repeat experiments. Microarray data files are available at http://www.sanger.ac.uk/PostGenomics/S_pombe/.

For quantification of mRNAs using real-time quantitative reverse transcription-PCR (RT-PCR), extracts from *S. pombe* cells were obtained. Part of the extract was immunoprecipitated with monoclonal antibodies directed against hemagglutinin (HA) (32). RNA from the precipitated material was obtained (QIAGEN), and equal amounts of material were used for reverse transcription with oligo(dT) (Promega). Quantitative PCR was done as described previously (13).

RESULTS

***S. pombe upf1*Δ cells are sensitive to oxidative stress.** To initiate investigations of a possible connection between NMD and the oxidative stress response in fission yeast, we first searched in silico for a gene that encodes Upf1, a conserved RNA helicase that is essential for NMD in all eukaryotic organisms examined thus far. The closest relative of Upf1 was encoded by the previously uncharacterized SPAC16C9.06c open reading frame (2). This ORF encodes a protein of 925 amino acids that shares 51% identity and 68% similarity with *S. cerevisiae* Upf1. Comparative sequence analysis revealed that SPAC16C9.06c, hereafter named Upf1, shares a high level of homology with Upf1 proteins from other organisms (Fig. 1A).

The complete open reading frame of *upf1*$^+$ was deleted and replaced by the *kanMx6* marker. This analysis revealed that Upf1 was not required for cell viability in fission yeast (Fig. 1B). This finding was consistent with reports that *S. cerevisiae upf1*Δ cells have no obvious growth defect (8). However, the fission yeast *upf1*Δ cells were observed to be unexpectedly sensitive to oxidative stress (Fig. 1B and C). In common with *spc1*Δ and *csx1*Δ cells, *upf1*Δ cells were unable to form colonies in agar medium containing 0.5 mM hydrogen peroxide, whereas wild-type cells were able to forms colonies in these conditions (Fig. 1B). The sensitivity of *upf1*Δ cells was specific to oxidative stress, as they were not at all sensitive to osmotic shock or UV irradiation (Fig. 1C). This phenotype is shared with *csx1*Δ cells (Fig. 1C) (32).

In view of the similar phenotypes of *upf1*Δ and *csx1*Δ mutants, we compared the hydrogen peroxide sensitivity of the *csx1*Δ *upf1*Δ double mutant to that of each of the single mutants (Fig. 1C). This analysis showed that each single mutant and the double mutant were equally sensitive to hydrogen peroxide. These findings indicated that Csx1 and Upf1 participate in the same pathway of resistance to oxidative stress.

Upf1 controls Atf1 mRNA expression. These findings prompted us to examine whether Upf1 has a role in controlling gene expression in response to oxidative stress. We first examined *ctt1*$^+$ (*cta1*$^+$), which encodes catalase, an enzyme that is required for the survival of oxidative stress (28). As observed previously (29), there was strong induction of *ctt1*$^+$ expression in response to oxidative stress in wild-type cells (Fig. 2A). This induction was substantially reduced in *upf1*Δ cells (Fig. 2A).

The abundance of *ctt1*$^+$ mRNA is regulated by the MAP kinase Spc1 and the transcription factors Atf1/Pcr1 and Pap1 (30). We have previously shown that the RNA-binding protein Csx1 controls *atf1*$^+$ mRNA decay (32), and here we show that Upf1 and Csx1 appear to work in the same pathway for resistance to oxidative stress (Fig. 1C). We hypothesized that *upf1*Δ cells might be defective in the accumulation of *atf1*$^+$ mRNA in response to hydrogen peroxide. To address that possibility, we measured the abundance of *atf1*$^+$ mRNA in wild-type and

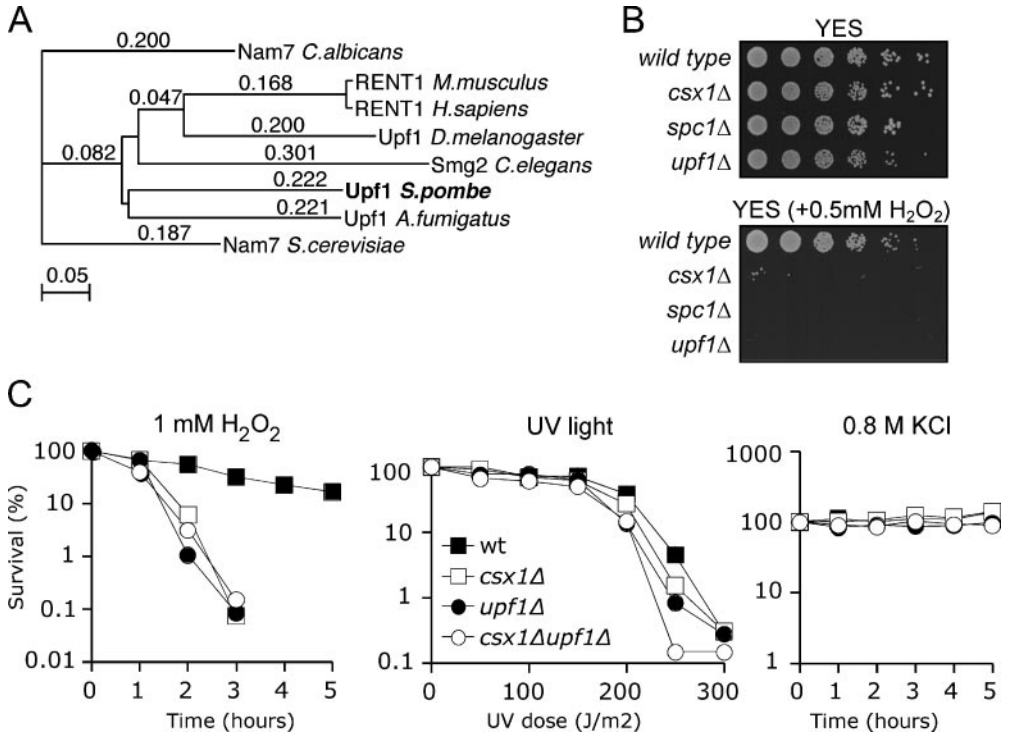

FIG. 1. Upf1 is essential for survival of oxidative stress. (A) Phylogenetic tree of the Upf1/Nam7 homologs. Sequences from *Candida albicans*, *Mus musculus*, *Homo sapiens*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Schizosaccharomyces pombe*, *Aspergillus fumigatus*, and *Saccharomyces cerevisiae* are shown on the tree. Branch lengths represent relative phylogenetic distances, as determined by Clustal X (17). (B) Fission yeast Upf1 is essential in response to oxidative stress. Serial dilutions (1/5) of the wild type and of *csx1*Δ, *spc1*Δ, and *upf1*Δ mutants were plated in rich medium (YES) in the absence or presence of 0.5 mM hydrogen peroxide. Plates were incubated at 30°C. (C) Genetic interaction of Upf1 with Csx1. The wild type (wt) and the indicated mutants were incubated in rich liquid medium in the presence of 1 mM hydrogen peroxide or 0.8 M KCl. At the indicated times, aliquots of the cell culture were plated on YES plates and incubated at 30°C. For the UV sensitivity assay, equal amounts of cells were plated on several YES plates. Each plate was irradiated with different doses of UV light. Each panel shows the average values from three experiments.

*upf1*Δ cells. In wild-type cells, we detected an approximately fourfold-higher expression of *atf1*$^+$ mRNA in response to oxidative stress, as shown previously (32). However, in *upf1*Δ cells, there was a smaller increase (approximately twofold) in *atf1*$^+$ mRNA in response to oxidative stress (Fig. 2B). This effect is very similar to that observed with *csx1*Δ cells (32) and is consistent with the genetic interaction involving *csx1*Δ and *upf1*Δ (Fig. 1).

To confirm and extend these analyses, we used real-time quantitative RT-PCR to measure *atf1*$^+$ mRNA abundance in wild-type, *upf1*Δ, and *csx1*Δ strains, and in an *upf1*Δ *csx1*Δ double mutant strain, during exposure to oxidative stress (1 mM H_2O_2). This analysis detected an approximately fivefold increase in *atf1*$^+$ mRNA abundance in the wild type at the 60-min time point, whereas the *upf1*Δ and *csx1*Δ strains showed only an approximately twofold increase (Fig. 2C). The behavior of the *upf1*Δ *csx1*Δ double mutant strain was not significantly different from the behavior of the *upf1*Δ and *csx1*Δ strains (Fig. 2C).

The similar phenotypes of the *upf1*Δ and *csx1*Δ single mutants and the *upf1*Δ *csx1*Δ double mutant strain suggested that Upf1 might control the half-life of *atf1*$^+$ mRNA, in a manner similar to that previously established for Csx1 (32). We tested this idea by treating wild-type and *upf1*Δ cells with hydrogen peroxide in the presence of the transcriptional inhibitor 1,10-phenanthroline. The rate of decay of *atf1*$^+$ mRNA under nonstressed conditions was identical in the wild type and *upf1*Δ mutants (Fig. 2D and E). However, treatment with hydrogen peroxide caused a dramatic reduction in *atf1*$^+$ mRNA half-life in *upf1*Δ cells (Fig. 2D and E). Csx1 has a similar effect on *atf1*$^+$ mRNA turnover rate (32). Together, these results indicate that Upf1, in coordination with Csx1, positively controls the stability of *atf1*$^+$ mRNA under oxidative stress conditions.

Upf1 is required for NMD in fission yeast. In view of the unexpected role of fission yeast Upf1 in the transcriptional response to oxidative stress, we sought to determine whether Upf1 is indeed required for NMD in fission yeast. We examined the transcript of the *ade6-M26* allele which carries a nonsense mutation (Fig. 3A) (26). First, we established that the abundance of *ade6*$^+$ mRNA after oxidative stress was independent of the activity of Upf1 or Csx1. After 60 min under oxidative stress, *ade6*$^+$ mRNA levels decreased to about 50% of the nontreated values, but this change was also observed in *upf1*Δ and *csx1*Δ mutants (Fig. 3B). In the *ade6-M26* mutant (*upf1*$^+$ *csx1*$^+$), we observed that full-length *ade6* mRNA was barely detectable, as expected of a mRNA that is degraded by the NMD machinery (26). The same reduction was observed in *csx1*Δ cells, indicating that Csx1 is not required in NMD. However, in *upf1*Δ cells, the amount of full-length *ade6-M26* mRNA was comparable to that of *ade6*$^+$ mRNA in the three

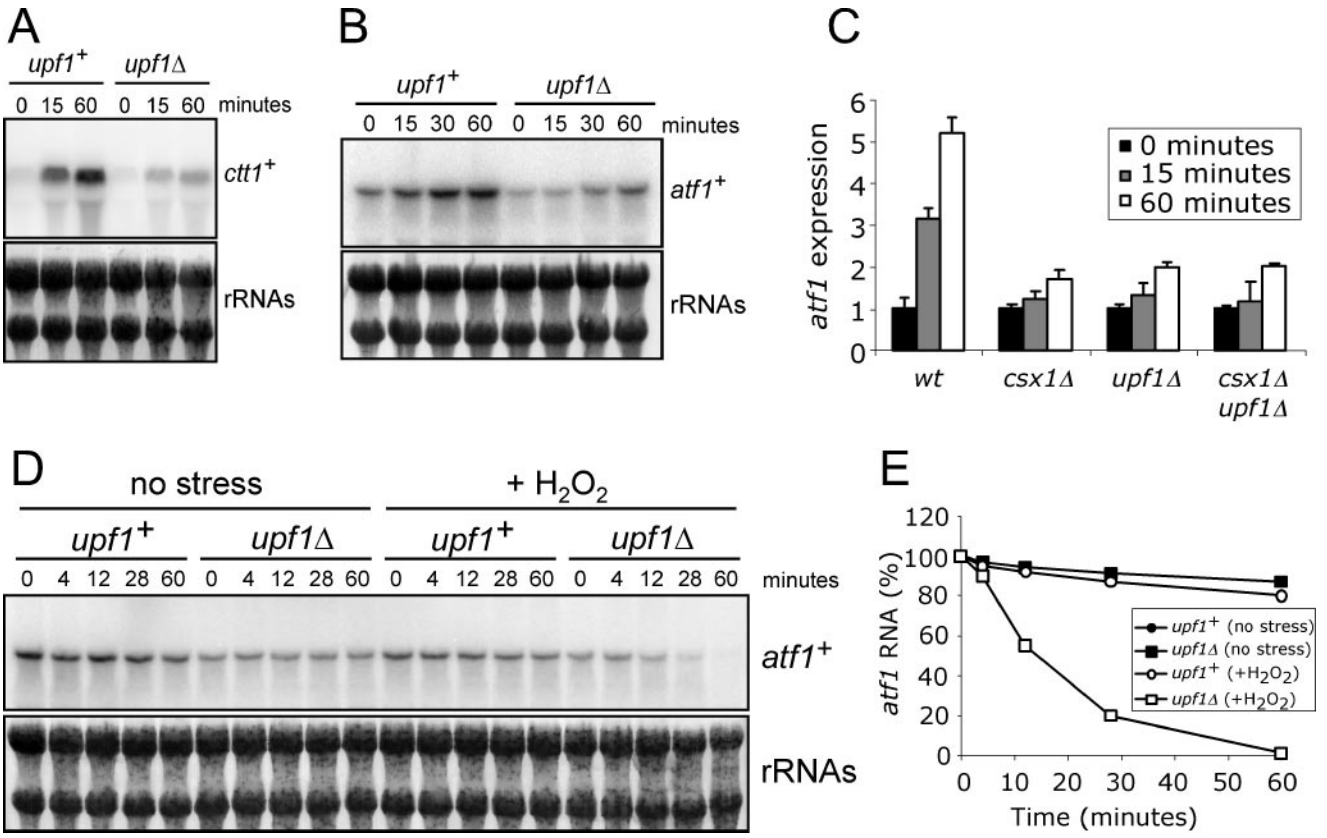

FIG. 2. Upf1 controls *ctt1*$^+$ and *atf1*$^+$ mRNAs in response to oxidative stress. (A) The wild type (*upf1*$^+$) and *upf1*Δ cells were treated with 1 mM hydrogen peroxide. At the indicated times, cells were collected, and total RNA was extracted. After electrophoretic separation and transfer to membranes, *ctt1*$^+$ mRNA was detected with a specific probe. (B) A similar experiment was performed to detect *atf1*$^+$ mRNA. (C) Quantitative real-time PCR analysis of *atf1*$^+$ mRNA in wild-type (wt), *csx1*Δ, *upf1*Δ, and *csx1*Δ *upf1*Δ cells exposed to 1 mM hydrogen peroxide. (D) Role of Upf1 on *atf1*$^+$ mRNA stability after oxidative stress. Wild-type (*upf1*$^+$) and *upf1*Δ cells were treated at time zero with 1,10-phenanthroline (300 μg/ml) in the absence of stress or in medium with 1 mM H_2O_2. Northern analysis was performed using a probe for *atf1*$^+$ mRNA. Methylene blue staining of the rRNAs is shown as a loading control. (E) Quantification of the data shown in panel D.

genetic backgrounds. These findings showed that Upf1 is required for degradation of the *ade6-M26* mRNA in *S. pombe*.

An interesting feature of the *ade6-M26* mutation is that it creates a CREB/ATF-type binding site (Fig. 3A). This binding site is used by Atf1/Pcr1 to promote expression of a short mRNA (16). This short *ade6-M26* mRNA was induced in the *ade6-M26 upf1*$^+$ *csx1*$^+$ cells that were exposed to hydrogen peroxide (Fig. 3B). Consistent with the role of Upf1 and Csx1 in stabilizing *atf1*$^+$ mRNA during oxidative stress, the short *ade6-M26* mRNA was not observed in *csx1*Δ or *upf1*Δ cells (Fig. 3B). These findings confirmed that Atf1 function is substantially impaired in *csx1*Δ and *upf1*Δ cells.

Upf1 does not associate with *atf1*$^+$ mRNA. Having established that Upf1 is required for stabilization of *atf1*$^+$ mRNA during oxidative stress, we undertook experiments to determine whether this mode of regulation involves a physical interaction between Upf1 and *atf1*$^+$ mRNA. RT-PCR was performed with mRNAs that coprecipitated with HA-tagged Upf1 expressed from the endogenous *upf1*$^+$ locus. These experiments were performed with *ade6*$^+$ and *ade6-M26* cells, with the *ade6-M26* mRNA serving as a positive control for mRNA that could be expected to coprecipitate with Upf1. Indeed, the amount of *ade6-M26* mRNA detected in the Upf1 precipitate was enriched approximately fourfold relative to the amount of *ade6*$^+$ mRNA (Fig. 3C). The relative enrichment of *ade6-M26* mRNA in the Upf1 precipitate from cells exposed to oxidative stress was even greater, approximately 10-fold in this experiment (Fig. 3C). As far as we know, this is the first report of the specific precipitation of Upf1 with a natural mRNA substrate. However, when we analyzed whether *atf1*$^+$ mRNA coprecipitated with Upf1, we could detect no enrichment of *atf1*$^+$ mRNA, either in the absence or presence of oxidative stress (Fig. 3C). The RT-PCR analysis of total RNA samples detected an approximately three- to fourfold increase in *atf1*$^+$ mRNA in cells exposed to oxidative stress. From these results, we conclude that the regulation of *atf1*$^+$ mRNA stability by Upf1 is unlikely to involve a direct interaction between Upf1 and *atf1*$^+$ mRNA.

Gene expression profiling in *upf1*Δ cells. Microarray analysis has indicated that about 5 to 10% of wild-type transcripts accumulate when the NMD pathway is inactivated in *S. cerevisiae* (15, 20), representing ~550 of the ORFs in the *S. cerevisiae* genome. It is uncertain how many of these transcripts are direct substrates of the NMD machinery. We decided to use whole-genome microarray analysis to better understand the role of Upf1 in controlling gene expression in *S. pombe*. Mi-

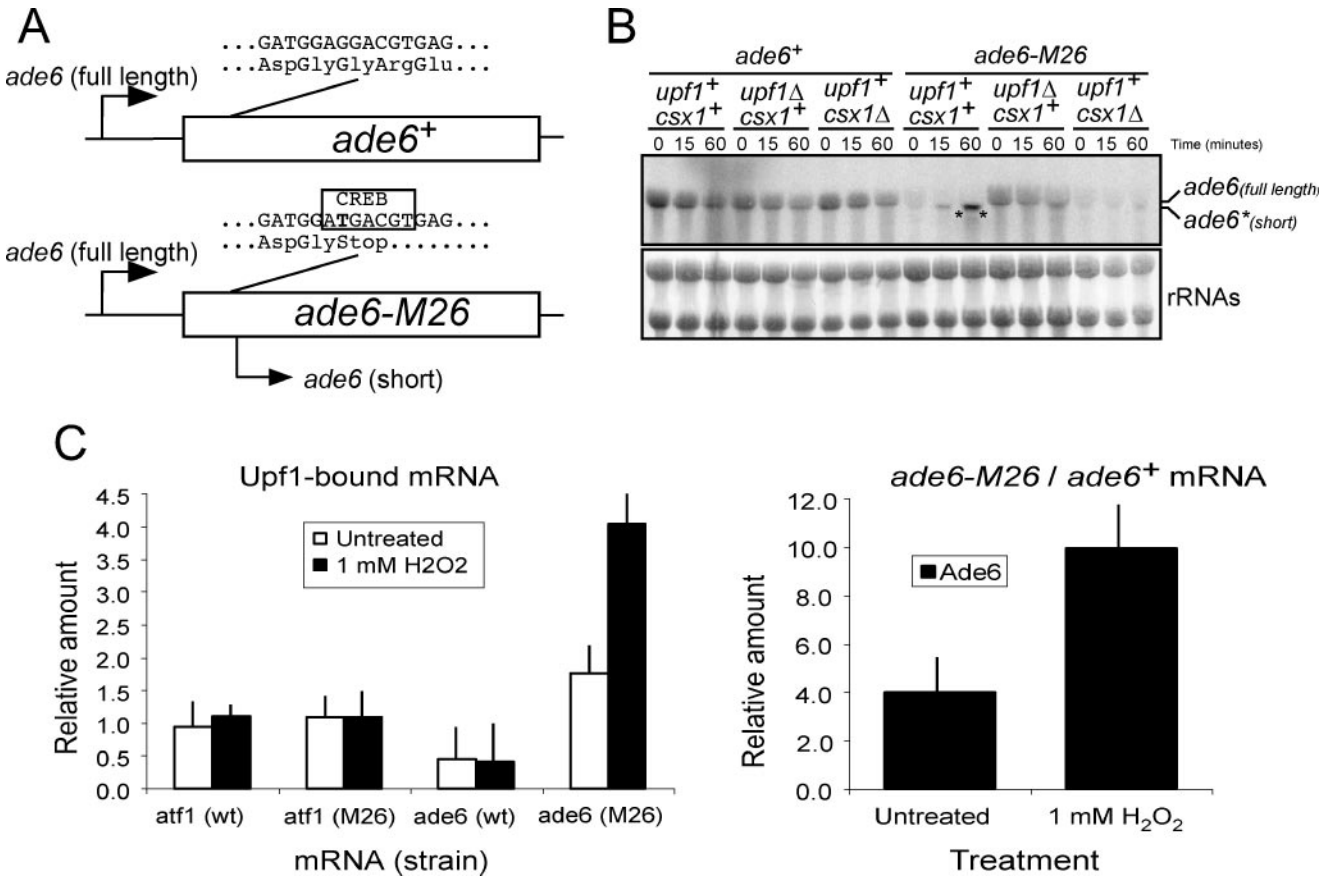

FIG. 3. Upf1 participates in NMD and associates with a PTC-containing mRNA but not *atf1*$^+$ mRNA. (A) Schematic representation of the *ade6*$^+$ and *ade6-M26* loci. Arrows indicate transcription initiation, and the box marks the Atf1/Pcr1 binding site. (B) Wild-type (*upf1*$^+$ *csx1*$^+$), *upf1*Δ (*upf1*Δ *csx1*$^+$) and *csx1*Δ (*upf1*$^+$ *csx1*Δ) strains (in *ade6*$^+$ or *ade6-M26* backgrounds) were treated with 1 mM hydrogen peroxide for the indicated times. An *ade6* probe was used to detect *ade6*$^+$ and *ade6-M26* mRNA. Methylene blue staining of the rRNAs is shown as a loading control. (C) Quantitation of RNA associated with Upf1 protein. Wild-type (wt) and Upf1::HA extracts (from *ade6*$^+$ or *ade6-M26* background) were immunoprecipitated with anti-HA monoclonal antibodies, and the associated Act1, Atf1, and Ade6 mRNAs were quantified. The left panel shows the relative amount of specific mRNA precipitated in the Upf1::HA strain compared with the untagged strain (normalized with Act1). The right panel shows the ratio between the amount of *ade6* mRNA bound to Upf1::HA in the *ade6-M26* strain versus the *ade6*$^+$ strain. The means and standard deviations from three experiments are shown.

croarray analysis of total RNA from *upf1*Δ cells showed that the expression of 27 genes was increased twofold or more relative to the wild type, while the expression of 16 genes was decreased to half or less in *upf1*Δ cells relative to the wild type (Table 1 and data not shown). The value of 27 genes up-regulated in *upf1*Δ cells is a much smaller fraction of the genome than measured for the *upf1*Δ mutant in *S. cerevisiae* (15). Of the 27 genes up-regulated in *upf1*Δ cells of fission yeast, 2 are pseudogenes and 14 have at least one ATG in the 80 nucleotides immediately upstream of the annotated translational ATG. It is likely, therefore, that the majority of the genes up-regulated in *upf1*Δ cells of fission yeast are direct targets of NMD.

We then used expression profiling to determine the effect of Upf1 on the expression of genes after treatment with oxidative stress. We obtained total RNA from wild-type and *upf1*Δ cells after 15 or 60 min of treatment with hydrogen peroxide. The microarray analysis of wild-type cells showed that the expression of 336 genes was increased at least twofold in one or both of the time points after exposure to oxidative stress. Of these 336 genes, 111 (33%) showed reduced expression (half or less of the wild type at one or both of the time points) in *upf1*Δ mutants. Included in this set of genes were *atf1*$^+$ and *pcr1*$^+$, as well as genes such as *gpx1*$^+$, which encodes a glutathione peroxidase that is predicted be important for survival of oxidative stress. Of those 111 Upf1-regulated genes, 98 (88%) showed reduced expression in *csx1*Δ mutants ($P < 0.003$), and 93 (84%) showed reduced expression in *atf1*Δ mutants ($P < 0.001$) (Fig. 4A and B). These results underscore the important functional relationship among Upf1, Csx1, and Atf1 in the regulation of the transcripts induced after oxidative stress.

Upf2 is also important for the response to oxidative stress. These findings indicated that Upf1 participates in a pathway with Csx1 that stabilizes *atf1*$^+$ mRNA during oxidative stress. To evaluate whether the role of Upf1 in the oxidative stress response is shared with other NMD factors in *S. pombe*, we examined whether *upf2*Δ mutants were sensitive to hydrogen peroxide. Upf2 is a known functional component of NMD in

TABLE 1. List of 27 genes whose expression is increased twofold or more in *upf1*Δ cells relative to the wild type[a]

Gene	Induction (fold)	Characteristic(s) or function
SPAC922.03[b]	8.3	1-Aminocyclopropane-1-carboxylate deaminase (predicted)
SPBC8E4.02c	3.7	Sequence orphan
SPAC27D7.11c	3.6	SPAC27D7.09c paralog; unknown function
SPAC56F8.14c	3.2	Sequence orphan
SPBC660.15	3.2	RNA-binding protein (predicted)
SPAC1039.02	3.0	SPBPB2B2.06c paralog; calcineurin-like phosphoesterase (predicted)
rex2	2.9	RNA exonuclease
SPAC23D3.12	2.8	Inorganic phosphate transporter (predicted)
SPCC18B5.02c	2.8	Pseudogene
spt4	2.7	Transcription elongation factor Spt4
SPAC25H1.03	2.7	Conserved hypothetical; unknown function
SPBP8B7.12c	2.6	Sequence orphan
SPBPB2B2.06c	2.6	SPAC1039.02 paralog; calcineurin-like phosphoesterase (predicted)
arg7	2.6	Argininosuccinate lyase
SPAC27D7.09c	2.4	SPAC27D7.11c paralog; unknown function
SPAC139.04c	2.3	Fructosyl amino acid oxidase (predicted)
SPCC663.07c	2.3	Pseudogene
SPBC1683.07	2.2	α-Glucosidase (predicted)
nic1	2.2	Heavy metal ion transporter
apn1	2.2	Apurinic endonuclease
thp1	2.2	Uracil DNA *N*-glycosylase activity
SPAC11D3.01c	2.1	Conserved hypothetical; unknown function
SPAC11G7.05c	2.1	S-malonyltransferase (predicted)
SPBC18E5.14c	2.1	Sequence orphan
SPCC1281.07c	2.1	Glutathione *S*-transferase (predicted)
mis12	2.0	Kinetochore protein
SPCC794.03	2.0	Amino acid permease family (predicted)

[a] The 16 genes whose expression was decreased to half or less in *upf1*Δ cells relative to wild type are SPAC1687.16c, SPBC21C3.19, SPBC3E7.06c, SPBC23G7.10c, SPBC887.17, *rpt6* (*let1*), SPAC922.04, *rds1*, SPBC8E4.01c, SPAC212.09c, SPAC750.08c, *mae2*, SPAPB24D3.07c, *abp2*, *hsp9*, and *upf1*.
[b] Genes that have at least one ATG in the 80 nucleotides immediately upstream of the annotated translational ATG are underlined.

fission yeast (26). We observed that the sensitivities of *upf1*Δ and *upf2*Δ mutants to oxidative stress were very similar and not additive (Fig. 4C), consistent with the tight functional relationship between both factors in NMD in other species. These results are consistent with a functional interaction between NMD factors and Csx1 in the control of the cellular response to oxidative stress in fission yeast.

The *cip1*Δ and *cip2*Δ mutations suppress the oxidative stress sensitivity of *upf1*Δ cells. Cip1 and Cip2 are two RNA recognition motif-containing proteins that associate with Csx1 (23). The physiological significance of this association was suggested by the ability of *cip1*Δ and *cip2*Δ mutations to suppress the oxidative stress sensitivity of *csx1*Δ cells. It appears that Cip1 and Cip2 participate in the pathway that degrade *atf1*$^+$ mRNA, and Csx1 counteracts this pathway (23). If Csx1 and Upf1 participate in the same mechanism that protects *atf1*$^+$ mRNA from degradation, we would anticipate that inactivation of Cip1 or Cip2 should also rescue the oxidative stress-sensitive phenotype of *upf1*Δ cells. Accordingly, we constructed *cip1*Δ *upf1*Δ and *cip2*Δ *upf1*Δ double mutant strains and compared their growth to the single mutants and the wild type on medium containing 1 mM hydrogen peroxide. This analysis revealed that compared to a *upf1*Δ strain, the *cip1*Δ *upf1*Δ and *cip2*Δ *upf1*Δ double mutant strains were substantially more resistant to the toxic effects of hydrogen peroxide (Fig. 5). These data support the conclusion that Upf1 activity in promoting survival of oxidative stress works to counteract the activities of Cip1 and Cip2.

DISCUSSION

The experiments undertaken in this study were motivated by the following observations. (i) Csx1 controls the transcriptional response to oxidative stress in fission yeast, in part through stabilization of *atf1*$^+$ mRNA (32). (ii) *S. pombe* Csx1 and *S. cerevisiae* Pub1 are structurally related proteins. (iii) Pub1 protects some transcripts from NMD (34). On the basis of these observations, we hypothesized that Csx1 might protect *atf1*$^+$ mRNA from NMD in fission yeast. A prediction of this model was that inactivation of NMD would stabilize *atf1*$^+$ mRNA in a *csx1*Δ mutant and suppress the sensitivity of these cells to oxidative stress. Here we have reported a number of findings that are contrary to this hypothesis and that instead suggest a role for NMD factors in promoting resistance to oxidative stress. The first finding, and perhaps the most striking, is that the *upf1*Δ mutation causes cells to be sensitive to oxidative stress (Fig. 1). This phenotype is observed for both prolonged and acute exposure to oxidative stress, that is, with cells grown for several days in agar medium containing H_2O_2 (Fig. 1B) or with cells grown for several hours in liquid medium in the presence of H_2O_2 (Fig. 1C). To our knowledge, this is the first study showing that an NMD protein is required for survival of oxidative stress; indeed, it may be one of the most striking phenotypes reported for an NMD mutant in any organism.

The unprecedented nature of this phenotype raises a number of questions. One question is whether we have correctly identified the SPAC16C9.06c open reading frame as encoding the actual Upf1 homolog in fission yeast. A number of facts

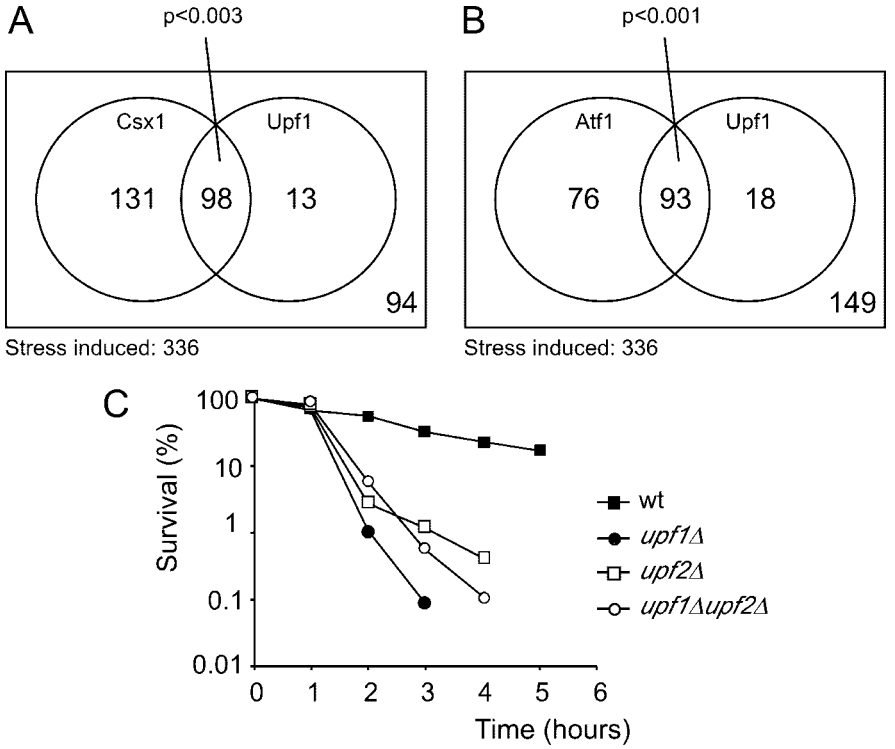

FIG. 4. Upf1 regulates global gene expression in response to oxidative stress. (A) Venn diagram representing genes induced at least twofold in response to 1 mM hydrogen peroxide in a Csx1- or Upf1-dependent manner. Dependency was determined when expression in the mutant was reduced at least 50% relative to the wild type. (B) Venn diagram representing genes induced at least twofold in response to 1 mM hydrogen peroxide in a Csx1- or Atf1-dependent manner. (C) The wild type (wt) and the indicated mutants were incubated in rich liquid medium in the presence of 1 mM hydrogen peroxide. At the indicated times, aliquots of the cell culture were plated on YES plates and incubated at 30°C. Each point represents an average from three experiments.

suggest that we have. The 925-amino-acid protein encoded by SPAC16C9.06c is similar in size to Upf1 protein in *S. cerevisiae* (971 amino acids) and humans (1,118 amino acids), and it is extensively conserved along almost its entire length to *S. cerevisiae* Upf1 (51% identity and 68% similarity) and human Upf1 (59% identity and 75% similarity). These amounts of sequence identity and similarity are typical among Upf1 homologs. The closer sequence relationship to the human homolog is statistically significant and is reflected in the phylogenetic tree of Upf1 homologs (Fig. 1A). After SPAC16C9.06c, there is a group of four predicted DNA or RNA helicases in fission yeast (SPCC737.07c, Sen1, SPBC29A10.10c, and Dna2) that display only ~30% identity and ~50% similarity over ~550 amino acids to *S. cerevisiae* Upf1. Therefore, sequence homology considerations strongly support the conclusion that SPAC16C9.06c encodes the true Upf1 homolog in fission yeast.

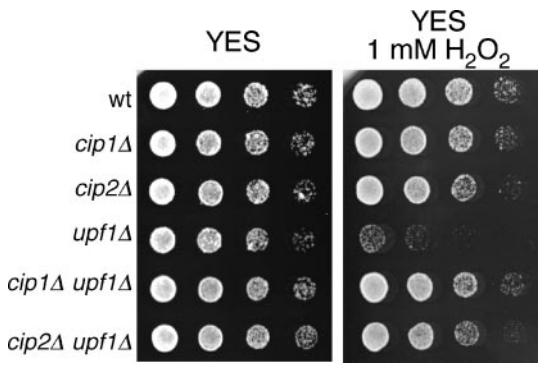

FIG. 5. Rescue of the oxidative stress-sensitive phenotype of *upf1*Δ cells by the *cip1D* and *cip2D* mutations. Serial dilutions of the indicated single and double mutant strains were plated on YES medium with or without 1 mM H_2O_2. wt, wild type.

This conclusion is further bolstered by our studies showing that Upf1 controls the abundance of mRNA containing premature termination codons (PTC) in fission yeast. We examined the *ade6-M26* allele, which has a point mutation that coincidentally introduces both a translation termination codon and a binding site for the Atf1/Pcr1 transcription factor (Fig. 3). Relative to the *ade6*$^+$ mRNA control, there was a large reduction in the amount of *ade6-M26* mRNA, and this effect required Upf1 (Fig. 3). From this result, we conclude that Upf1 is required for the degradation of a PTC-containing mRNA, and thus, it appears that Upf1 is required for NMD in fission yeast.

A previous study analyzed the role of a Upf2 homolog in nonsense-mediated decay in fission yeast (26). As we observed with Upf1, this study showed that the steady-state level of *ade6-M26* mRNA was strongly reduced in a manner that required Upf2. This study showed the same effect for another PTC-containing mRNA from the *ade6* locus (*ade6-M375*), and it also used the transcription inhibitor thiolutin to show that the half-life of *ade6-M375* mRNA was strongly reduced rela-

tive to the *ade6*$^+$ mRNA control. This effect was also dependent on Upf2 (26). These data constitute persuasive evidence Upf2 is required for NMD in fission yeast. As we have shown here, *upf2*Δ cells are sensitive to exposure to hydrogen peroxide (Fig. 4). Taken together, these data provide strong support for the conclusion that two NMD factors, Upf1 and Upf2, are required for survival of oxidative stress in *S. pombe*.

Whole-genome expression profiling in *S. cerevisiae* has shown the NMD factors control the abundance of a surprisingly large number of transcripts, estimated to be between 5 and 10% (~550) of the total wild-type transcripts (15, 20). Fewer than 20 of these wild-type transcript mRNAs are known NMD targets, and the majority do not fit into any known category of transcripts predicted to be regulated by NMD. In contrast to these findings, we have found that a relatively small number of transcripts (27) are increased twofold or more in unstressed *upf1*Δ cells relative to the wild type (Table 1). Among the transcripts up-regulated in *upf1*Δ cells, there is an overrepresentation of genes with ATGs in the region 80 nucleotides immediately upstream of the annotated ATG, which is one of the categories of predicted NMD-regulated transcripts, but the correlation is by no means absolute. More studies will be needed to determine which of these are natural substrates of the NMD pathway in fission yeast, but the difference between the number of NMD-regulated transcripts in budding and fission yeast is nevertheless quite striking. The difference is even starker when one considers that *S. cerevisiae* NMD mutants have a relatively subtle phenotype, being partially defective at respiratory growth at cold temperatures (1). This defect is thought to be linked, at least in part, to an inability to properly regulate the accumulation of the *ADR1* transcript (38), which encodes a transcription activator of genes for generation of acetyl coenzyme A and NADH from nonfermentable substrates (43).

Because our initial model was that NMD factors are involved in regulating the turnover of *atf1*$^+$ mRNA, we examined the abundance of *atf1*$^+$ mRNA in the *upf1*Δ strain. Contrary to our initial model, but consistent with the observation that *upf1*Δ cells are sensitive to oxidative stress, we found that the increase in *atf1*$^+$ mRNA that occurs in response to oxidative stress was strongly dampened in *upf1*Δ cells relative to the wild type (Fig. 2B and C). There was also a modest decrease in the abundance of *atf1*$^+$ mRNA in *upf1*Δ cells prior to the exposure to growth medium containing hydrogen peroxide (Fig. 2B). This difference may reflect the fact that in unstressed conditions Upf1 is involved in controlling *atf1*$^+$ transcript abundance, or more likely, that even in the standard rich growth medium (YES) there is a modest amount of oxidative stress or other forms of cytotoxic stress. Importantly, we found that during oxidative stress, the half-life of *atf1*$^+$ mRNA is reduced in *upf1*Δ cells relative to the wild type (Fig. 2D and E), indicating that Upf1 has a role in stabilizing *atf1*$^+$ mRNA. However, it should be noted that both *csx1*Δ and *upf1*Δ cells are more sensitive to oxidative stress than are *atf1*Δ cells (32) (our unpublished data). Therefore, the stress sensitivity of both *csx1*Δ and *upf1*Δ cells cannot be entirely attributed to defects in Atf1-dependent transcription.

The exact mechanism by which Upf1 promotes the stabilization of *atf1*$^+$ mRNA is for the moment a mystery. However, our genetic data strongly suggest that it collaborates with Csx1. In studies that measure the survival of oxidative stress, we have found that *csx1*Δ and *upf1*Δ cells display similar levels of increased sensitivity to hydrogen peroxide, and in these assays the *csx1*Δ *upf1*Δ double mutant cells are similar to the respective single mutants (Fig. 1C). Thus, by genetic epistasis analysis, Upf1 and Csx1 appear to be involved in a common mechanism of stress survival. In a previous study we found that the oxidative stress sensitivity of *csx1*Δ cells can be effectively suppressed by deletions of *cip1*$^+$ or *cip2*$^+$, which encode predicted RNA-binding proteins that can be found in a complex with Csx1 (23). Interestingly, Cip1 and Cip2 have the same relationship with Upf1; that is, the *cip1*Δ and *cip2*Δ mutations are able to suppress the oxidative stress phenotype of *upf1*Δ cells (Fig. 5). These data are consistent with the idea that Upf1 and Csx1 work in the same pathway to counteract the activities of Cip1 and Cip2 in promoting the turnover of *atf1*$^+$ mRNA.

Although our data support a model in which Upf1 and Csx1 act in a nonredundant fashion to stabilize of *atf1*$^+$ mRNA, Upf1 and Csx1 clearly do not have identical functions. In particular, Csx1 is not involved in nonsense-mediated decay of mRNA (Fig. 3B). The PTC-containing *ade6-M26* mRNA, which is destabilized in a Upf1-dependent mechanism, is not restored in a *csx1*Δ strain (Fig. 3B). Furthermore, genome-wide expression profiling has shown that while *csx1*Δ and *upf1*Δ cells have a large overlap in the transcripts that fail to properly accumulate in response to oxidative stress, the patterns are by no means identical. In particular, there are about 131 transcripts that appear to be regulated by Csx1 but not by Upf1 (Fig. 4A). Some of these differences may be threshold effects, but even accounting for this possibility, there appears to be a significant difference between the transcripts that are regulated by Csx1 and Upf1.

Our previous analysis showed that *atf1*$^+$ mRNA was specifically enriched in immunopurified Csx1 complexes (32). In contrast, we were unable to detect *atf1*$^+$ mRNA in complex with Upf1 (Fig. 3C). These are technically difficult experiments, and therefore, we are reluctant to firmly conclude that the effect of Upf1 on *atf1*$^+$ mRNA abundance involves an indirect interaction. However, we were able to detect an association between Upf1 and *ade6-M26* mRNA (Fig. 3C), so it appears that the experiment is sufficiently sensitive to detect interactions involving Upf1 and NMD substrates. It therefore seems likely that Upf1 does not directly associate with *atf1*$^+$ mRNA. This conclusion is further supported by the failure to detect a coprecipitation between Upf1 and Csx1 by mass spectrometry analysis of Csx1 complexes (23).

We have shown that on one hand, Upf1 is required for the proper transcriptional induction of many genes that are responsive to oxidative stress, while on the other hand, it is required for degradation of the *ade6-M26* PTC-containing mRNAs. The former function is carried out in conjunction with Csx1 and is mediated, at least in part, by stabilization of *atf1*$^+$ mRNA. On the basis of our expression profiling studies, it is likely that *pcr1*$^+$ mRNA, which encodes the other subunit of the Atf1/Pcr1 heterodimeric transcription factor, is also regulated by Upf1. How can Upf1 be required to stabilize some mRNAs and degrade others? One possibility is that Upf1 positively regulates Csx1-dependent gene expression by promoting the degradation of a PTC-containing mRNA that encodes a negative effector of the pathway. Our expression profiling stud-

ies have provided a list of candidates for this postulated negative effector of the Csx1 pathway of mRNA stabilization (Table 1). Future studies will address whether any of these genes are specifically involved in the oxidative stress response.

Whatever the mechanism by which Upf1 contributes to stabilization of *atf1*$^+$ mRNA, our studies clearly show that *upf1*Δ and *upf2*Δ mutants are acutely sensitive to oxidative stress. Exposure to reactive oxygen species, which can be derived from exogenous sources but are also formed as normal byproducts of aerobic metabolism, is a routine experience in eukaryotes. Indeed, a failure to keep reactive oxygen species under control is thought to underlie some of the most common diseases, particularly those associated with aging (4). All organisms have multiple mechanisms of coping with oxidative stress caused, many of which appear to be substantially conserved from yeasts to humans, such as the p38/Jun N-terminal protein kinase and Spc1 (Sty1) MAP kinase pathways in mammals and fission yeast, respectively (18, 33). Our findings lead us to predict that the role of Upf1 and Upf2 in tolerance of oxidative stress may be one of the selective pressures for conservation of these proteins in eukaryotes.

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