Molecular Biology of the Cell
Vol. 13, 2977–2989, September 2002

Role of Fission Yeast Tup1-like Repressors and Prr1 Transcription Factor in Response to Salt Stress

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Submitted December 4, 2001; Revised April 29, 2002; Accepted June 13, 2002
Monitoring Editor: Elizabeth Craig

In *Schizosaccharomyces pombe*, the Sty1 mitogen-activated protein kinase and the Atf1 transcription factor control transcriptional induction in response to elevated salt concentrations. Herein, we demonstrate that two repressors, Tup11 and Tup12, and the Prr1 transcription factor also function in the response to salt shock. We find that deletion of both *tup* genes together results in hypersensitivity to elevated cation concentrations (K^+ and Ca^{2+}) and we identify *cta3*$^+$, which encodes an intracellular cation transporter, as a novel stress gene whose expression is positively controlled by the Sty1 pathway and negatively regulated by Tup repressors. The expression of *cta3*$^+$ is maintained at low levels by the Tup repressors, and relief from repression requires the Sty1, Atf1, and Prr1. Prr1 is also required for KCl-mediated induction of several other Sty1-dependent genes such as *gpx1*$^+$ and *ctt1*$^+$. Surprisingly, the KCl-mediated induction of *cta3*$^+$ expression occurs independently of Sty1 in a *tup11*Δ *tup12*Δ mutant and so the Tup repressors link induction to the Sty1 pathway. We also report that in contrast to a number of other Sty1- and Atf1-dependent genes, the expression of *cta3*$^+$ is induced only by high salt concentrations. However, in the absence of the Tup repressors this specificity is lost and a range of stresses induces *cta3*$^+$ expression.

INTRODUCTION

Exposure of cells to environmental stress triggers a rapid increase in the transcription of genes whose products have protective functions (Toone and Jones, 1998). Key to this response are stress-activated protein kinase (SAPK) pathways that transmit the signal from stress sensors to the transcription factors that regulate gene expression. These pathways are evolutionarily conserved, and homologs of the mammalian SAP kinases, p38/RK/CSBP (Marshall, 1994), are present in both *Saccharomyces cerevisiae* (Hog1) (Brewster *et al.*, 1993) and *Schizosaccharomyces pombe* (Sty1/Spc1) (Miller *et al.*, 1995; Shiozaki and Russell, 1995). The Hog1 pathway in *S. cerevisiae* is activated essentially by hyperosmolarity (Brewster *et al.*, 1993), whereas the *S. pombe* Sty1 pathway, like mammalian p38, is activated by a range of adverse conditions (Millar *et al.*, 1995; Shiozaki and Russell, 1996; Degols and Russell, 1997; Buck *et al.*, 2001).

Models of SAPK-dependent regulation of transcription have been almost exclusively based upon the positive control of activators. However, recent analysis of *S. cerevisiae* has demonstrated that the Sko1 repressor regulates the expression of Hog1-dependent osmostress genes, such as *ENA1* and *GRE2*, via recruitment of the Ssn6(Cyc8)-Tup1 global corepressor complex (Marquez *et al.*, 1998; Proft and Serrano, 1999; Garcia-Gimeno and Struhl, 2000; Proft *et al.*, 2001). Ssn6-Tup1 mediates its function via the organization of repressive chromatin structures (Cooper *et al.*, 1994; Edmondson *et al.*, 1996; Watson *et al.*, 2000; Bone and Roth, 2001; Wu *et al.*, 2001) and by inhibition of the basal transcription machinery (Redd *et al.*, 1997; Papamichos-Chronakis *et al.*, 2000; Zaman *et al.*, 2001). This global repressor controls the expression of numerous genes through interaction with a variety of site-specific DNA binding proteins (Smith and Johnson, 2000). Relief from this repression is achieved by control of the proteins that serve to tether the complex to DNA; for example, Sko1 is phosphorylated by Hog1 at three sites in its N-terminal region, disrupting the

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.01–12–0568. Article and publication date are at www.molbiolcell.org/cgi/doi/10.1091/mbc.01–12–0568.

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Table 1. Strains used in this study

Strain	Genotype	Reference
NT4	*h⁺ ade6-M210 leu1-32 ura4-D18*	Zhu *et al.* (1994)
NT5	*h⁻ ade6-M216 leu1-32 ura4-D18*	Zhu *et al.* (1994)
SW54	*h⁺ ade6-M210 leu1-32 ura4-D18 tup11::ura4⁺*	Janoo *et al.* (2001)
BSP03	*h⁺ ade6⁻ leu1-32 ura4-D18 tup12::ura4⁺*	Janoo *et al.* (2001)
RJP12	*h⁻ ura4::fbp1-lacZ tup11::ura4⁺ tup12::ura4⁺*	Janoo *et al.* (2001)
SW76	*h⁺ ade6⁻ tup11::ura4⁺ tup12::ura4⁺ ura4-D18 leu1-32*	This study
HAI003	*h⁻ ade6⁻M216 leu1-32 ura4-D18 cta3-lacZ::ura4⁺*	Nishikawa *et al.* (1999)
SW107	*h⁻ ade6⁻ leu1-32 ura4-D18 tup11::ura4⁺ tup12::ura4⁺ cta3-lacZ::ura4⁺*	This study
KS14709	*h⁻ atf1HA6H::ura4⁺ leu1-32 ura4-D18*	Shiozaki & Russell (1996)
JM1144	*h⁻ sty1-1 leu1-32 ura4-D18*	Millar *et al.* (1995)
JM1160	*h⁻ ade6-M216 leu1-32 ura4-D18 sty1::ura4⁺*	Millar *et al.* (1995)
NT147	*h⁹⁰ leu1-32 ura4-D18 atf1::ura4⁺*	Takeda *et al.* (1995)
JX125	*h⁹⁰ ade6⁻ leu1-32 ura4-D18 pcr1::ura4⁺*	Kanoh *et al.* (1996)
RJP59	*h⁺ his7-366 ura4::fbp1-lacZ pcr1::ura4⁺ tup11::ura4⁺ tup12::ura4⁺*	Janoo *et al.* (2001)
SW89	*h⁺ ade6⁻ leu1-32 ura4-D18 tup11::ura4⁺ sty1-1*	This study
SW88	*h⁺ leu1-32 ura4-D18 tup12::ura4⁺ sty1-1*	This study
SW90	*h⁺ ade6⁻ leu1-32 ura4-D18 tup11::ura4⁺ tup12::ura4⁺ sty1-1*	This study
SW91	*h⁻ leu1-32 ura4-D18 tup11::ura4⁺ tup12::ura4⁻ sty1::ura4⁺*	This study
SW95	*h⁻ ade6-M216 leu1-32 ura4-D18 cta3::ura4⁺*	This study
SW93	*h⁺ ade6⁻ leu1-32 ura4-D18 cta3::ura4⁺ tup11::ura4⁺ tup12::ura4⁺*	This study
SW92	*h⁺ leu1-32 ura4-D18 atf1::ura4⁺ tup11::ura4⁺ tup12::ura4⁺*	This study
RJP59	*h⁺ ura4::fbp1-lacZ pcr1::ura4⁺ tup11::ura4⁺ tup12::ura4⁺ his7-336*	Janoo *et al.* (2001)
SW97	*h⁺ ade6-M216 ura4-D18 his7-336 leu1-32 prr1::ura4⁺*	This study
SW96	*h⁺ ade6⁻ ura4-D18 leu1-32 prr1::ura4⁺ tup11::ura4⁺ tup12::ura4⁺ his7-336*	This study

interaction with Ssn6-Tup1 (Proft *et al.*, 2001). Therefore, a component of the osmotic induction of some genes occurs via derepression rather than by activation.

In fission yeast, Sty1 operates via the transcriptional activators Atf1/Gad7 (Takeda *et al.*, 1995; Kanoh *et al.*, 1996) and Pap1 (Toda *et al.*, 1991). Atf1 is phosphorylated in a Sty1-dependent manner and loss of Atf1 results in hypersensitivity to osmotic stress, high levels of calcium, and an inability to respond to deteriorating nutritional conditions (Takeda *et al.*, 1995; Kanoh *et al.*, 1996; Ohmiya *et al.*, 1999b). In addition, Atf1 forms a heterodimeric complex with Pcr1, a related ATF/CREB factor, which is also required for transcriptional induction of some stress genes (Watanabe and Yamamoto, 1996). Pap1 activates transcription in response to oxidative stress, and its subcellular localization is regulated in a Sty1-dependent manner (Toone *et al.*, 1998). Recently, Prr1, a homolog of Skn7 in *S. cerevisiae* (Brown *et al.*, 1993), has also been implicated in the transcriptional response to oxidative stress (Ohmiya *et al.*, 1999a). Skn7 and Prr1 have heat-shock factor-like DNA binding domains and also share homology with bacterial "two-component" response regulators that are controlled by histidine-to-aspartate phosphorelay systems (Appleby *et al.*, 1996).

Herein, we have addressed the roles of the Tup-like repressors Tup11 and Tup12 (Mukai *et al.*, 1999; Janoo *et al.*, 2001) in the response to stress in *S. pombe*. We find that deletion of both *tup* genes in combination results in hypersensitivity to KCl and $CaCl_2$, and we also identify *cta3⁺* as a novel stress gene that is negatively regulated by Tup11-Tup12. The expression of *cta3⁺* is rapidly and specifically induced in response to salt shock in a Sty1- and Atf1-dependent manner, but the dependence on the Sty1 pathway for induction is lost in a *tup11*Δ *tup12*Δ mutant. Furthermore, Tup11 and Tup12 proteins function as specificity factors by preventing induction of *cta3⁺* in response to inappropriate stresses such as heat and oxidative stress. We also reveal a new role for the "response regulator" protein Prr1 and demonstrate that it is required for proper KCl-mediated transcriptional induction of Sty1-dependent genes such as *cta3⁺*, *ctt1⁺*, and *gpx1⁺*.

MATERIALS AND METHODS

Strains

Routine culture of *S. pombe* and general genetic methods were performed as described in Moreno *et al.* (1991). The strains used in this study are described in Table 1. The *cta3⁺* gene was disrupted using a polymerase chain reaction (PCR)-based approach as described by Bahler *et al.* (1998). Oligonucleotides 5′ KO (5′-TTTGATTTTACTTATATTTCTCCCCTTCTACTCATCCCGATATATTCTTACTTCCTTGATTCAATCTCAAATATTGTTCAGCTTAGCTACAAATCCCACT-3′) and KO 3′ (5′-ATAAATCCTTTACGATTTGTCGGTTCTGTGAAAACGATACACTCACGCATATTCATATACATATTCATGGCAAGAAAACATCTGACATAAAACGCCTAGG-3′) were used to amplify a 1.6-kb *ura4⁺*-containing fragment from pRep42. The amplified fragment was used to transform strain NT5 strain to Ura⁺, creating strain SW95. Integration at the correct locus was confirmed by PCR analysis.

Plasmids

The *tup11⁺* coding sequence was amplified by PCR from a cDNA library by using the following primers: 5′-GCACGGATCCCATGGCGTCAGTGGAGGATGC-3′ and 5′-CTAGGGATCCAATTCAAGGAGATGCAGGGTC-3′. The *tup12⁺* coding sequence was amplified using primers 5′-GCACGGATCCCATGATTACTGTCCGCCAATC-3′ and 5′-CTGCTAGGCATATGGCGCTCATGAAACAAACCG-3′. Fragments were cleaved with *Bam*HI and cloned into the *Bam*HI site of derivatives of pRep41 and pRep42 vectors

that allow the expression of proteins as N-terminal HA or 6HisMyc fusions (Craven *et al.*, 1998).

RNA Analysis

RNA samples were prepared from 0.25 to 0.5 × 10^9 cells. Pellets were washed in H_2O and resuspended in 200 μl of RNA buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 50 mM EDTA pH 8.0, and 0.25% SDS) with 200 μl of phenol/chloroform. Cells were disrupted with 0.75 ml of glass beads (0.5 mm; Biospec Products, Bartlesville, OK) in a Ribolyser (Hybaid, Middlesex, United Kingdom). A further 0.75 ml of RNA buffer was added followed by spinning in a microfuge for 10 min. The aqueous layer was subjected to two further phenol/chloroform extractions before the RNA was precipitated with 0.1 volume of sodium acetate, pH 5.2, and 0.6 volume of isopropanol. RNA pellets were washed in 70% ethanol and resuspended in H_2O. RNA analysis was as described by White *et al.* (1986). Briefly, a 10–15-μg sample of total RNA was denatured with glyoxal, separated on a 1.2% agarose gel prepared in 15 mM sodium phosphate, pH 6.5, and transferred to a GeneScreen hybridization membrane (PerkinElmer Life Sciences, Boston, MA). The *his3*$^+$ probe has been described previously (Baum *et al.*, 1997). Other gene-specific probes were produced by PCR amplification from genomic DNA by using the appropriate primers. All probes were labeled with [α-^{32}P]dCTP by using a Prime-a-Gene labeling kit (Promega, Madison, WI). Transcript levels were quantified relative to the loading control using a PhosphorImager BAS-1500 (Fuji Photo Film, Tokyo, Japan).

β-Galactosidase Assays

Assays were performed as described previously (Takeda *et al.*, 1995).

Coprecipitations

Whole cell extracts were prepared as described by Whitehall *et al.* (1999) with some modification. Cultures were grown to mid-log phase (OD_{595} = 0.25–0.5) in EMM medium. Cells were harvested washed once and snap frozen. Pellets were washed in 1 ml of lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% NP-40, 10 mM imidazole, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 100 μg/ml phenylmethylsulfonyl fluoride). Cells were disrupted with 2 ml of glass beads by vortexing twice for 45 s with 1-min incubation on ice in between. Protein extracts were recovered and centrifuged at 13,000 rpm for 10 min at 4°C. Protein precipitations were performed by adding 25 μl of nickel-agarose (50% slurry in lysis buffer) to 1 mg of whole protein extract and incubating at 4°C for 1 h with gentle agitation. Precipitates were recovered by centrifugation and washed four times with lysis buffer containing 200 mM NaCl and 20 mM imidazole. Samples were analyzed by SDS-PAGE and proteins were transferred to nitrocellulose membrane and subjected to Western blotting by using monoclonal hemagglutinin (HA) (12CA5) antibody (Babco, Berkeley, CA).

Electrophoretic Mobility Shift Assays (EMSAs)

Whole cell extracts were prepared as described above except that cells were grown in YE5S medium and extracts were prepared in buffer containing 25 mM HEPES pH 7.6, 0.1 mM EDTA, 150 mM KCl, 0.1% Triton X-100, 25% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride 2 μg/ml pepstatin, 2 μg/ml leupeptin, and 2 μg/ml aprotinin. Radiolabeled DNA fragments were prepared using PCR amplification as described in Zhu *et al.* (1994). The oligonucleotides used for amplification of probe 1 were 5′-TAAAACACCGACATGTAGCC-3′ and 5′-TTGAGAGAAACTAACCAAGG-3′. The oligonucleotides for probe 2 were 5′-CTCTGTCATGGAAATCCACAC-3′ and 5′-ATAAGCAGCAAAGCTTGCCTG-3′. Binding reactions were performed by adding 15 μg of whole cell extract to 20-μl reactions containing 25 mM HEPES pH 7.6, 34 mM KCl, 5 mM $MgCl_2$, and 2 μg of poly[d(I-C)]. Reactions were incubated for 10 min at room temperature before the addition of ~0.5 ng of radiolabeled probe DNA followed by a further 20-min incubation. Samples were analyzed by electrophoresis through 4% polyacrylamide gels run in 0.5× Tris-borate-EDTA buffer. Antibody supershift was performed by adding 0.2 μg of monoclonal HA antibody (12CA5) (Babco) 10 min after the addition of probe DNA.

RESULTS

To address the role of repressors in the transcriptional response to stress in fission yeast we examined whether cells lacking the *tup* genes *tup11*$^+$ and *tup12*$^+$ exhibited any stress-related phenotypes. We found that single and double *tup* mutant strains exhibited an increased tolerance to cadmium but that the *tup11*Δ *tup12*Δ mutant strain had decreased tolerance to elevated levels of Ca^{2+} and K^+ ions. The degree of sensitivity to these salt stresses was similar to that

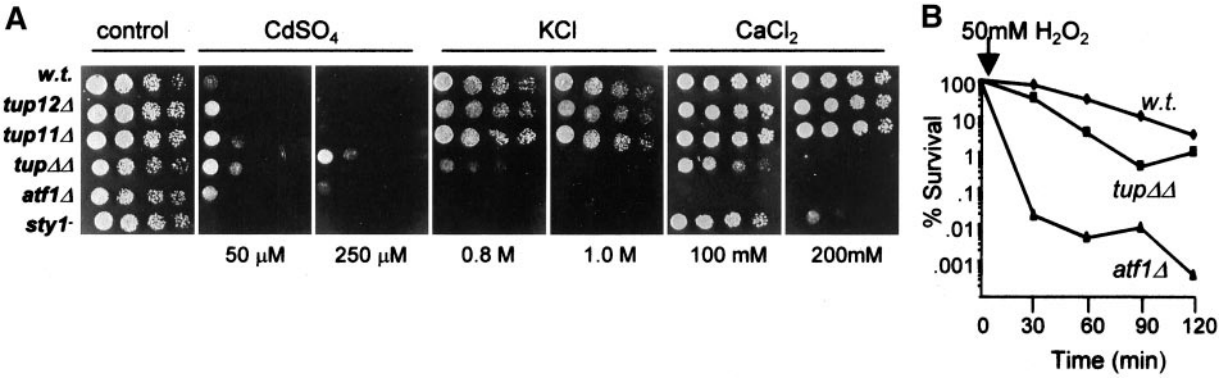

Figure 1. Stress-related phenotypes of cells lacking Tup repressors. (A) Exponentially growing cultures (~0.4 × 10^7 cells/ml) were diluted serially, spotted onto YE5S agar, and incubated for 2–3 d at 30°C or spotted onto YE5S agar supplemented with $CaCl_2$, KCl, or $CdSO_4$ at the indicated concentration and incubated for 3–4 d at 30°C. (B) Exponentially growing cultures of wild type (w.t.) (NT4), *atf1*Δ (NT147), and *tup11*Δ *tup12*Δ (SW76) strains were treated with H_2O_2 (to a final concentration of 50 mM), and viable cell numbers were determined by plating onto YE5S agar.

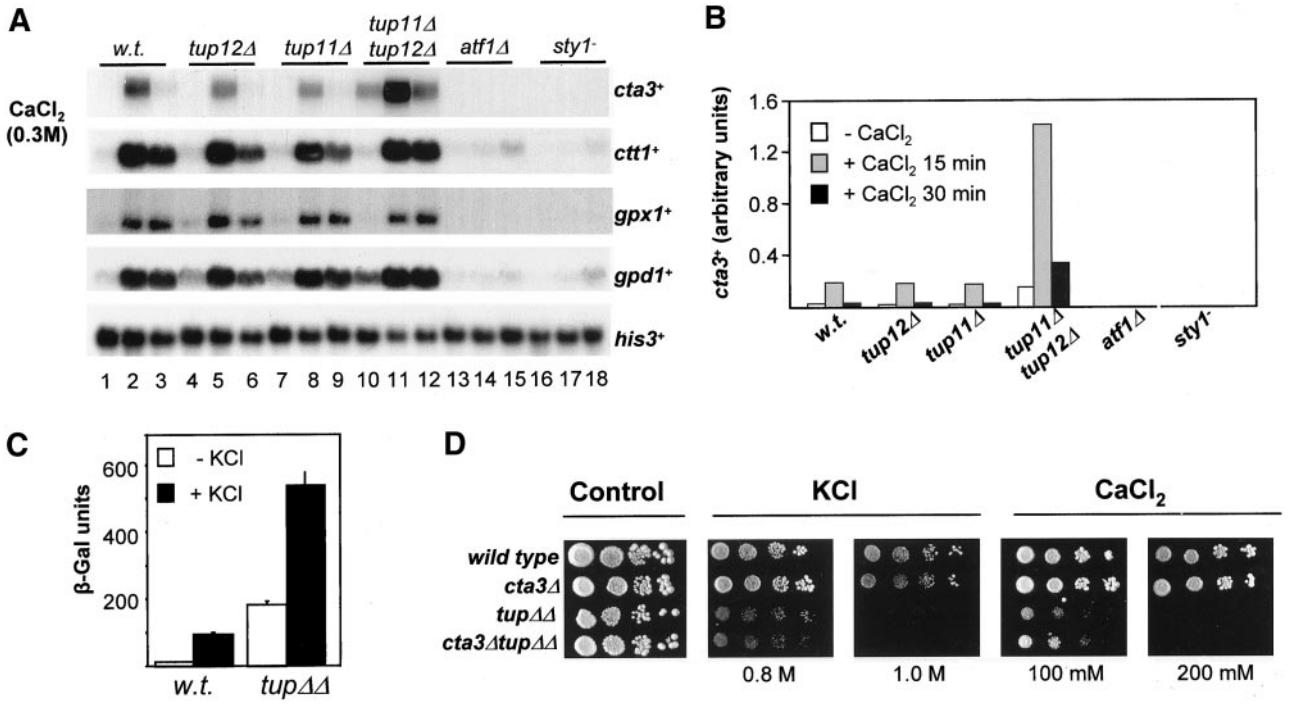

Figure 2. Tup11 and Tup12 repress the transcription of the salt stress gene *cta3*$^+$. (A) Strains used are indicated above the lane and were wild type (w.t.) (NT4), *tup12*Δ (BSP03), *tup11*Δ (SW53), *tup11*Δ *tup12*Δ (SW76), *atf1*Δ (78 T147), and *sty1-1* (JM1144). Log phase cultures growing at 30°C in YE5S (lanes 1, 4, 7, 10, 13, and 16) were incubated with $CaCl_2$ (to final concentration of 0.3 M) for 15 min (lanes 2, 5, 8, 11, 14, and 17) and 30 min (lanes 3, 6, 9, 12, 15, and 18). Total RNA was extracted, separated by electrophoresis, and Northern blots were analyzed with the indicated probes. The level of *his3*$^+$ mRNA was used as a loading control. (B) Quantification of *cta3*$^+$ mRNA levels in A. (C) Influence of Tup proteins on the activity of a *cta3-lacZ* reporter. β-Galactosidase assays were performed on extracts derived from exponentially growing cells (open bars) and cells treated with KCl to 0.6 M for 60 min (black bars). The strains used were wild type (w.t.) (HAI003) and *tup11*Δ *tup12*Δ (SW107). Data is the mean of three independent cultures. (D) Deletion of *cta3*$^+$ does not rescue the salt sensitivity of *tup11*Δ *tup12*Δ cells. Exponentially growing cultures (~0.4×10^7 cells/ml) were diluted serially, spotted onto YE5S agar and incubated for 2–3 d at 30°C or spotted onto YE5S agar supplemented with $CaCl_2$ or KCl (at the indicated concentration) and incubated for 3–4 d at 30°C. Strains used were wild type (w.t.) (NT4), *tup11*Δ *tup12*Δ (SW76), *cta3*Δ (SW95), and *cta3*Δ *tup11*Δ *tup12*Δ (SW93).

associated with loss of either the Atf1 transcription factor or the Sty1 MAP kinase that are known to control the induction of genes in response to elevated cation concentrations (Shiozaki and Russell, 1996; Wilkinson *et al.*, 1996). The *tup11*Δ *tup12*Δ double mutant strain was only slightly less sensitive to KCl than the *sty1-1* and *atf1*Δ strains and the *tup11*Δ *tup12*Δ strain was actually more sensitive to $CaCl_2$ than strain lacking Sty1 (Figure 1A). In contrast, the double *tup* mutant strain had wild-type levels of tolerance to high sorbitol concentrations, indicating that although they are K^+- and Ca^{2+}-intolerant they are not osmosensitive (our unpublished data).

The similarity in the sensitivities of *tup11*Δ *tup12*Δ and *atf1*Δ strains to elevated K^+/Ca^{2+} ions was unexpected because Tup11 and Tup12 have been previously demonstrated to be repressors (Mukai *et al.*, 1999; Janoo *et al.*, 2001), whereas Atf1 is primarily a transcriptional activator. We therefore investigated whether *tup11*Δ *tup12*Δ cells shared any other phenotypes with *atf1*Δ cells. It has recently been demonstrated that *atf1*Δ cells are sensitive to an acute oxidative stress (Nguyen *et al.*, 2000; Quinn *et al.*, 2002). When challenged with a high dose of H_2O_2 (50 mM) *atf1*Δ cells rapidly lose viability (Figure 1B). In contrast, *tup11*Δ *tup12*Δ cells were only slightly more sensitive than wild-type cells in this assay. Furthermore, although *atf1*Δ cells conjugate poorly (Takeda *et al.*, 1995), a *tup11*Δ *tup12*Δ strain conjugates in nutrient-rich media (Janoo *et al.*, 2001). Hence, *tup11*Δ *tup12*Δ cells and *atf1*Δ cells share only a subset of phenotypes. Taken together, these findings are consistent with Tup11 and Tup12 having overlapping functions and indicate that Tup11 and Tup12 play roles in the cellular response to stress.

Tup11 and Tup12 Negatively Regulate Expression of Salt-Stress gene cta3$^+$

Cells lacking *atf1*$^+$ and both *tup* genes have similar sensitivities to salt stress and so we examined whether the expression of genes known to be induced by exposure to salt stress, via Atf1, were also regulated by the Tup repressors (Figure 2, A and B). We found that expression of *cta3*$^+$, which encodes a cation-transporting P-type ATPase (Ghislain *et al.*, 1990; Halachmi *et al.*, 1992; Benito *et al.*, 2002), was markedly influenced by loss of the Tup proteins; deletion of both *tup* genes together resulted in a large increase in the basal level of expression (Figure 2, A and B). Loss of both Tup proteins

also resulted in a large increase in expression after exposure to $CaCl_2$ (Figure 2, A and B) and KCl (Figures 5, 7, and 8). Thus, Tup11 and Tup12 function in a partially redundant manner to repress *cta3*$^+$ expression and limit the level of induction. As previously observed (Nishikawa *et al.*, 1999), the induction of *cta3*$^+$ expression is completely dependent upon both the Sty1 MAP kinase and the Atf1 transcription factor, and thus *cta3*$^+$ displays a novel pattern of stress regulation that is positively controlled by Sty1 and Atf1 but negatively regulated by the Tup repressors. Indeed, deletion of the *tup* genes either singly or in combination had only minor effects on the expression levels of other Sty1-dependent genes such as *gpd1*$^+$, *ctt1*$^+$, and *gpx1*$^+$ in unstressed cells and in cells subjected to a $CaCl_2$ shock (Figure 2, A and B).

To confirm that increased level of *cta3*$^+$ transcripts associated with deletion of *tup* genes was due to an effect on transcription and not mRNA stability, we measured the expression of an integrated *cta3*$^+$ promoter—*lacZ* reporter (Nishikawa *et al.*, 1999). It is highly unlikely that Tup repressors would specifically influence the stability of *lacZ* transcripts. Consistent with the Northern analysis, deletion of both the *tup* genes resulted in 14-fold increase in expression of the *lacZ* reporter relative to the wild-type control (Figure 2C). Furthermore, exposure of cells to high KCl concentrations (0.6 M for 1 h) increased the level of expression sevenfold in wild-type cells and threefold in the cells lacking the Tup repressors (Figure 2C). These results suggest that *S. pombe* Tup proteins exert their effects at the level of transcription.

To determine whether the high level of *cta3*$^+$ expression observed in the *tup11*Δ *tup12*Δ double mutant confers the increased sensitivity of this strain to elevated K^+ and Ca^{2+} concentrations we examined the effect of deleting the *cta3*$^+$ gene in the presence or absence of the *tup* genes. Loss of Cta3 function has previously been reported to result in increased sensitivity to elevated Ca^{2+} concentrations (Ghislain *et al.*, 1990). In contrast, Nishikawa et al. (1999) found that *cta3* null cells did not exhibit any detectable change in resistance to K^+, Ca^{2+}, or Na^+ ions. In agreement with the latter study our *cta3*Δ mutant exhibited wild-type levels of resistance to both Ca^{2+} and K^+ (Figure 2D). Moreover, deletion of the *cta3*$^+$ gene in a *tup11*Δ *tup12*Δ strain did not rescue the salt-sensitive phenotype associated with the loss of the *tup* genes (Figure 2D), and plasmid-mediated overexpression of *cta3*$^+$ in wild-type cells did not result in any increased sensitivity to KCl or $CaCl_2$ (our unpublished data). These results indicate that the salt sensitivity of *tup*$^-$ cells is not simply due to the elevated expression of the *cta3*$^+$ gene.

To date, the *fbp1*$^+$ gene encoding fructose 1,6-bisphosphatase is the only gene that has been identified as a target gene for Tup11-Tup12–mediated repression (Mukai *et al.*, 1999; Janoo *et al.*, 2001). The expression of *fbp1*$^+$ is also positively regulated by the Sty1 pathway (Takeda *et al.*, 1995; Kanoh *et al.*, 1996; Stettler *et al.*, 1996), but its expression is induced by carbon limitation (Hoffman and Winston, 1991) and not by other acute stresses that activate Sty1, such as heat shock, oxidative stress, and osmotic shock. Furthermore, the cAMP pathway negatively regulates the expression of *fbp1*$^+$, and mutations that disrupt this pathway result in increased expression under repressing (glucose-rich) conditions (Hoffman and Winston, 1991). In contrast, growing cells under carbon-limiting conditions did not induce the expression of *cta3*$^+$ nor were mRNA levels influenced by a deletion of *git2*$^+$ that encodes adenylate cyclase (our unpublished data).

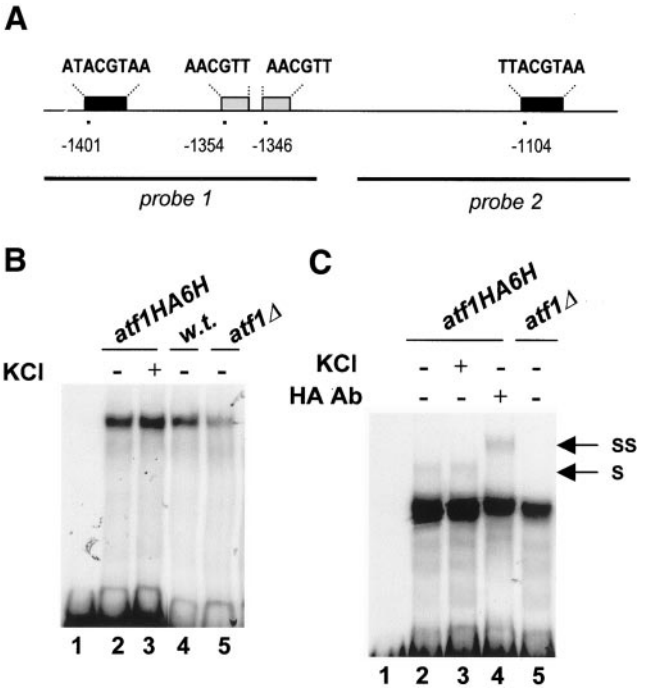

Figure 3. Complexes formed on the *cta3*$^+$ promoter. (A) Schematic of the CRE-like elements in the *cta3*$^+$ promoter and the probes used for EMSAs. Consensus or near consensus CRE elements are shaded black, and the CRE-like sequences containing an ACGT core are shaded gray. Their locations are given relative to the initiation codon. (B) EMSAs were performed with probe 1 by using whole cell extracts derived from exponentially growing *atf1HA* (KS14709), w.t. (NT4), or *atf1*Δ (NT147) cells as indicated above the lanes. The extract used in lane 3 was derived from *atf1HA6H* cells treated with KCl (0.6 M for 15 min) immediately before extract preparation. Lane 1 is a probe alone control. (C) EMSAs were performed with probe 2 by using extracts derived from exponentially growing cells (lanes 2, 4, and 5) or cells treated with KCl (0.6 M for 15 min) immediately before extract preparation (lane 3). The identity of the extract is indicated above the lane. The reaction in lane 4 contained 0.2 μg of HA antibody. Lane 1 is a probe alone control. Arrows mark the positions of the Atf1-dependent shifted (S) and supershifted (SS) complexes.

Formation of Protein Complexes on the cta3$^+$ *Promoter*

Studies have indicated that Atf1 binds constitutively to a CRE binding site in the *gpd1*$^+$ promoter (Wilkinson *et al.*, 1996). In contrast, EMSAs of the *fbp1*$^+$ promoter have demonstrated that Atf1 associates with a CRE-like element in UAS1 only under activating (glucose-limiting) conditions (Neely and Hoffman, 2000). We therefore examined the ability of Aft1 to bind to the *cta3*$^+$ promoter. Inspection of the DNA sequence revealed the presence of a number of potential CRE-like Atf1 binding sites located between −1111 and −1401 relative to the initiation codon (Figure 3A). We per-

formed EMSAs by using whole cell extracts and a DNA fragment corresponding to the −1477 to −1297 region of the promoter. This region includes a near consensus CRE element and two CRE-like sequences containing the highly conserved ACGT core sequence. A major slow-migrating complex was formed on this probe (Figure 3B). This binding activity was not changed by subjecting cells to stress (KCl 0.6 M for 15 min) before extract preparation. The complex was also present in extracts derived from *atf1*Δ cells, indicating that it does not require Atf1. Furthermore, the mobility of the complex was unchanged when HA antibody was included in reactions containing HA epitope-tagged Atf1 (our unpublished data). Next, we examined the ability of complexes to form on a probe corresponding to the −1249 and −1058 region of the promoter that contains a single CRE element. In this case, we also observed a binding activity that was Atf1-independent (Figure 3C). However, we also detected a slow-migrating complex that was absent in reactions lacking Atf1. Also, the mobility of this complex was reduced by the addition of the HA antibody to reactions containing HA epitope-tagged Atf1. This Atf1-dependent complex was present in reactions using extracts derived from unstressed and stressed cells, indicating that at least under these experimental conditions Atf1 binds constitutively to this region of the *cta3*$^+$ promoter. We were unable to properly assess the role of Tup proteins on DNA binding activity; when *tup*$^-$ extracts were used a marked reduction in the level of complex formation on both probes was observed. However, this seemed to be due to difficulties in preparing extracts from these cells rather than a specific effect because we found that extracts lacking Tup proteins also showed a reduced ability to form complexes on a DNA probe unrelated to the *cta3*$^+$ promoter (our unpublished data).

Tup11 and Tup12 Interact

Our data indicate that Tup11 is capable of repressing *cta3*$^+$ expression in the absence of Tup12 and vice versa. It is also known that *S. cerevisiae* Tup1 tetramerizes through its N-terminal domain (Varanasi *et al.*, 1996; Jabet *et al.*, 2000) and based on homology it is very likely that the *S. pombe* Tup proteins form homotetramers. However, it is possible that in addition to functioning in homomeric complexes Tup11 and Tup12 may also function in a heteromeric complex. Therefore, we investigated the ability of Tup11 and Tup12 to interact using a coprecipitation assay. Whole cell extracts were prepared from wild-type cells that expressed 6His-tagged Tup11 (or Tup12) and coexpressed HA-tagged Tup11 (or Tup12). Ni^{2+}-agarose was then used to precipitate His-tagged Tup proteins, and the presence of HA-tagged Tup proteins was examined by Western blotting (Figure 4). In these experiments, Tup11 copurified with Tup12 and vice versa, indicating that Tup11 and Tup12 physically interact. The specificity of this interaction was demonstrated by the absence of HA-tagged Tup proteins in control precipitates derived from cells extracts expressing the empty 6His vector (Figure 4, lanes 7 and 8). Thus, Tup11 and Tup12 have the potential to regulate gene expression in the same protein complex.

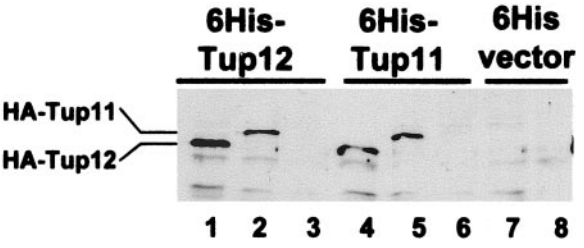

Figure 4. Tup11 and Tup12 coprecipitate. Whole cell extracts were prepared from wild-type cells containing plasmids expressing epitope-tagged Tup proteins: pRep42-HisMycTup12 (lanes 1–3), pRep42-HisMycTup11 (lanes 4–6), pRep42-HisMyc empty vector (lanes 7 and 8), pRep41-HATup12 (lanes 1, 4, and 7), and pRep41-HATup11 (lanes 2, 5, and 8). Extracts were precipitated with Ni^{2+}-agarose analyzed on 8% SDS polyacrylamide gels and subjected to Western blotting by using HA monoclonal antibody.

Tup Repressors Link Transcriptional Induction to the Sty1 Pathway

To test whether the high level of *cta3*$^+$ expression observed in the absence of the Tup repressors was dependent upon the Sty1 MAP kinase *cta3*$^+$ mRNA levels were examined in a strain that lacks both Sty1 and Tup function (*sty1-1 tup11*Δ *tup12*Δ). In this mutant the level of *cta3*$^+$ transcripts was similar to that observed in *tup11*Δ *tup12*Δ cells, indicating that Sty1 is not required for basal levels of expression (Figure 5, A and B). Surprisingly, exposure of this strain to a KCl-mediated shock resulted in induction of *cta3*$^+$ expression, indicating that in the absence of the Tup proteins the Sty1 MAP kinase is not required for the stress-mediated induction of *cta3*$^+$. Consistent with these observations the expression of *cta3*$^+$ was also induced by salt shock (0.6 M KCl) in a *sty1*Δ *tup11*Δ *tup12*Δ strain (our unpublished data). The expression of other genes such as *pyp2*$^+$ and *gpd1*$^+$ was not induced in the *sty1-1 tup11*Δ *tup12*Δ triple mutant, although deletion of the *tup* genes did restore the basal level of expression in *sty1*$^-$ cells (Figure 5A). The kinetics of KCl-mediated induction of *cta3*$^+$ were similar in wild-type and *tup11*Δ *tup12*Δ cells, with mRNA levels peaking at 20 min but elevated mRNA levels persisted for a greater length of time in cells lacking the Tup proteins (Figure 5, C and D). In the *sty1-1 tup11*Δ *tup12*Δ triple mutant strain induction was delayed and peak mRNA levels were not observed until 30 min after the addition of KCl.

We next determined whether removal of Tup11 and Tup12 rescued any of the other phenotypes associated with loss of Sty1. We examined the ability of cells to grow on medium supplemented with cadmium. Deletion of the *tup* genes in a *sty1*$^+$ background increases resistance to cadmium (Figure 1A) but unexpectedly deletion of *tup11*$^+$ and *tup12*$^+$ in a *sty1-1* background reduced cadmium tolerance (Figure 6A). Thus, the resistance of *tup*$^-$ cells to cadmium depends on Sty1 function and in its absence they become hypersensitive. The elongated cell morphology of *sty1-1* cells that is indicative of a G2 cell cycle delay was slightly exacerbated by deletion of the *tup* genes (Figure 6B). Furthermore, deletion of the *tup* genes in an *aft1*Δ or a *sty1-1* background resulted in a small increase in sensitivity to KCl

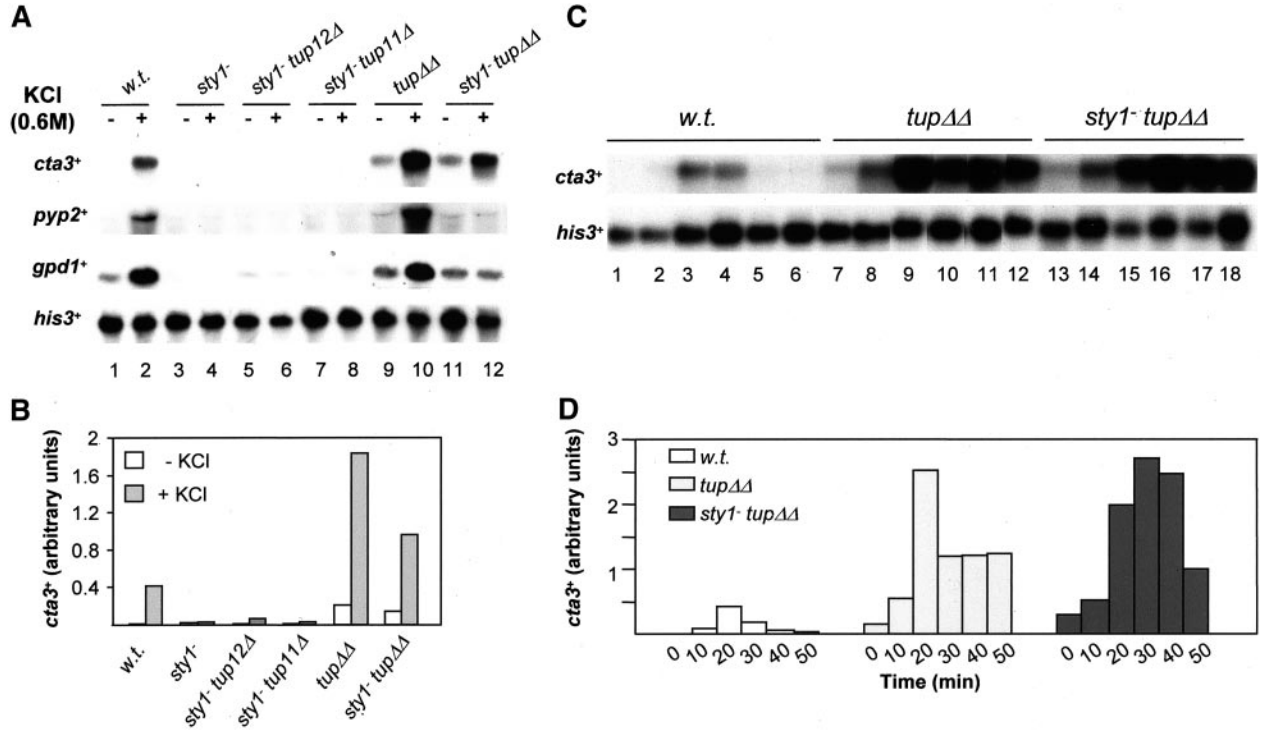

Figure 5. Deletion of *tup11*$^+$ and *tup12*$^+$ allows Sty1-independent transcriptional induction of *cta3*$^+$. (A) Strains used are indicated above the lanes and were wild type (w.t.) (NT4), *sty1-1* (JM1144), *sty1-1 tup12*Δ (SW88), *sty1-1 tup11*Δ (SW89), *tup11*Δ *tup12*Δ (SW76), and *sty1-1 tup11*Δ *tup12*Δ (SW90). Log phase cultures growing at 30°C in YE5S (lanes 1, 3, 5, 7, 9, and 11) were incubated with KCl (to a final concentration of 0.6 M) for 15 min (lanes 2, 4, 6, 8, 10, and 12). Total RNA was extracted, separated by electrophoresis, and analyzed by Northern blotting with the indicated probes. The level of *his3*$^+$ mRNA was used as a loading control. (B) Quantification of the *cta3*$^+$ mRNA levels in A. (C) Kinetics of the induction of *cta3*$^+$ mRNA in response to KCl shock. Log phase cultures growing at 30°C in YE5S (lanes 1, 7, and 13) were incubated with KCl (to a final concentration of 0.6 M) for 10 min (lanes 2, 8, and 14), 20 min (lanes 3, 9, and 15), 30 min (lanes 4, 10, and 16), 40 min (lanes 5, 11, and 17), or 50 min (lanes 6, 12 and 18). The strains used were wild type (w.t.) (NT4), *tup11*Δ *tup12*Δ (SW76), and *sty1-1 tup11*Δ *tup12*Δ (SW90). Total RNA was extracted, separated by electrophoresis, and analyzed by Northern blotting with the indicated probes. (D) Quantification of the *cta3*$^+$ mRNA levels in C.

and the *tup11*Δ *tup12*Δ *atf1*Δ triple mutant strain was slightly less tolerant to $CaCl_2$ than the parental strains (Figure 6C).

We next addressed whether removal of Tup11-Tup12 repression rendered the induction of *cta3*$^+$ independent of Atf1. Deletion of *atf1*$^+$ in a *tup11*Δ *tup12*Δ mutant strain resulted in a further increase in *cta3*$^+$ transcript levels in unstressed cells, suggesting that nonactivated Atf1 may have a repressive effect on transcription that is independent of Tup11 and Tup12 (Figure 7, A and B). A similar effect has been observed previously; the decrease in the basal level of *ctt1*$^+$ mRNA associated with loss of Sty1 function is suppressed by deletion of *atf1*$^+$ (Degols and Russell, 1997). In the *atf1*Δ *tup11*Δ *tup12*Δ background, *cta3*$^+$ mRNA levels did not increase after exposure to KCl (0.6 M), indicating that Atf1 is absolutely required for induction of *cta3*$^+$ in response to a salt shock.

The bZIP transcription factor Pcr1 that can heterodimerize with Atf1 (Kanoh *et al.*, 1996) is also required for stress-mediated induction of *cta3*$^+$ expression (Figure 7, C and D). Examination of *cta3*$^+$ mRNA levels in a *pcr1*Δ *tup11*Δ *tup12*Δ strain revealed that Pcr1 is not required for the high level of basal expression, and furthermore in this strain expression of *cta3*$^+$ was partially induced in response to a salt shock. Thus, the Tup repressors ensure that induction of *cta3*$^+$ remains dependent upon the Sty1 MAP kinase and to a lesser extent, the activator Pcr1.

Prr1 Is Involved in Regulation of Gene Expression in Response to Elevated K$^+$ Ions

Our analysis suggests that another factor may regulate transcription of *cta3*$^+$ independently of Sty1. The Prr1 transcription factor is known to regulate oxidative stress responsive genes (Ohmiya *et al.*, 1999a), but there is no evidence that Sty1 regulates its activity directly. Therefore, we analyzed mRNA levels in a *prr1*Δ strain and found that the level of *cta3*$^+$ transcripts after exposure to KCl was significantly reduced in comparison with the wild-type strain (Figure 8, A and B). Furthermore, the influence of Prr1 was not confined to the *cta3*$^+$ gene because KCl-mediated induction of both *ctt1*$^+$ and *gpx1*$^+$ expression was also significantly reduced in the *prr1*Δ mutant strain. This was surprising because Prr1 has previously been reported not to be involved in the transcriptional response to high salt (Ohmiya *et al.*,

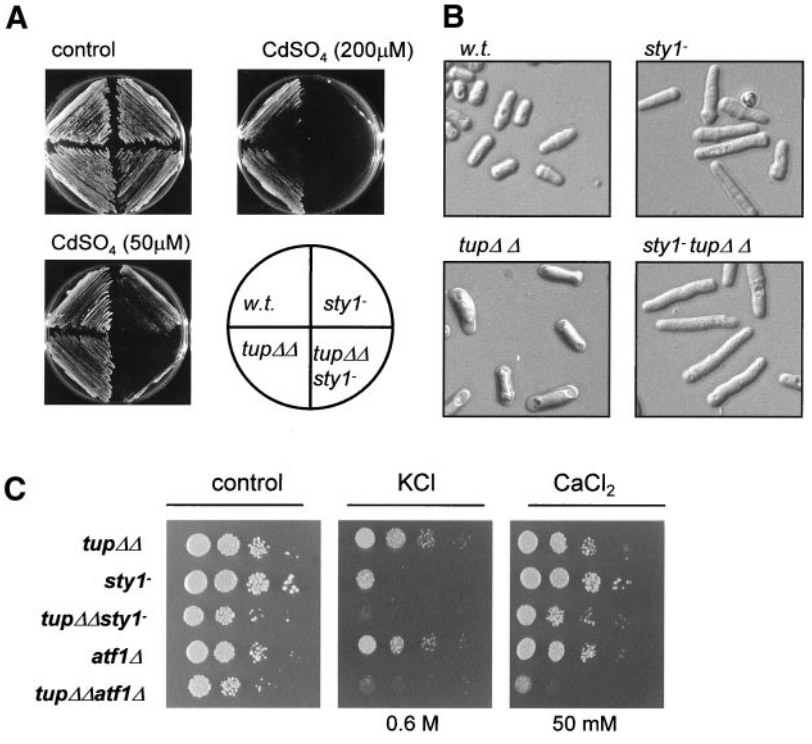

Figure 6. Genetic interactions. (A) Deletion of the *tup* genes does not rescue phenotypes associated with the *sty1-1* mutation. The indicated strains were subcultured onto YE5S agar (control) or subcultured onto YE5S agar supplemented with $CdSO_4$ (to the indicated concentration) and incubated at 30°C for 3–4 d. (B) Comparison of the morphology of wild type (w.t.) (NT4), *sty1-1* (JM1144), *tup11*Δ *tup12*Δ (SW76), and *sty1-1 tup11*Δ *tup12*Δ (SW90) cells. (C) Exponentially growing cultures were diluted serially, spotted onto YE5S agar or YE5S agar supplemented with $CaCl_2$ or KCl (at the indicated concentration), and incubated for 2 d at 30°C. The strains used were wild type (w.t.) (NT4), *tup11*Δ *tup12*Δ (SW76), *sty1-1* (JM1144), *sty1-1 tup11*Δ *tup12*Δ (SW90) *atf1*Δ (NT147), and *atf1*Δ *tup11*Δ *tup12*Δ (SW92).

1999a) and indeed KCl-mediated induction of some genes such as $gpd1^+$ occurs independently of Prr1 (Ohmiya *et al.*, 1999a; Figure 8, A and B). To determine the role that Prr1 plays in control of $cta3^+$ expression, we measured $cta3^+$ mRNA levels in a *tup11*Δ *tup12*Δ *prr1*Δ triple mutant strain. In this background the expression of $cta3^+$ was induced by exposure to high concentrations of KCl (0.6 M) (Figure 8, C and D). However, the deletion of $prr1^+$ in a *tup11*Δ *tup12*Δ background resulted in a decrease in the basal level of $cta3^+$ mRNA (Figure 8, C and D). Thus, Prr1 activity contributes to the high basal level of expression that is associated with loss of the Tup repressors.

In vitro experiments have demonstrated that recombinant Prr1 binds to a heat shock-like element in the $ste11^+$ promoter (Ohmiya *et al.*, 1999b). Analysis of the $cta3^+$ promoter revealed the presence of such an element (GGAAAATTC) located at −2068 relative to the initiation codon. However, in assays using this region of the promoter and whole cell extracts we were unable to detect a Prr1-dependent binding activity (our unpublished data). Therefore, we cannot exclude the possibility that the role of Prr1 in regulation of $cta3^+$ expression is indirect.

Tup11 and Tup12 Prevent Induction in Response to Inappropriate Stresses

Sty1, and thus in turn Atf1-Pcr1, is activated in response to a number of environmental insults. Accordingly, the expression of Atf1- and Pcr1-dependent genes such as $ctt1^+$, $pyp2^+$, and $gpx1^+$ are induced in response to a variety of stresses such as UV irradiation, heat shock, and hyperosmolarity and an oxidative stress elicited by exposure to high concentrations of H_2O_2 (Shiozaki and Russell, 1996; Wilkinson *et al.*, 1996; Degols and Russell, 1997; Nguyen *et al.*, 2000; Quinn *et al.*, 2002). However, some Atf1-Pcr1 target genes are induced only by a subset of these stresses. For example, we found that $cta3^+$ expression was induced specifically in response to salt shock but not by oxidative stress (6 mM H_2O_2) or by heat shock (15 min at 42°C) (Figure 9, A and D). In contrast, $ctt1^+$ and $gpx1^+$ mRNA levels were both induced by these treatments, indicating that Atf1 (and Pcr1) were active under these conditions. This indicates that activation of the Sty1 pathway per se is not sufficient to induce the expression of $cta3^+$ and mechanisms must exist to prevent induction of gene expression in response to such "inappropriate stresses." We wanted to examine the possibility that Tup11 and Tup12 play a role in this process. Therefore, we measured the levels of $cta3^+$ mRNA after exposing a *tup11*Δ *tup12*Δ strain to an oxidative stress (6 mM H_2O_2) and a heat shock (15 min at 42°C). In contrast to the wild-type strain, the expression of $cta3^+$ was significantly induced by heat stress and by exposure to high levels of H_2O_2. In addition, the level of $cta3^+$ transcripts was induced by hypotonic conditions in a *tup11*Δ *tup12*Δ strain but not in a wild-type strain (our unpublished data). We also examined $cta3^+$ transcript levels in a *sty1*Δ *tup11*Δ *tup12*Δ triple mutant strain (Figure 9, B and D). This revealed that the induction in expression in response to heat shock was partly independent of the MAP kinase. In contrast, the induction of $cta3^+$ expression in response to oxidative stress mediated by H_2O_2 was completely dependent upon Sty1, suggesting a difference in the mechanism of induction. Further analysis indicated that the response to heat shock was independent of Prr1 (Figure 9, C and D) but dependent upon Atf1 (our unpublished data).

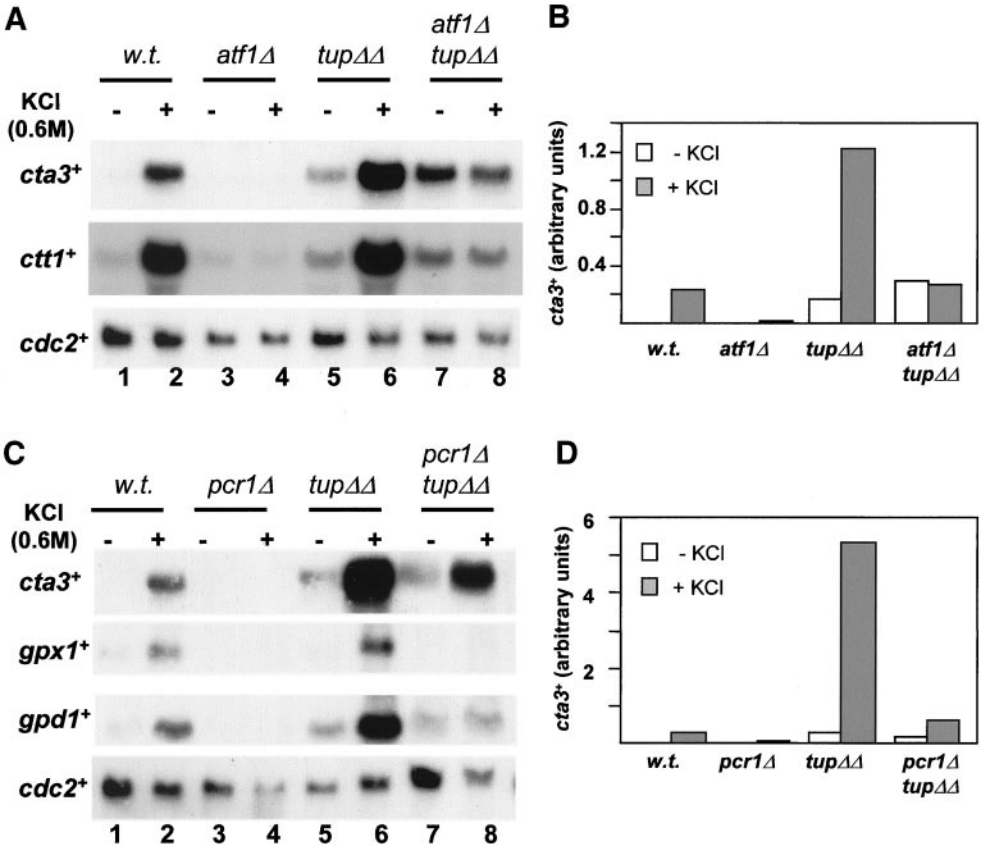

Figure 7. Transcription induction of *cta3*$^+$ in cells lacking Tup11 and Tup12 is Atf1 dependent. (A) Strains used are indicated above the lanes and were wild type (w.t.) (NT4), *atf1*Δ (NT147), *tup11*Δ *tup12*Δ (SW76), and *atf1*Δ *tup11*Δ *tup12*Δ (SW92). Log phase cultures growing at 30°C in YE5S (lanes 1, 3, 5, and 7) were incubated with KCl (to a final concentration of 0.6 M) for 15 min (lanes 2, 4, 6, and 8). Total RNA was extracted, separated by electrophoresis and subjected to Northern analysis with the indicated probes. The level of *cdc2*$^+$ mRNA was used as a loading control. (B) Quantification of *cta3*$^+$ mRNA levels in A. (C) As for A, except strains used were wild type (w.t.) (NT4), *pcr1*Δ (JX25), *tup11*Δ *tup12*Δ (SW76), and *pcr1*Δ *tup11*Δ *tup12*Δ (RJP59). (D) Quantification of *cta3*$^+$ mRNA levels in C.

Taken together, these findings indicate that the Tup repressors function as part of the mechanism that ensures the specificity of stress-mediated transcriptional induction at the *cta3*$^+$ promoter.

DISCUSSION

In this study, we reveal roles for *S. pombe* Tup11 and Tup12 in the cellular response to elevated K^+ and Ca^{2+} levels. We identify *cta3*$^+$ as a novel stress-induced gene whose transcription is coregulated by the Sty1 MAP kinase pathway and the Tup repressors. Our results indicate that the Tup repressors fulfill a number of functions in the control of *cta3*$^+$ expression. First, they maintain low levels of basal expression and limit the level of induction. Second, they ensure that induction of expression is linked to the Sty1 pathway. And third, they maintain the specificity of induction. We also reveal a new role for the response regulator Prr1 and demonstrate that it functions to regulate gene expression in response to elevated salt concentrations. Prr1 is known to contribute to the regulation of several genes whose expression is induced by oxidative stress via the Pap1 transcription factor (Ohmiya *et al.*, 1999a), and so Prr1 regulates gene expression in response to a number of stresses.

Tup11–Tup12 Interaction

Our data and that of others (Mukai *et al.*, 1999; Janoo *et al.*, 2001) suggest that Tup11 and Tup12 can function in homomeric complexes. In addition, we demonstrate that Tup11 and Tup12 have the potential to form a heteromeric complex. This is significant because full function requires both repressors; some derepression of a *fbp1::lacZ* reporter is observed upon deletion of a single *tup* gene (Janoo *et al.*, 2001). Furthermore, single *tup* mutants have demonstrable phenotypes such as increased resistance to cadmium. Thus, regulation of some genes may depend upon both repressors and the formation of heteromeric Tup complexes.

Relief of Tup11-Tup12–mediated Repression

The Hog1 MAP kinase in *S. cerevisiae* plays a direct role in relieving Ssn6-Tup1–mediated repression at osmostress genes; Hog1 phosphorylates Sko1, reducing its affinity for the corepressor complex (Proft *et al.*, 2001). It is possible that the Sty1 MAP kinase may similarly antagonize the action of Tup11-Tup12; however, our results demonstrate that the Atf1, Pcr1, and Prr1 transcription factors are required for relief from Tup-mediated repression at the *cta3*$^+$ promoter. It is probable that the *S. pombe* Tup proteins function, at least in part, through the organization of repressive chromatin structures (Mukai *et al.*, 1999), and therefore it is possible that Atf1-Pcr1 and Prr1 overcome this repression by recruiting positive-acting chromatin remodeling complexes such as Swi-Snf or histone acetylase complexes (HATs). In support of this, DNA binding by the Atf1-Pcr1 heterodimer is known to alter local nucleosome positioning at the *ade6-M26* hotspot and thereby promote meiotic recombination (Mizuno *et*

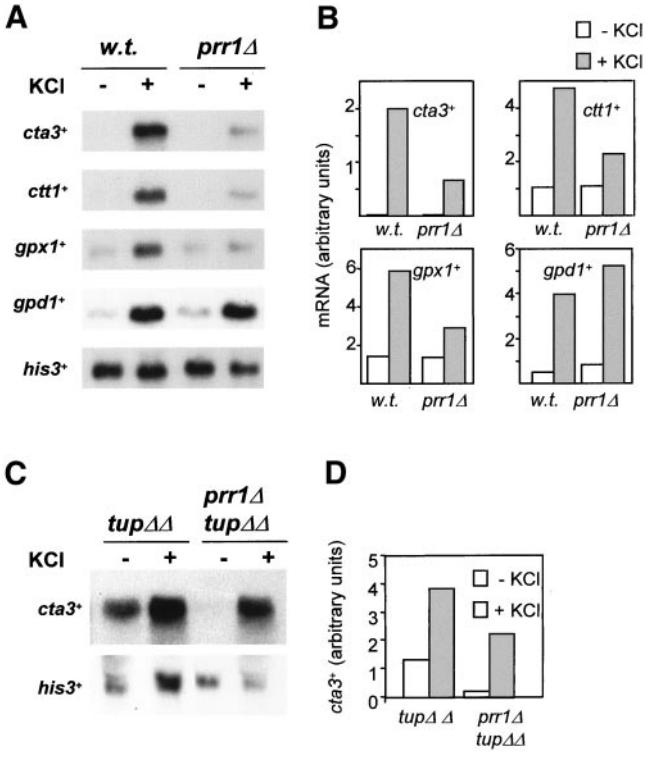

Figure 8. Prr1 is involved in the regulation of gene expression in response to high salt. (A) Strains used are indicated above the lanes and were wild type (w.t.) (NT4) and *prr1*Δ (SW97). Log phase cultures growing at 30°C in YE5S (lanes 1 and 3) were incubated with KCl (to a final concentration of 0.6 M) for 15 min (lanes 2 and 4). Total RNA was prepared and subjected to Northern analysis with the indicated probes. The level of *his3*$^+$ mRNA was used as a loading control. (B) Quantification of the mRNA levels in A. (C) Strains used are indicated above the lanes and were *tup11*Δ *tup12*Δ (SW76) and *prr1*Δ *tup11*Δ *tup12*Δ (SW96), and the treatment was as described in A. (D) Quantification of the mRNA levels in C.

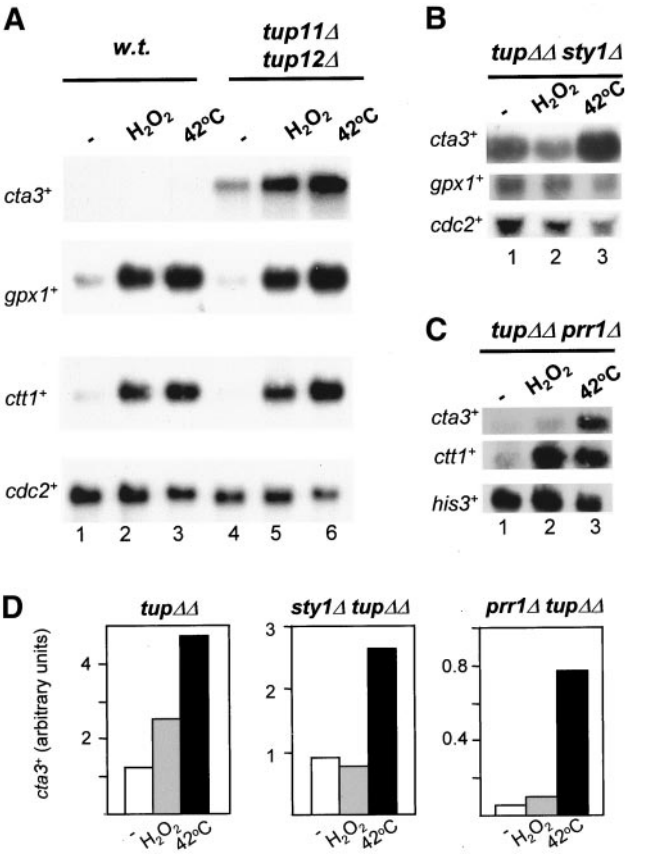

Figure 9. Tup11 and Tup12 prevent induction in response to inappropriate stresses. (A) Strains used were wild type (w.t.) (NT4) and *tup11*Δ *tup12*Δ (SW76). Mid log cultures growing at 30°C in YE5S (lanes 1 and 4) were incubated with H_2O_2 (final concentration 6 mM) for 15 min (lanes 2 and 5) or shifted to 42°C for 15 min (lanes 3 and 6). Total RNA was prepared and subjected to Northern analysis with the indicated probes. The level of *cdc2*$^+$ mRNA was used as a loading control. (B) Strain used was *sty1*Δ *tup11*Δ *tup12*Δ (SW91) and the treatments were as described in A. (C) Strain used was *prr1*Δ *tup11*Δ *tup12*Δ (SW91) and the treatments were as described in A. (D) Quantification of the *cta3*$^+$ mRNA levels in A, B, and C. The strains are indicated above the graphs and the treatments are indicated below.

al., 1997; Kon *et al.*, 1998). Moreover, genes such as *SUC2* in *S. cerevisiae* are regulated by the interplay between Ssn6-Tup1 repression and Swi-Snf–mediated activation (Gavin and Simpson, 1997).

In *S. cerevisiae*, Hog1-dependent transcriptional induction of *HAL1* requires the Gcn4 activator that relieves Tup1-Ssn6–mediated repression by competing with Sko1 for the occupancy of a single CRE binding site (Pascual-Ahuir *et al.*, 2001). This CRE element functions as a dual control element and integrates both positive and negative regulatory signals. Furthermore, analysis of the *S. pombe* *fbp1*$^+$ promoter, which is regulated by both Atf1-Pcr1 and Tup11-Tup12, has demonstrated the presence of a control element (called UAS2) that contains a CRE-like sequence and is bound by multiple activators and repressors (Neely and Hoffman, 2000). Interestingly, the Atf1-Pcr1 transcription factor does not bind to UAS2 directly, but it does influence the protein complexes that assemble on it (Neely and Hoffman 2000). The *cta3*$^+$ promoter contains a number of CRE-like elements at least one of which mediates Atf1 binding (Figure 3C). It will be interesting to determine the contributions of these elements to activation and repression of *cta3*$^+$ transcription.

Tup Repressors Maintain Specificity of Induction

The advent of stressful conditions results in the rapid and Sty1-dependent phosphorylation of Atf1 (Shiozaki and Russell, 1996; Wilkinson *et al.*, 1996). Although the precise role of Atf1 phosphorylation remains obscure, it is evident that transcriptional activation by Atf1 is dependent upon Sty1. However, deletion of the *tup* genes allows transcriptional induction of *cta3*$^+$ to occur in *sty1*$^-$ cells. Furthermore, in a *tup11*Δ *tup12*Δ mutant induction of *cta3*$^+$ expression does not require Pcr1 or Prr1. Thus, the Tup repressors function to "wire" induction to the Sty1 pathway, insulating it from

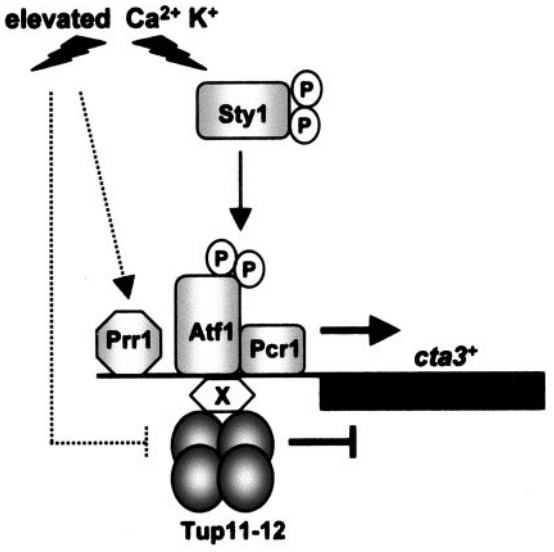

Figure 10. Model for the regulation of *cta3*$^+$ expression. Under nonstress conditions, *cta3*$^+$ expression is repressed by Tup11 and/or Tup12 that are tethered to the promoter through interaction with a site-specific DNA binding protein "X." Activation of the Sty1 pathway alone is insufficient to induce expression, and the Tup repressors prevent activation by Atf1-Pcr1 and Prr1. Elevated Ca^{2+} or K^+ concentrations trigger the activity of other pathways (indicated by dashed lines) that interfere with Tup repression and/or facilitate activation via the response regulator Prr1 and Atf1-Pcr1. In cells lacking the Tup repressors, specificity is lost and expression is induced in response to a range of stresses.

interfering signals. These results also suggest that Atf1 activity can be "uncoupled" from Sty1 in this specific case and that an additional mechanism for activating transcription that requires Atf1 exists. The finding that Prr1 also controls expression of *cta3*$^+$ suggests that it may function as part of this mechanism.

The Sty1 pathway in *S. pombe* is fundamentally different to the Hog1 pathway in *S. cerevisiae* because it is triggered by exposure to a wide range of adverse environmental conditions. As a consequence, a large number of Sty1 target genes are up-regulated by multiple stresses. The products of such genes may comprise a set of "general stress response proteins" that are necessary because a single environmental insult may result in multiple classes of intracellular stress (Rep *et al.*, 2001). Nonetheless, discrete stimuli also produce distinct transcriptional outputs, because there are subsets of Sty1-dependent genes, such as *cta3*$^+$, that are induced only by specific stresses. A major question to be addressed is the mechanism by which Sty1 signaling is integrated into the regulation of such genes. Expression of *cta3*$^+$ is not induced by oxidative stress, heat shock, carbon limitation, or sexual differentiation (Figure 6; our unpublished data), and furthermore *cta3*$^+$ is only poorly induced by an osmotic shock mediated by high sorbitol concentrations (Nishikawa *et al.*, 1999). Thus, the transcriptional response is triggered essentially by elevated intracellular cation concentrations rather than by an osmotic effect (i.e., decrease in turgor pressure across the plasma membrane). The *cta3*$^+$ gene encodes a putative intracellular P-type ATPase transporter that is involved in cation extrusion or sequestration into intracellular compartments. Loss of function leads to an accumulation of cytoplasmic Ca^{2+} levels (Ghislain *et al.*, 1990; Halachmi *et al.*, 1992), although recent evidence suggests that Cta3 is primarily a K^+ ion pump (Benito *et al.*, 2002). It is thus consistent that it is salt stress that specifically that triggers its transcriptional induction. However, removal of the constraints imposed by Tup repressors allows *cta3*$^+$ to be induced in response to other stresses such as elevated temperature and oxidative stress. Thus, the Tup repressors function as a part of a mechanism that adds specificity to Sty1-dependent transcriptional induction.

Our results also indicate that activation of the Sty1 pathway alone is insufficient to induce *cta3*$^+$ expression and implies that an elevated cation concentration triggers an additional pathway that is required to circumvent repression (Figure 10). In this respect it may be significant that Prr1 is involved in the regulation of *cta3*$^+$ expression because its structure suggests that it may be one part the target of a histidine-aspartate phosphorelay pathway. Recent work has identified several of these pathways in fission yeast (Nguyen *et al.*, 2000; Buck *et al.*, 2001), and current experiments are addressing its contribution to the regulation of Prr1 in the response to stress.

ACKNOWLEDGMENTS

We thank Janet Quinn, Mark Toone, and Burk Braun for advice on the manuscript. We also thank Burk Braun, Hirofumi Aiba, and Jonathan Millar for providing strains. This work was supported by a National Institutes of Health grant GM-46226 (to C.S.H.), by a Medical Research Council Ph.D. research studentship (to P.M.), a Medical Research Council Career Establishment Grant (to B.A.M.), and by a Biotechnology and Biological Sciences Research Council project grant (13/P11981) (to S.K.W.).

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