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Functional Comparison of the Tup11 and Tup12 Transcriptional Corepressors in Fission Yeast†

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Gene duplication is considered an important evolutionary mechanism. Unlike many characterized species, the fission yeast *Schizosaccharomyces pombe* contains two paralogous genes, *tup11*$^+$ and *tup12*$^+$, that encode transcriptional corepressors similar to the well-characterized budding yeast Tup1 protein. Previous reports have suggested that Tup11 and Tup12 proteins play redundant roles. Consistently, we show that the two Tup proteins can interact together when expressed at normal levels and that each can independently interact with the Ssn6 protein, as seen for Tup1 in budding yeast. However, *tup11*$^-$ and *tup12*$^-$ mutants have different phenotypes on media containing KCl and $CaCl_2$. Consistent with the functional difference between *tup11*$^-$ and *tup12*$^-$ mutants, we identified a number of genes in genome-wide gene expression experiments that are differentially affected by mutations in the *tup11*$^+$ and *tup12*$^+$ genes. Many of these genes are differentially derepressed in *tup11*$^-$ mutants and are over-represented in genes that have previously been shown to respond to a range of different stress conditions. Genes specifically derepressed in *tup12*$^-$ mutants require the Ssn6 protein for their repression. As for Tup12, Ssn6 is also required for efficient adaptation to KCl- and $CaCl_2$-mediated stress. We conclude that Tup11 and Tup12 are at least partly functionally diverged and suggest that the Tup12 and Ssn6 proteins have adopted a specific role in regulation of the stress response.

In the budding yeast, *Saccharomyces cerevisiae*, the Tup1-Ssn6 corepressor, which is important for repression of many genes involved in a wide range of physiological processes, has been studied extensively (7, 32). Recent work has suggested that Tup1-Ssn6 also plays an important role during derepression of at least some target genes (22, 26). The active form of the corepressor is thought to consist of a protein complex containing four Tup1 subunits that together interact with a single Ssn6 subunit (30, 38). Molecular dissection of the Tup1 protein has revealed three functionally defined domains. The C-terminal contains a sevenfold repeated WD 40 domain that can form a β-transducin-like propeller structure important for protein interactions and tetramerization (33), whereas the N terminus is involved in the interaction with Ssn6 (4, 43, 44). The third, central domain is required for the repression activity of the complex (10, 37). Repression of target genes has been reported to occur by different molecular mechanisms. First, Tup1-Ssn6 can recruit histone deacetylases to genes, which results in deacetylation of histones and thereby a repressive chromatin structure (10, 39, 41). Second, Tup1-Ssn6 interacts with hypoacetylated N-terminal tails of histones H3 and H4 that have been programmed for repression by the action of histone deacetylases (8–10). Third, Tup1-Ssn6 has also been reported to interfere directly with the transcriptional machinery by interacting with factors important for the repressive activity of the mediator subcomplex of the RNA polymerase II holoenzyme (21, 25).

The Tup1-Ssn6 complex is recruited to the promoters of target genes by interaction with DNA bound transcriptional repressor proteins that recognize specific sequences within target gene promoters. Examples of such repressor proteins include the Matα2 repressor that regulates mating-type-specific genes (19) and the Mig1 repressor that regulates glucose-repressed genes (35), as well as Crt1 and Sko1, which are involved in the control of DNA repair (15) and hyperosmotic stress (28), respectively. Recently, this gene-specific role has been complemented by observations that Tup1 might be involved in establishing domains of heterochromatin structure in the subtelomeric regions of chromosomes (31). These so-called HAST domains contain clusters of Tup1 and Ssn6 repressed genes and coincide with regions that are deacetylated by the histone deacetylase Hda1. It has thus been suggested that Tup1-Ssn6 establishes formation of heterochromatin in these regions by recruiting Hda1. HAST domains are distinct from adjacent heterochromatin regions that are established via Tup1-independent recruitment of the Sir2 histone deacetylase.

In the evolutionarily distant fission yeast (*Schizosaccharomyces pombe*) there are a number of differences in the mechanisms involved in gene-specific repression and the formation of transcriptionally silent regions of heterochromatin compared to *S. cerevisiae*. Most notably, fission yeast have an interfering RNA (RNAi)-based mechanism for establishment and maintenance of silent heterochromatin similar to other eukaryotes that is apparently absent from *S. cerevisiae* (11, 40). In spite of these differences, the Tup1-Ssn6 corepressor is conserved throughout fungi and there are also related corepressors in higher eukaryotes such as HIRA, TLE1, and Groucho, which are functionally homologous to the yeast corepressor (6, 7). The fission yeast is unusual compared to other fungi because it contains two genes encoding Tup1-like corepressors. Evidence that the *S. pombe* proteins function similarly to the Tup1-Ssn6

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TABLE 1. *S. pombe* strains used in this study

Strain	Genotype	Source or reference
JY741	*h⁻ ura4-D18 leu1-32 ade6M216*	24
JY741 (Δ*tup11*)	*Δtup11::ura4⁺ h⁻ ura4-D18 leu1-32 ade6M216*	24
JY741 (Δ*tup12*)	*Δtup12::LEU2⁺ h⁻ ura4-D18 leu1-32 ade6M216*	24
JY741 (Δ*tup11,12*)	*Δtup11::ura4⁺ Δtup12::LEU2⁺ h⁻ ura4-D18 leu1-32 ade6M216*	24
Fy367	*h⁺ ura4-D18 leu1-32 ade6M210*	3
Hu494	*ssn6-HA kanMX h⁺ ura4-D18 leu1-32 ade6-210*	This study
Hu497	*ssn6-GFP kanMX h⁺ ura4-D18 leu1-32 ade6-210*	This study
Hu853	*tup11-GFP kanMX h⁺ ura4-D18 leu1-32 ade6-M210*	This study
Hu851	*tup12-GFP kanMX h⁺ ura4-D18 leu1-32 ade6-M210*	This study
FFB21	*tup11-ha kanMX h⁺ ura4-D18 leu1-32 ade6-M210*	This study
FFB23	*tup12-ha kanMX h⁺ ura4-D18 leu1-32 ade6-M210*	This study
Hu855	*tup11-GFP kanMX tup12-HA kanMX ura4-D18 leu1-32 ade6-M210*	This study
Hu856	*ssn6-GFP kanMX tup12-HA kanMX ura4-D18 leu1-32 ade6-M210*	This study
FFB13	*ssn6-GFP kanMX tup11-HA kanMX ura4-D18 leu1-32 ade6-M210*	This study
FFB34	*ssn6-GFP kanMX tup11-HA kanMX Δtup12::LEU2⁺ ura4-D18 leu1-32 ade6-M210*	This study
FFB37	*ssn6-GFP kanMX tup12-HA kanMX Δtup11::ura4⁺ ura4-D18 leu1-32 ade6-M210*	This study

complex in *S. cerevisiae* has been reported recently (12, 14, 16, 23, 24, 27, 45). Tup11 has been shown to repress target genes containing LexA binding sites when fused to the LexA DNA-binding domain, and binding studies have shown that Tup11 is able to interact with the *S. cerevisiae* transcription factor Matα2 (24). Studies with Tup11 and Tup12 have also revealed that they both act as negative regulators of the *S. pombe fbp1⁺* gene in a redundant fashion (16). As in *S. cerevisiae*, the *tup11⁻* and *tup12⁻* deletion phenotypes are also associated with flocculation in liquid media, defective mating, and defects in stress responses. Examples of known target genes for Tup11 and Tup12 are the intracellular cation transporter *cta3⁺*, which is activated under high-salt conditions (12). Tup11 has also been reported to interact directly with Fep1, a transcription factor that represses the expression of the iron transport genes *fio1⁺* and *fip1⁺* in response to high iron concentrations (27, 45).

Gene duplication is thought to be an important mechanism involved in the evolution of biological diversity. The apparent gene duplication that has given rise to the *tup11⁺* and *tup12⁺* genes, located close to the end of chromosome 1 in fission yeast, therefore offers an interesting opportunity to study this process. As expected, Tup11 and Tup12 have been shown to play at least partly redundant roles. However, no comparative study has been made to determine whether and to what extent the significant sequence divergence between the two proteins is associated with differences in their function. We show here that both proteins have retained the ability to interact with each other and with the fission yeast Ssn6 protein but that Tup12 has evolved a specialized role in the regulation of the stress response.

MATERIALS AND METHODS

Strains and growth conditions. All *S. pombe* strains used in the present study are listed in Table 1. Strains were cultivated at 30°C in rich yeast extract medium YES containing 0.5% yeast extract and 3% glucose supplemented with 75 mg of required amino acids per liter or in synthetic minimal MM medium as described previously (1). Spotting assays were performed by spotting 5-fold serial dilutions of cells on rich medium supplemented with KCl or $CaCl_2$. Plasmids encoding full-length *tup11* and *tup12* cloned into pRep42 as described previously (12) were transformed by electroporation and selected on MM medium. The *tup11⁺* and *tup12⁺* open reading frame (SPAC18B11.10 and SPAC620.14c, respectively) and the *ssn6⁺* open reading frame (SPAC23E6.09) were tagged by a PCR-based approach described previously (2). The following gene specific primer pairs were used for immunotagging the strains in Table 1: Tup12, 5′-GCTGTTAGCCCGAATGGCCATTGCTTTGCTACTGGTAGTGGTGACTTACGAGCAAGAATTTGGTCTTATGAGGATCTGCGGATCCCCGGGTTAATTA-3′ and 5′-TTGTCGGGGATTGTATGGAAGTATCAAAAATTAAAAAAAGGGAATTGAAATGATTCAAGAATTAGCAAAAACATCTGGGAATTCGAGCTCGTTTAAAC-3′; Tup11, 5′-AGCCCAGATGGTAGGCAATTTGCTTCGGGAAGTGGTGATTTACGTGCTCGCATATGGTCAATTGACCCTCATCTCCTCGGATCCCCGGGTTAATTAA-3′ and 5′-ATTAAGGAATTTTTTATTAATTGTTTATTTTTTAAATAAAAAGTGTCTCAGTTAATTTATGAGAGAAGCCATTCTCCAGAATTCGACCTCGTTTAAAC-3′; and Ssn6, 5′-CCAAAGCAAGCAGCAAGGACTCTCGATATTGATGAAAATTACGATGATGATGAGGGAGAAAAAGAAACCGTGTCAGTTCGGATCCCCGGGTTAATTAA-3′ and 5′-TTCCCGCGTATCAGCTACACCAGTATCATCAATTTTTAAAATATGTATGACTATTGTAAGCAAATTTCAAATGTGAGCGAATTCGAGCTCGTTTAAAC-3′.

Oligonucleotides were used to amplify hemagglutinin (HA) or green fluorescent protein (GFP) containing KanMX fragments from the pFA6-HA-KanMX and pFA6-GFP-KanMX vectors, respectively. The amplified fragments were electroporated into the wild-type Fy367 strain to generate immunotagged strains containing the KanMX marker. Integration at the correct locus was confirmed by PCR.

Western blotting and coimmunoprecipitation. Chromosomally tagged yeast strains were grown in YES medium for 18 h to mid-log phase (10^7 cells/ml). Cells were harvested and washed in NP-40 lysis buffer (0.150 M NaCl, 0.050 M Tris [pH 8], 1% NP-40 with protease inhibitors [Complete cocktail inhibitors; Roche catalog no. 1873580], leupeptin at 2 μg/ml, and 1 mM phenylmethylsulfonyl fluoride). Cell extract was made with glass beads in a bead beater. For immunoprecipitation experiments, the extract was precleared with protein A (Sigma catalog no. P-1406) coupled beads and incubated with a polyclonal rabbit α-GFP antibody (Clontech catalog no. 8372-2). For 500 μl of extract, 1 μl of α-GFP antibody was used. The antibodies were precipitated with protein A-coupled beads as described above. The precipitated beads were washed three times and resuspended in sodium dodecyl sulfate (SDS) loading buffer. Resuspended protein extracts were resolved on SDS-polyacrylamide gel electrophoresis (PAGE) and subjected to Western blot analysis. The membrane was probed with monoclonal mouse α-HA antibody (Roche catalog no. 12CA5).

Immunofluorescence microscopy. Chromosomally double-tagged strains were grown for 18 h to mid-log phase (10^7 cells/ml), harvested, fixed with formaldehyde, and incubated with primary and secondary antibodies as described previously (3). The following primary antibodies were used: α-HA monoclonal mouse (Roche catalog no. 12CA5) and α-GFP polyclonal rabbit (Molecular Probes catalog no. 11121). Secondary antibodies conjugated with Texas red or fluorescein isothiocyanate (Jackson Immunoresearch Laboratories) were used. Nuclear staining was performed with DAPI (4′,6′-diamidino-2-phenylindole). The cells were subjected to confocal microscopy with a Zeiss Axioscope II microscope. Digital deconvolution was performed with Openlab software by using 0.3-μm z spacing and nearest-neighbor deconvolution.

Protein alignments and phylogenetic clustering. Protein sequences were subjected to CLUSTAL W alignment with Macvector 6.5 software by using the CLUSTAL W blosum matrix with a gap penalty of 20. The following sequences were handled and analyzed: *S. cerevisiae*, Tup1 (P16649); *Saccharo-*

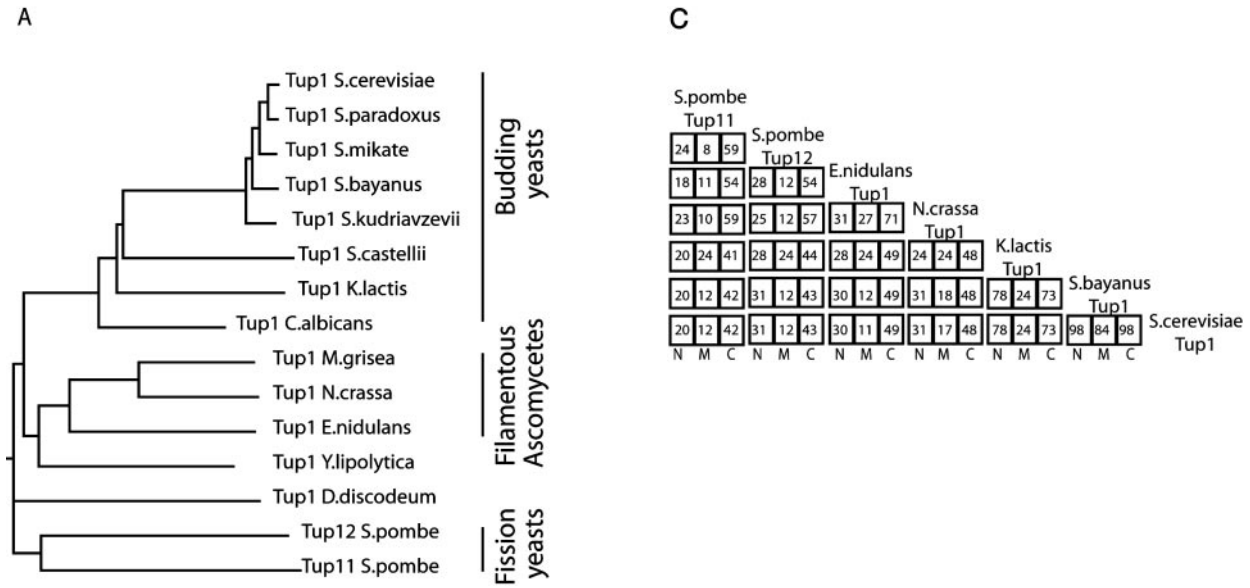


FIG. 1. Phylogenetic relationships within the Tup1 family. (A) Phylogenetic dendrogram of the Tup1 protein family. Full-length protein sequences were compared and ordered by their relationship into phylogenetic classes. The different Tup1 proteins are indicated in the figure and relate to the following species: Tup1, *S.cerevisiae*; Tup1, *S, paradoxus*; Tup1, *S. mikate*; Tup1, *Saccharomyces bayanus*; Tup1, *S. kudriavzevii*; Tup1, *S. castellii*; Tup1, *K. lactis*; Tup1, *C. albicans*; Tup1, *M. grisea*; Tup1, *N. crassa*; Tup1, *E. nidulans*; Tup1, *Y. lipolytica*; Tup1, *D. discodeum*; Tup11, *S. pombe*; and Tup12, *S. pombe.* (B) Amino acid sequence alignment of Tup1 homologues. The figure shows sequence comparison of the full-length *S. pombe* Tup11 (614 amino acids [aa]) and Tup12 (586 aa) with *E. nidulans* Tup1 (619 aa), *N. crassa* Tup1 (604 aa), *K. lactis* Tup1 (682 aa), *S. bayanus* Tup1 (717 aa), and *S. cerevisiae* Tup1 (713 aa). Identical residues are highlighted with dark shading, and similar residues are highlighted with light shading. The different Tup protein domains are indicated. Fungal Tup proteins share the same domain architecture and a general high sequence similarity. (C) Cross comparison of the individual Tup protein domain sequences. Sequences identified as N-terminal domain (N), intermediate domain (M), or C-terminal domain (C) in panel B were subjected to a cross comparison between the different species. The boxed numbers in the figure correspond to the percentage identical protein residues over the defined amino acid stretch. The phylogenetic tree in panel A and the comparisons in panels B and C were generated with a CLUSTAL W multiple sequence program (see Materials and Methods).

myces paradoxus, Tup1 (MIT_Spar_c90_2709); *Saccharomyces mikate*, Tup1 (Smik_Contig2778.4); *Saccharomyces bayanus*, Tup1 (Sbay_Contig666.45); *Saccharomyces kudriavzevii*, Tup1 (Skud_Contig1764.4); *Saccharomyces castellii*, Tup1 (ScTup1 as_Contig629.12); *Kluyveromyces lactis*, Tup1 (P56094); *Candida albicans*, Tup1 (P56093); *Magnaporthe grisea*, Tup1 (AACUO1001716.1); *Neurospora crassa*, Tup1 (P78706); *Emericella nidulans*, Tup1 (AAB63194); *Yarrowia lipolytica*, Tup1 (CAC81004); *Dictostelium discodeum*, Tup1 (AAC29438); *S. pombe*, Tup11 (Q09715); and *S. pombe*, Tup12 (Q9UUG8). Full-length protein sequences were used in Fig. 1A and B. The cross comparison in Fig. 1C was generated by comparing the different Tup1 domain as defined in Fig. 1B.

Microarray analysis. Cells from Δ*tup11* and Δ*tup12* strains were grown in YES medium at 30°C for 18 h to early mid-log phase (0.5×10^7 cells/ml), followed by a medium change for 45 min. For normal conditions the cells were transferred to YES medium, and for stress induction the cells were transferred to YES medium containing 1 M KCl for 45 min. Cells were harvested and immediately placed in lysis buffer and phenol. Two RNA samples from independent cultures were prepared for each condition, and 25 μg of RNA was subjected to reverse transcription (RT; 11904-018 Invitrogen) and labeled with CY3 (CY3 dCTP 53021; Amersham) or CY5 (CY5 dCTP 55021; Amersham) prior to hybridization on *S. pombe* gene microarrays from Eurogentec SA, Seraing, Belgium (42). Altogether four microarrays spotted in duplicate were used, which generated four datum points for each condition and gene. The microarray signals were visualized with a ScanExpress laser scanner and quantified with the Imagene 4.2 software. The data were analyzed and normalized with the Lowess per-spot per-chip method with the GeneSpring software (Silicon Genetics). Data with a standard deviation of >80% of the mean value were excluded. To assess the significance of the twofold differences identified, we performed a one-sample, one-tailed *t* test to test the null hypothesis that observed changes are not significant. Stress data for the individual Tup11/12 differentiated genes and control groups were extracted from the Sanger Institute database (www.sanger.ac.uk/PostGenomics/S_pombe/projects/stress/) (5). To calculate the enrichment of genes found in core environmental stress response (CESR) clusters and to determine the significance for observed results by chance, we used the hypergeometric distribution test in the GeneSpring software.

RT-PCR. RNA from JY741, JY741(Δ*tup11*), JY741(Δ*tup12*), Y741(Δ*tup11,12*) was extracted independently as described above and was subjected to DNase treatment and RT (11904-018; Invitrogen) for synthesis of cDNA. Samples were subjected to duplex PCR, and the generated products were separated with electrophoresis on a 1.5% agarose gel and visualized with ethidium bromide. Gels were digitalized and quantified with the Imagauge 4.0 software.

RESULTS

Comparison of Tup1 family proteins. In order to view the differences between the two Tup1-like corepressor proteins in fission yeast with a broader perspective, we compared the primary sequences of these proteins with homologues found in other fungi and the slime mold (*Dictostelium discodeum*). Of the species we have investigated, fission yeast is the only case in which Tup1 corepressors are encoded by more than one gene. The relative similarities between the Tup1 proteins from different species (Fig. 1A) generally reflect the overall phylogenetic differences between the species. Thus, Tup1 proteins from budding yeasts and filamentous fungi are found in different clades. The two fission yeast Tup1-like proteins are found in a clade that is separate from both of these groups consistent with the separate phylogenetic classification of fission yeast in the Archaeascomycetes. The similarity relationships between the different Tup1-like proteins are therefore consistent with a duplication of the Tup1 gene specifically in the Archaeascomycetes, but they also suggest that the duplication was an early event in the evolution of this clade.

The fungal Tup proteins are similar to each other through-

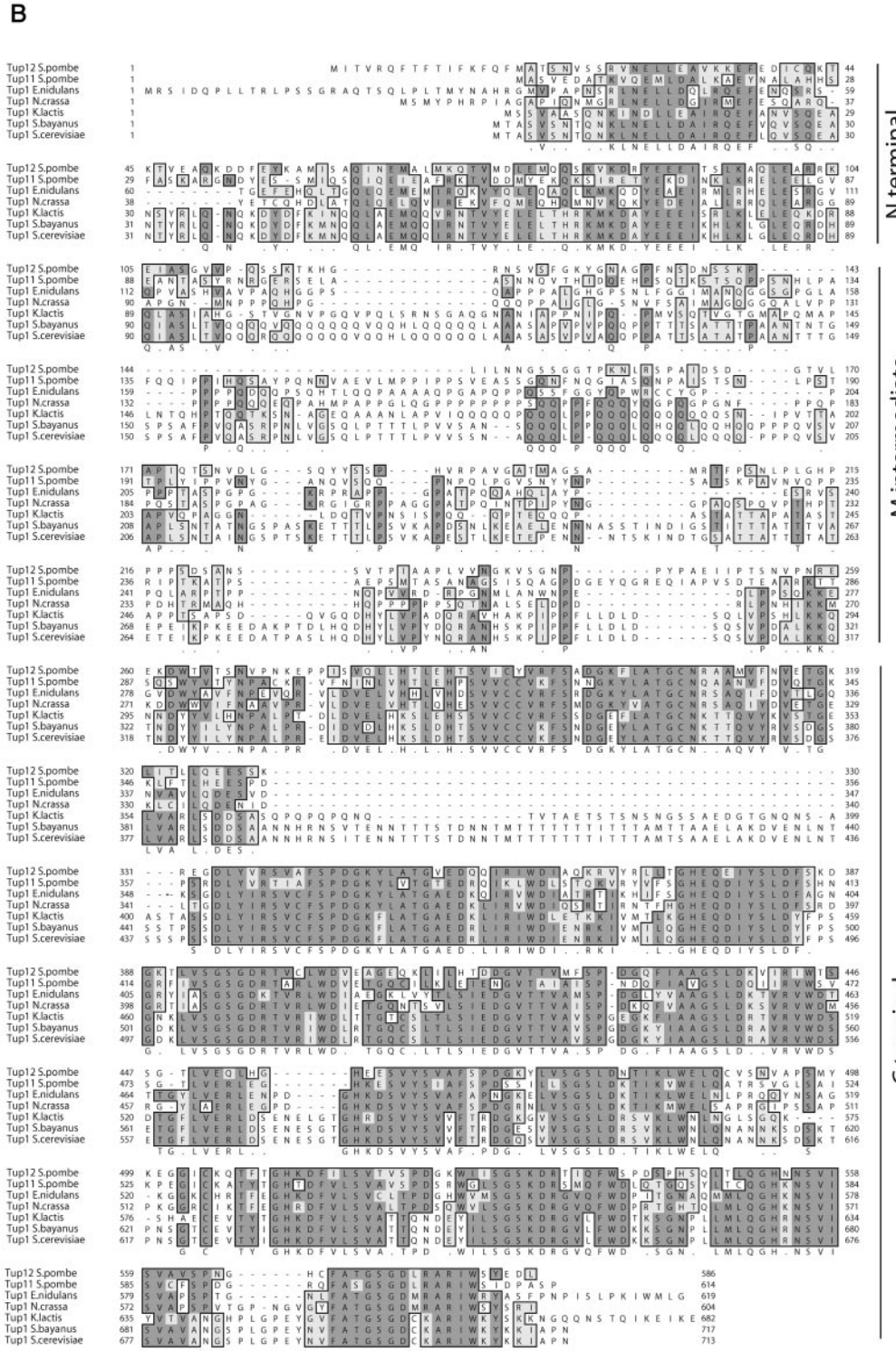


FIG. 1—*Continued*.

out their protein sequence, and they share the same domain architecture (Fig. 1B). The proteins are all highly homologous in the WD40 C-terminal domain and less conserved in the N-terminal Ssn6-binding domain and the variable central domain, proposed to play a role in the repression activity of the protein. Sequence alignments of the individual domains show that these three different domains are differentially conserved in relation to evolutionary distance (Fig. 1C). For example, Tup11 and Tup12 that are most similar to each other overall show the lowest degree of similarity in the central domain of any of the species compared in Fig. 1C. This suggests that the different domains have evolved as separate functional units.

Relative expression levels, interaction, and localization of the Tup11 and Tup12 proteins. Since Tup11 and Tup12 are most conserved in the C-terminal domain that has been implicated in tetramerization of Tup1-like proteins, we investigated aspects significant to interaction between Tup11 and Tup12. Previous work (12) has shown that Tup11 and Tup12 can interact in coimmunoprecipitation experiments with extracts prepared from cells that overexpress the proteins. We created a strain in which the chromosomal loci encoding Tup11 and Tup12 were modified so that the proteins would be expressed in an epitope-tagged form from their endogenous promoters. Using cell extracts produced from this strain, we showed that at least a portion of Tup12-HA could be immunoprecipitated by using antibodies directed against Tup11-GFP (Fig. 2A). The ability of Tup11 and Tup12 to interact combined with the model in which Tup1-like proteins function in the form of a tetramer creates potential complications for the acquisition of independent functional roles by the two proteins during evolution. This is because if the two proteins are expressed at similar levels and freely associate, the vast majority of resulting tetramers would contain both Tup11 and Tup12. To measure the relative expression levels of the Tup11 and Tup12 proteins, we created modified strains in which the chromosomal loci have been modified to express Tup11 and Tup12, respectively, fused to the HA epitope tag. Extracts from these strains and a wild-type, untagged strain were analyzed by Western blotting. Figure 2B shows that there is no large difference in the expression level of the Tup11 and Tup12 proteins. We reasoned that if the vast majority of Tup1-like oligomers contain both Tup11 and Tup12, there would be a high degree of colocalization of the two proteins in the nuclei of cells. To test this, we studied the strain in which both *tup11*$^+$ and *tup12*$^+$ loci were tagged with the HA and GFP epitope tags, respectively, by using immunofluorescence microscopy (Fig. 2C). Images from immunostained cells were captured and deconvolved. Both proteins are distributed in a somewhat punctate fashion throughout the nucleus, and there is a significant degree of overlap in their distribution. However, it was clear that the two proteins were not completely colocalized since there were nuclear regions in which each protein was found in the absence of the other. We conclude that there are regions within the nucleus that exclusively contain either Tup11 or Tup12 but not both.

Tup11 and Tup12 independently interact with the Ssn6 homologue in fission yeast. The relative conservation of the N terminal, Ssn6-binding domain of Tup1-like proteins suggests that Tup11 and/or Tup12 might interact with a homologue of the Ssn6 protein in fission yeast. A database search for proteins with homology to the *S. cerevisiae* Ssn6 protein revealed a single clear orthologue in fission yeast (SPBC23E6.09). In order to test whether the two Tup proteins can bind Ssn6, we modified the fission yeast *ssn6*$^+$ genomic locus to create a strain that expressed the fission yeast Ssn6 protein fused to GFP at its C terminus. This strain was crossed to the strains expressing HA-tagged Tup11 and Tup12 proteins to create double-tagged strains. Using cell extracts prepared from these strains, we could specifically coimmunoprecipitate both Tup11-HA and Tup12-HA by using antibodies directed against GFP (Fig. 3A). Since these strains expressed both Tup11 and Tup12, it remained a formal possibility that Ssn6 interacts with only one of the Tup proteins and that coimmunoprecipitation

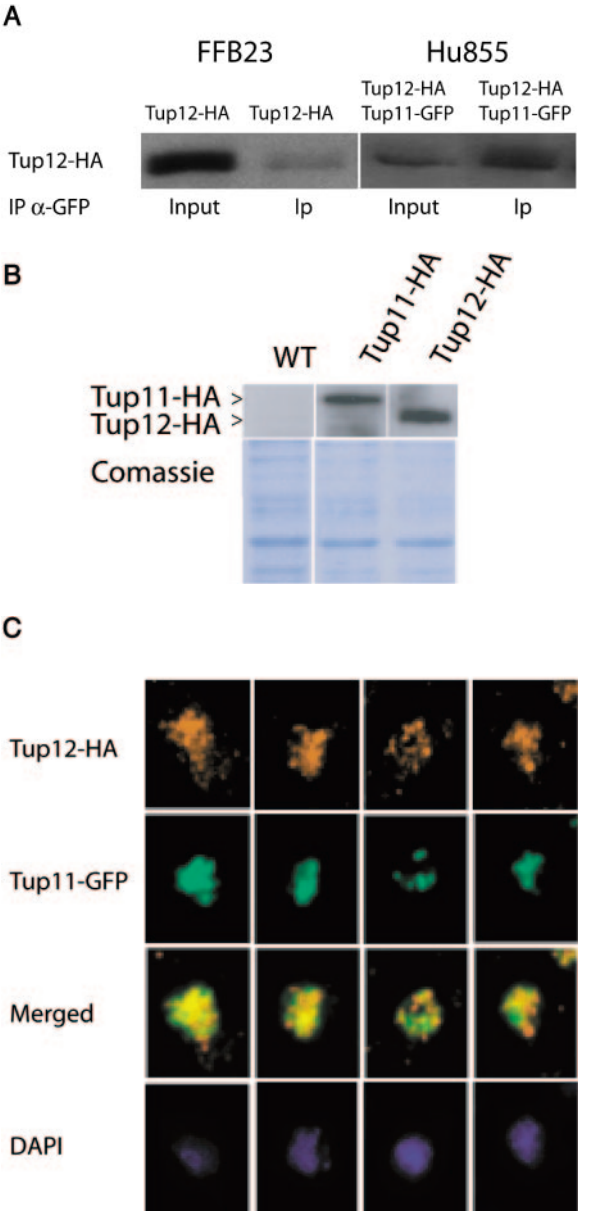


FIG. 2. Tup11 and Tup12 interact in vivo. (A) Immunoprecipitation of epitope-tagged Tup12-HA and Tup11-GFP from yeast whole-cell extracts (FFB23 and Hu855) indicates that Tup11 and Tup12 can interact. Whole-cell extracts were incubated with a polyclonal rabbit α-GFP antibody and precipitated with protein A-coupled beads. Samples were subjected to SDS-PAGE and Western blot analysis and detected by using a mouse α-HA antibody. Input fraction corresponds to 1/25 of the total immunoprecipitated sample. (B) Western blot showing the expression levels of in vivo-tagged Tup12-HA (FFB23) and Tup11-HA (FFB21). Yeast protein extracts were prepared from chromosomally tagged Tup12-HA, Tup11-HA and wild-type untagged cells. Cells were grown to mid-log phase, and extracts were subjected to SDS-PAGE and probed with a α-HA antibody. Coomassie blue staining of the extracts used in the Western blot showed the total protein concentrations in the samples used. (C) Immunofluorescence microscopy analysis showing the nuclear staining patterns of the chromosomally double-tagged Tup11-GFP (green) and Tup12-HA (red) Hu855 strain. A series of images of individual immunostained nuclei were captured in different focal planes and subjected to deconvolution (z = 0.3 μm [see Materials and Methods]). Nuclear DNA staining with DAPI (blue) is shown. The merged pictures show colocalization of Tup11-GFP and Tup12-HA in yellow.

of the other is due to its presence in oligomeric complexes containing both proteins. To address this issue, we repeated the analysis with strains expressing the HA fusion proteins in which the gene encoding the nontagged protein had been deleted. Figure 3B shows that both Tup11 and Tup12 interact with fission yeast Ssn6 independently of each other. We conclude that the divergence that has occurred in the sequence of the Ssn6-interaction domain has not resulted in a qualitative difference in the ability of the Tup11 and Tup12 proteins to interact with the Ssn6 partner protein, which is present in only one version in fission yeast. Thus, it is possible that corepressor tetramers consisting of only Tup11 or Tup12 could interact with the Ssn6 protein and regulate different programs of gene expression.

Tup11 and Tup12 have distinct functional roles. Previous reports indicate that Tup11 and Tup12 play at least partly redundant roles (12, 16, 27). To investigate whether they may also have distinct physiological roles, we investigated the *tup11*Δ, *tup12*Δ, and double-knockout *tup11*,*12*Δ deletion phenotypes under various conditions that have been reported to affect the growth of *tup11*,*12* deletion strains (12, 16). Interestingly, we observed differential phenotypes for the *tup11* and *tup12* deletion strains on medium containing 1 to 1.5 M KCl and 0.25 M $CaCl_2$ (Fig. 4A and B). Figure 4A shows that cells lacking Tup12 grow equally poorly on KCl as cells lacking both Tup proteins. Thus, under these conditions there are at least some Tup12 functions that are important for growth that cannot be performed by Tup11. The same is true for growth on 0.25 M $CaCl_2$ (Fig. 4B). However, at lower levels of $CaCl_2$ (0.1 M), Tup11 can replace the critical functions of Tup12, and only the double mutant shows a reduced growth phenotype. These observations support the view that Tup11 and Tup12 are functionally redundant under some conditions but that under other conditions they are functionally diverged. Since there was a theoretical possibility that the phenotypes we observed for *tup12* mutant could be due to secondary modifier mutations in the strain that we used, we backcrossed the mutant to a wild strain to test for cosegregation of the salt sensitivity phenotype with the mutated *tup12* locus. Cosegregation was observed in all spores analyzed from the cross (data not shown). To further investigate this issue, we overexpressed Tup11 and Tup12 in a Δ*tup12* background to see whether expression of either protein could rescue the phenotype associated with the *tup12* deletion. Expression of Tup12 from a plasmid rescued the KCl and $CaCl_2$ sensitivity phenotypes associated with defects in Tup12, as expected (Fig. 4C). However, overexpression of the wild-type *tup11*$^+$ allele from the same plasmid could not complement the phenotype of Δ*tup12* strains. We therefore conclude that at least some critical aspects of $CaCl_2$ and KCl adaptation are associated with a specific function of the Tup12 protein.

Identification of genes differentially affected by deletion of the *tup11*$^+$ or *tup12*$^+$ genes by using DNA microarrays. The differential phenotypes observed in Fig. 4 suggest that Tup11 and Tup12 may have distinct gene targets. To investigate this, we used DNA microarrays described previously (42) to compare the effect of gene knockouts of Tup11 and Tup12 on genome-wide gene expression patterns. We prepared duplicate cultures of Δ*tup11* and Δ*tup12* mutant strains under both normal (YES at 30°C) and KCl-stressed (YES at 30°C incubated with 1 M KCl for 45 min) growth conditions. RNA samples

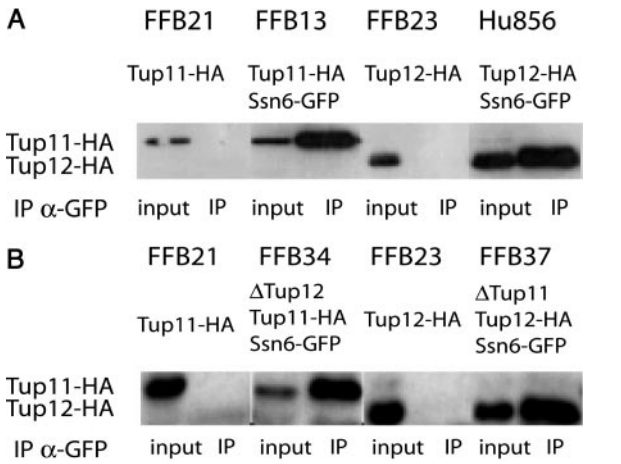


FIG. 3. Tup11 and Tup12 interact with Ssn6 in vivo by immunoprecipitation. (A) Immunoprecipitation of chromosomally tagged Tup11-HA, Tup12-HA, and Ssn6-GFP from yeast whole-cell extracts (FFB21, FFB13, FFB23, and Hu856). Whole-cell extracts were incubated with a polyclonal rabbit α-GFP antibody and precipitated with protein A-coupled beads. Samples were separated by SDS-PAGE, subjected to Western blot analysis, and detected with a mouse α-HA antibody. The input fraction corresponds to 1/25 of the total immunoprecipitated sample. (B) Tup11 and Tup12 interact independently with Ssn6. Cell extracts from chromosomally tagged Tup11-HA and Ssn6-GFP from *tup12*$^-$ cells (FFB34) and Tup12-HA and Ssn6-GFP from *tup11*$^-$ cells (FFB37) were incubated with a polyclonal rabbit α-GFP antibody and precipitated with protein A-coupled beads. Samples were subjected to Western blot analysis, separated by a SDS-PAGE, and visualized by using a mouse α-HA antibody. The input fraction corresponds to 1/25 of the total immunoprecipitated sample.

were prepared from each culture, and labeled probes were made by incorporation of Cy3 and Cy5 fluorescent nucleotides into cDNA during RT prior to hybridization to duplicate microarrays for each growth condition. The duplicate pairs of RNA from Δ*tup11* and Δ*tup12* strains were each labeled in both alternative dye orientations to reduce putative dye bias artifacts from the final combined set of data. The results of the experiments in the absence or presence of KCl are summarized in the ratio-intensity plots shown in Fig. 5. We detected 49 of 4,905 measurable genes under normal growth conditions and 29 of 4,931 measurable genes under KCl stress that showed a differential effect of >2-fold (red spots). Ten genes are common between these two groups. A complete list of the differentially regulated genes in Fig. 5 is available in the supplemental material. Tables 2 and 3 list twofold differentially affected genes in the absence and presence of KCl, respectively, for which the fold change values are statistically significant ($P < 0.05$). Interestingly, within these more stringently selected gene sets, there is a clear bias toward genes whose expression is increased in the Δ*tup12* strain relative to the Δ*tup11* mutant. This could be due to the fact that under the physiological conditions studied here most of the reproducible differential effects reflect genes that are specifically repressed by Tup12.

Validation and characterization of differentially affected Tup-targeted genes. Before we further characterized genes identified by microarray analysis, it was necessary to confirm and validate the results for some of the genes of interest by RT-PCR. Since the majority of the reproducible significant expression changes involved a relative increase in expression in

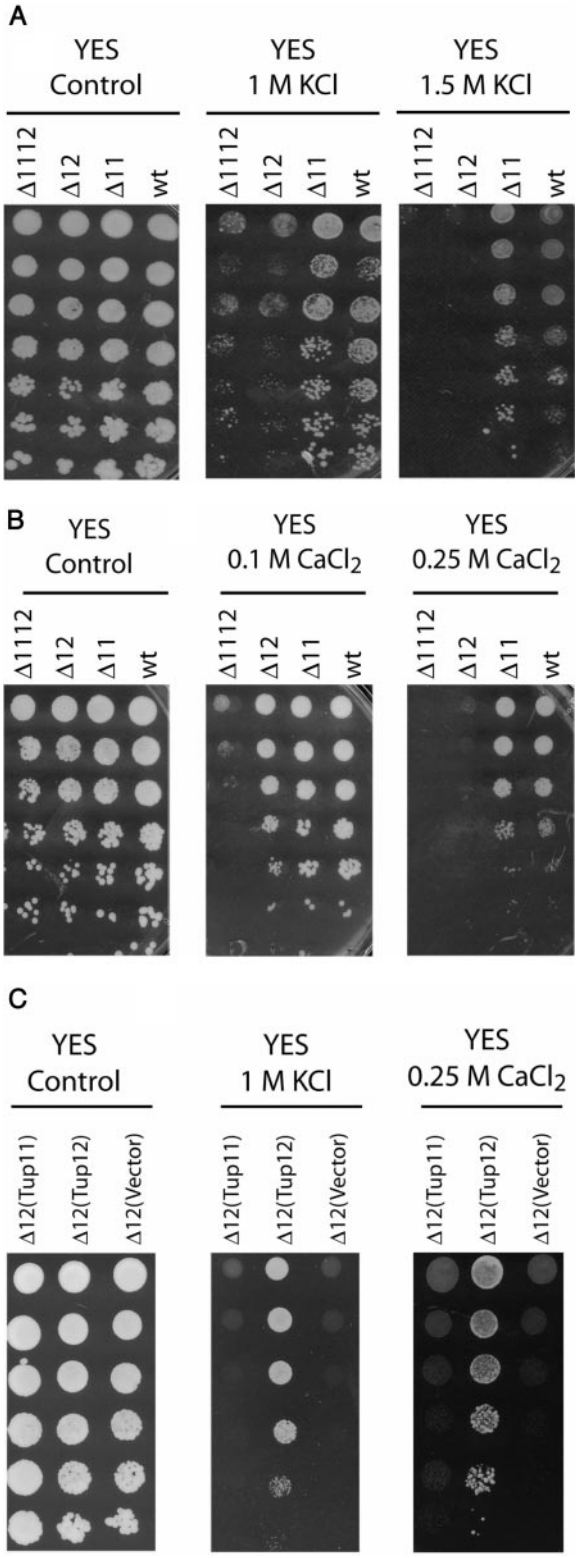


FIG. 4. Spotting assay of *tup* deletion mutants show differential salt sensitivity phenotypes. Different *tup* deletion strains were subjected to growth assays. Strains were spotted in fivefold dilutions on YES agar plates supplemented with 1.0 or 1.5 M KCl (A) or 0.1 or 0.25 M $CaCl_2$ (B) as indicated and grown at 30°C for 3 days before pictures were obtained. The following controls and *tup* mutant strains were used: wild-type strain JY741, deletion mutant JY741(Δ*tup11*), deletion mutant JY741(Δ*tup12*), and double-deletion mutant JY741(Δ*tup11,12*). (C) A plasmid expressing *tup12*$^+$ complements the Δ*tup12* stress phenotype. Spotting assays showed the effect of a high-copy plasmid expressing Tup11 and Tup12, respectively, in a Δ*tup12* background. The expression of Tup11 cannot reverse the growth phenotype of the Δ*tup12* strain, whereas Tup12 expression can. Deletion mutant JY741 (Δ*tup12*) was transformed with pRep42-Tup11, pRep42-Tup12, and the empty pRep42 vector. Transformant cells were spotted in fivefold dilutions on YES agar plates supplemented as indicated and grown at 30°C for 3 days before pictures were obtained.

the Δ*tup12* strain, we selected a number of genes with this behavior for validation both in the absence or presence of KCl-induced stress. All nine of nine tested genes and conditions in which the Δ*tup11*/Δ*tup12* expression ratio was <0.5 were validated (Tables 1 and 2). To determine whether the ratios of <0.5 are due to a defect in repression by Tup12 or a hypothetical defect in activation by Tup11, it was necessary to compare transcript levels for differentially altered genes in the two mutant strains with their levels in wild-type and double-mutant strains. In all cases tested, the expression difference was due to a defect in Tup12-dependent repression (Fig. 6A). Figure 6B shows negative controls confirming that the RT-PCR amplified bands reflect RNA levels present in the samples. Generally, there was a good quantitative correspondence with the ratio calculated from the arrays. We infer that Tup12 plays a functionally diverged role in repressing a number of genes under normal and KCl-induced stress conditions. The KCl sensitivity associated with Tup12-defective strains could be due to inappropriate expression of one or more of these genes during KCl-induced stress.

To determine whether the Ssn6 protein is involved in regulation of Tup12-dependent genes, we constructed a *ssn6*Δ strain. However, our results (to be published elsewhere) show that *ssn6*$^+$ is an essential gene in fission yeast. Characterization of different tagged strains that we have constructed showed that insertion of an HA tag at the C terminus of *ssn6*$^+$ leads to stress phenotypes similar to those observed for Tup12-defective strains. As shown in Fig. 6C, the *ssn6-HA* strain grew less well than the wild type on 1 M KCl at 30°C, and the growth defect was enhanced at 36°C. This phenotype is specific to the *ssn6-HA* strain since it was not observed in the *ssn6-GFP* strain at either temperature (Fig. 6C). Next, we wanted to investigate transcript levels from Tup12-dependent genes in the *ssn6-HA* strain by RT-PCR. Figure 6D shows that all of the genes that were Tup12 dependent in Fig. 6A were also derepressed in the *ssn6-HA* strain at 30°C. We conclude that the Ssn6 protein is important for regulation of Tup12-dependent target genes.

Genes differentially regulated by Tup11 and Tup12 are overrepresented in genes induced by environmental stress. We noted that several of the genes that are differentially affected by Δ*tup11* and Δ*tup12* are known stress response genes. To investigate this further, we used the hypergeometric distribution to test the significance of the overlap with the 237 CESR genes identified previously (5). The top 100 Tup12-specific genes identified in Fig. 5A shows a highly significant ($P = 10^{-16}$) overlap with the CESR genes. A significant overlap ($P = 0.03$) was also obtained with the 24 genes in Table 2. The CESR genes were defined as genes that responded >2-fold to at least four of five stress conditions tested (5). These were oxidative stress (H_2O_2), heavy metal toxicity (Cd), elevated

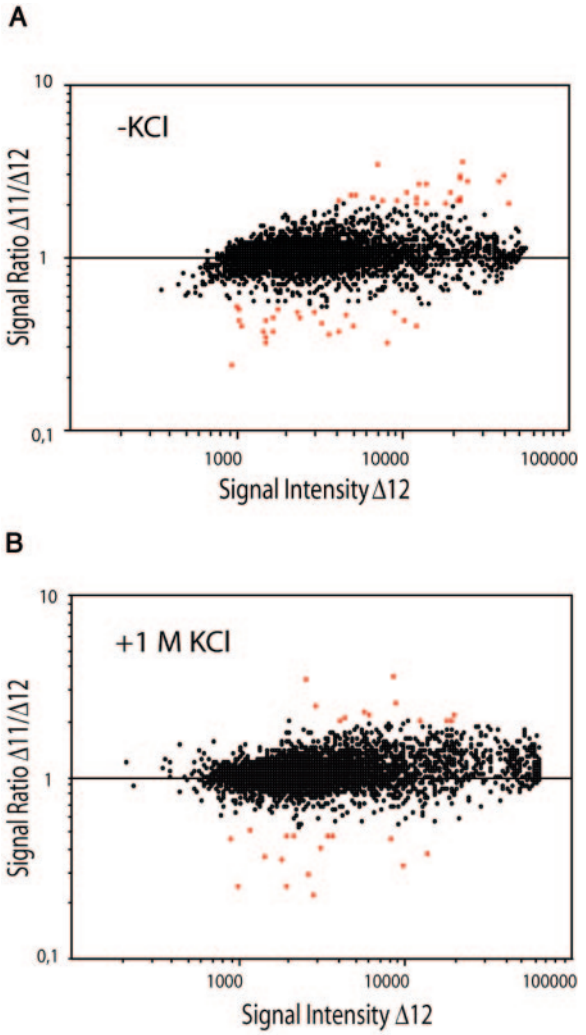


FIG. 5. Ratio intensity plots of microarray data show different regulation of targets. Plots of log intensity ratios versus log intensity averages show the gene regulation effect of the Δ*tup11* deletion compared to the *tup12*Δ deletion. The mean signal intensity ratios of the Δ*tup11* and Δ*tup12* signals were plotted against the signal intensity of the Δ*tup12* signal after normalization (see Materials and Methods). Data are mean signal values (four measurements) from two independent dye swap experiments. Data with a standard deviation of >80% of the mean have been excluded and are not shown. Genes in red represent twofold differentially affected genes up or down. (A) Data generated under normal conditions (YES at 30°C). A total of 49 genes (red) were affected ≥2-fold out of 4,905 genes totally. (B) Data generated under stress induction for 45 min (YES plus 1 M KCl at 30°C). A total of 29 genes (red) were affected ≥2-fold out of 4,931 genes totally.

temperature (39°C), osmotic stress (sorbitol), and DNA damage (MMS). Figure 7A shows the frequency distribution for the number of stresses that induce each of the Tup12-specific genes by >2-fold (note that 2 of 24 genes are omitted due to a lack of data). Only two genes were not induced by any stress. The remaining genes appear to reflect two distributions. The first consists of CESR genes, whereas the second is made up of genes that respond more specifically to stress, most often to only one stress condition. Figure 7B shows the frequency distribution of stress responses for the non-CESR genes. Most frequent are responses to H_2O_2, Cd, and heat, whereas sorbitol-induced stress responses are less frequent. None of these genes responded to MMS. We conclude that during the functional divergence of Tup11 and Tup12 during evolution the two proteins adopted overlapping but distinct roles and that Tup12 has become specialized in the regulation of a subset of stress response genes. These genes include both CESR genes and stress-specific response genes.

TABLE 2. Differentially affected genes under normal conditions

Name	Description	Δ*tup11*/Δ*tup12* ratio[a]	*P*
SPBC3E7.02c	*hsp16*$^+$ heat shock protein	0.46*	0.0034
SPBC839.06	*cta3*$^+$ cation transporter	0.24*	0.0323
SPCC757.07c	*cta1*$^+$ catalase	0.41†	0.0246
SPBC359.06	*class II* aldolase	0.36*	0.0042
SPCC1739.08c	Sorbitol utilization protein	0.46*	0.0235
SPAC343.12	*rds1*$^+$ involved in stress response	0.47	0.0102
SPCC1223.03c	*gut2*$^+$ glycerol-3-phosphate dehydrogenase	0.34	0.0159
SPAC17G6.03	Calcineurin-like phosphoesterase	0.42	0.0426
SPAC27D7.10c	Glycoprotein *S. pombe* specific	0.42	0.0009
SPAC27D7.11c	Glycoprotein *S. pombe* specific	0.39	0.0012
SPBC1105.01	rRNA processing	3.53	0.0185
SPBC15D4.02	Zinc finger protein	0.47	0.0314
SPBC19C7.04c	Conserved hypothetical	0.31	0.0019
SPBC646.06c	Glucanase, glycosyl hydrolase family	2.21	0.0225
SPMIT.05	*cob*$^+$ cytochrome *b*	2.12	0.0413
SPAC13F5.03c	Glycerol dehydrogenase	0.45	0.0068
SPAC23D3.12	Inorganic phosphate transporter	0.37	0.0022
SPAC2E1P3.05c	Conserved hypothetical	2.23	0.0088
SPBC713.12	Squalene epoxidase	0.47	0.0129
SPBC106.02c	ParB-like nuclease	0.39	0.0344
SPBP4G3.02	*pho1*$^+$ acid phosphatase	0.40	0.0029
SPBPB2B2.01	Amino acid permease family	0.42	0.0018
SPCC622.11	Conserved hypothetical transmembrane	0.44	0.0001
SPCC622.12c	NADP glutamate dehydrogenase	0.47	0.0018

[a] *, validated by RT-PCR (Fig. 6A); †, validated by RT-PCR (data not shown).

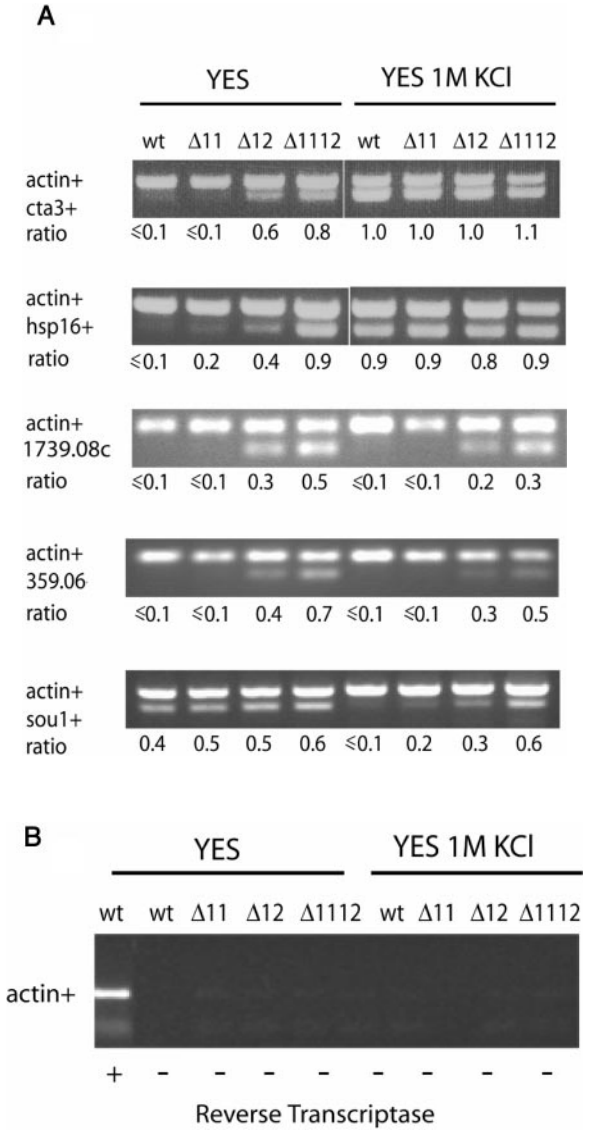
DISCUSSION

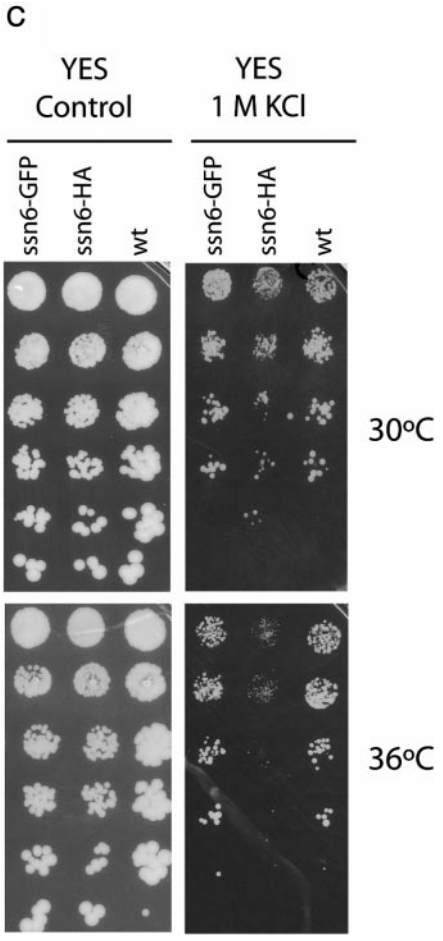
Primary sequence comparison of the Tup11 and Tup12 proteins from fission yeast with Tup family members from other fungi suggests that a gene duplication may have occurred in

TABLE 3. Differentially affected genes under 1 M KCl salt induction

Name	Description	Δ*tup11*/Δ*tup12* ratio[a]	*P*
SPAC8E11.10	*sou1*$^+$ sorbitol utilization protein	0.22*	0.0028
SPBC359.06	Class II aldolase	0.25*	0.0148
SPCC1739.08c	Sorbitol utilization protein	0.25*	0.0215
SPAC1F7.07c	*fip1*$^+$ iron permease	0.35†	0.0053
SPAC1002.17c	*fur1*$^+$ uracil phosphoribosyltransferase	2.45‡	0.0254
SPAPJ760.03c	Hypothetical glycoprotein	2.11‡	0.0391
SPAC1002.19	GTP cyclohydrolase	2.15	0.0073
SPAC1039.02	Calcineurin-like phosphoesterase	0.35	0.0027
SPAC30D11.01c	*agl*$^+$ alpha-glucosidase	0.46	0.0475
SPBC337.03	Conserved hypothetical	2.20	0.0322
SPBC887.17	Uracil permease	0.47	0.0081
SPAC23D3.12	Inorganic phosphate transporter	0.29	0.0135
SPAC2E1P3.05c	Conserved hypothetical	3.51	0.0009
SPBC713.12	Squalene epoxidase	0.45	0.0007
SPBP4G3.02	*pho1*$^+$ histidine acid phosphatase	0.37	0.0135
SPBPB2B2.01	Amino acid permease family	0.44	0.0130
SPCC622.11	Conserved hypothetical transmembrane	0.47	0.0123
SPCC622.12c	NADP glutamate dehydrogenase	0.47	0.0023

[a] *, validated by RT-PCR (Fig. 6A); †, validated by RT-PCR (data not shown); ‡, failed validation.





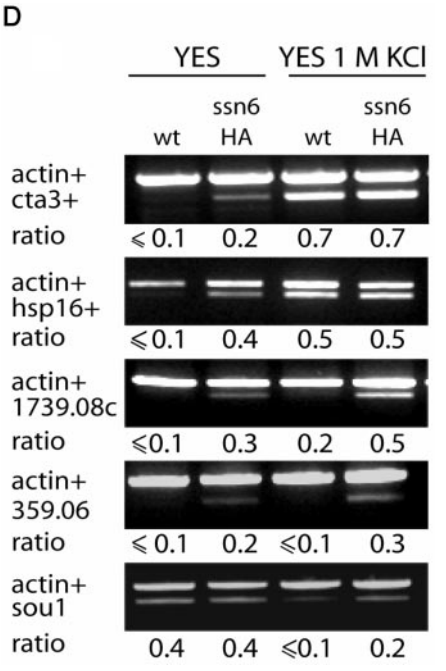


FIG. 6. Validation and characterization of differentially affected genes by RT-PCR. (A) RNA samples from wild-type JY741, JY741 (Δ*tup11*), JY741(Δ*tup12*), and JY741(Δ*tup11,12*) extracted both under normal conditions (YES at 30°C) and under salt induction conditions (YES at 30°C plus 1 M KCl) were subjected to DNase treatment, RT, and PCR with gene-specific primers. PCR products were separated, digitalized, and quantified. The indicated numbers correspond to the transcript ratios in relation to the individual actin control. (B) Samples from wild-type JY741, JY741(Δ*tup11*), JY741(Δ*tup12*), and JY741 (Δ*tup11,12*) were controlled for contaminant DNA content. Cells were subjected to DNase treatment, RT, and PCR. No *act1*$^+$ signals could be detected in samples not subjected to RT. (C) Spotting assay showing the growth defect of the Ssn6-HA-tagged strain on 1 M KCl at 30 and 36°C. Cells were spotted in fivefold dilutions and were grown at the indicated temperature for 3 days. (D) RT-PCR showing derepression of Tup12 target genes in the *ssn6-HA* strain. RNA extracted from wild-type JY741 and *ssn6-HA* Hu494 under normal conditions (YES at 30°C) and under salt induction conditions (YES plus 1 M KCl at 30°C) were subjected to RT-PCR procedures described above. The indicated numbers correspond to the transcript ratios in relation to the actin internal control.

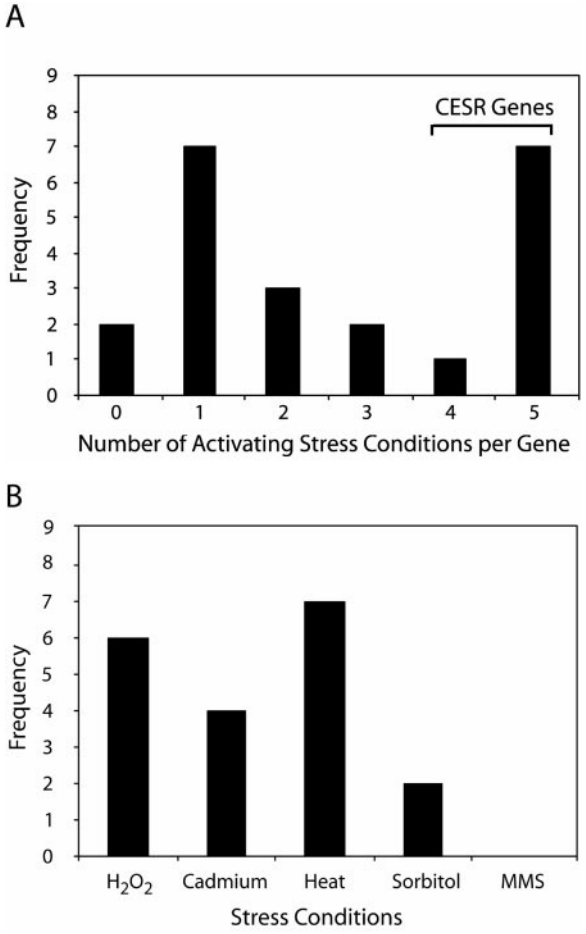


FIG. 7. Tup12-specific genes are over-represented in genes regulated by environmental stress. (A) Frequency with which the Tup12-specific genes listed in Table 2 are subject to regulation by different environmental stresses. Stress data were taken from the Sanger Institute database (5). Note that *cta3*$^+$ and *pho1*$^+$ were omitted from this analysis due to insufficient stress data. (B) Frequency with which non-CESR, Tup12-specific stress response genes are regulated by different environmental stress conditions.

fission yeast close to the divergence of the Archaeascomycetes from other fungi. Our observation that Tup11 and Tup12 have some functions distinct from each other is therefore not surprising. The existence of distinct roles is also completely consistent with the conservation of other functional attributes of the proteins. Thus, in some contexts the proteins may have redundant roles while in others they may have distinct functions. Proteins encoded by duplicated genes can diverge either by each protein acquiring new functional attributes or by differential loss of attributes that were present in the progenitor protein (20). It is not possible to determine the relative extent to which these mechanisms have contributed to the functional divergence of Tup11 and Tup12.

The redundant and distinct functions of Tup11 and Tup12 occur in highly related physiological contexts. For example, our data show that on 0.1 M $CaCl_2$ the two proteins appear to be functionally redundant, whereas on 0.25 M $CaCl_2$ Tup11 is completely unable to rescue the nongrowth phenotype associated with defects affecting Tup12. This could be accounted for either by a threshold in the ability of Tup11 to correctly regulate a set of Tup12-regulated genes as $CaCl_2$ levels increase or by considering the two conditions as physiologically separate with at least partially different sets of Tup-targeted genes. The sensitivity of Δ*tup12* mutants to KCl is more clear-cut. In this case, we have not seen any rescue of the *tup12* deletion phenotype by Tup11 under any condition tested. These phenotypes are also manifested by the *ssn6-HA* loss-of-function allele, suggesting that Tup12 functions together with Ssn6 in contexts in which it has a different function from Tup11. The phenotypic differences caused by defects in Tup11 and Tup12 in the present study are somewhat more pronounced than those reported for similar conditions previously (12). The differences may reflect differences in the way the experiments were performed. It is, however, important to note that the phenotypes we observed cosegregate with the *tup12*Δ mutation through meiosis and that they can be rescued by plasmid-mediated expression of Tup12 but not by plasmid-mediated expression of Tup11.

The existence of diverged functions for Tup11 and Tup12 has to be viewed in the context of what is known about the structure of the Tup1-Ssn6 corepressor complex. A complicating feature here is that Tup1 in budding yeast is thought to build a tetrameric complex that binds to a single Ssn6 subunit. In fission yeast maximum diversification would be created if the Tup11 and Tup12 subunits were unable to participate in the same tetrameric structures and that these had a differential ability to interact with the fission homologue of Ssn6. Using coimmunoprecipitation of epitope-tagged proteins expressed at endogenous levels, we have confirmed the previous observation that Tup11 and Tup12 can interact together (12) even when they are expressed at normal levels. In addition, we have shown that there is no large difference in the affinity of either protein for Ssn6. Indeed, all of the Tup12-specific genes we tested also require Ssn6, strongly suggesting that Tup12-specific repression is mediated by Tup-Ssn6 corepressor complexes and not by a putative alternative mechanism in which Tup12 functions alone or together with an alternative partner protein. Thus, these assays do not detect a functional difference between the C-terminal oligomerization domain and the N-terminal Ssn6-interacting domains of these two proteins that are 41 and 76% diverged in sequence, respectively. Tup11 has in addition been shown to interact with Ssn6 in the two-hybrid system previously (24). Since Ssn6 is thought to be involved in targeting corepressor complexes to promoters (36, 37), our results suggest that the difference between Tup11 and Tup12 may be the result of differences in the repression activity of recruited complexes. Consistent with this the repression domains of the proteins are highly divergent in sequence with only 8% of residues conserved between them. Further studies will be required to address this issue. It is unclear whether the distinct repression activities of Tup11 and Tup12 are manifested by corepressor complexes containing a mixture of the two proteins or by complexes containing only Tup11 and Tup12. Since Tup11 and Tup12 are expressed at similar levels, one might predict that most corepressor complexes would contain a mixture of Tup11 and Tup12 subunits interacting with Ssn6. However, we cannot exclude the existence of components in vivo that favor the assembly of corepressors containing exclusively Tup11 or Tup12 proteins. Our immunofluorescence data suggest that such a mechanism may exist since the local-

ization of the proteins in the nucleus is partially distinct (Fig. 2C).

Using DNA microarrays we identified a number of genes that are differentially affected by defects in Tup11 and Tup12. The most reproducible of these showed a relative expression increase in Δ*tup12* compared to Δ*tup11* that could be validated with RT-PCR. *cta3*$^+$ and *hsp16*$^+$ are genes that are repressed on normal medium by Tup12-Ssn6 but that are physiologically derepressed by KCl-induced stress. On KCl, expression levels in the mutant strains are similar to those seen in the wild type, and therefore it is difficult to attribute the KCl sensitivity phenotype to inappropriate regulation of genes with this behavior. SPCC1739.08c and SPBC359.06 are normally repressed in the absence or presence of KCl, and Tup12-Ssn6 defects cause derepression under both conditions. Genes showing this pattern could cause sensitivity to KCl if inappropriate expression of the genes on KCl media has a more severe consequence for growth than in its absence. *sou1*$^+$ represents a third pattern of regulation in which Tup12-Ssn6 specific repression is only seen in the presence of KCl. Inappropriate expression of such genes could be associated with the KCl sensitivity seen in the Δ*tup12* and *ssn6-HA* strains.

The RT-PCR experiments depicted in Fig. 6 demonstrate that pure Tup12-containing corepressors are fully active on Tup12-specific genes because repression of all five genes tested is as efficient in tup11Δ cells as in the wild type. However, the occurrence of Tup11 in complexes regulating these genes in wild-type cells cannot be excluded and, indeed, in the absence of Tup12, pure Tup11 complexes can partially repress Tup12-specific genes. The partial repression effect seen in *tup12*Δ cells could of course be due to a Tup-independent repression function of the Ssn6 protein.

We cannot formally exclude that some of the genes shown in Fig. 5 are due to loss of an activator activity associated with Tup11. However, since all of the examples we have studied by RT-PCR are due to a loss of Tup12-specific repression activity, it is likely that the vast majority of these genes represent targets for Tup12-specific repression. This supposition is further supported by the fact that the differentially affected genes are enriched in genes that are known to be derepressed during environmental stress. Sequence analysis of the Tup12-dependent promoters also indicate the presence of ATF/CRE binding sites and T-rich elements at similar frequencies to those described earlier for the so-called CESR genes that are regulated >2-fold in at least four of the five stress conditions tested previously by others (5). We have not found any other DNA motifs that could account for Tup12-specific repression.

A small number of genes targeted by Tup11 and Tup12 have been characterized in fission yeast previously. The *fbp1*$^+$ gene has been shown to be a target for both Tup11 and Tup12, which appear to function in a redundant fashion to repress its expression (16). Consistent with this view, we did not identify *fbp1*$^+$ as a differentially affected gene in our DNA microarray experiments. While scrutinizing a previous report (27), we noticed that the *fio1*$^+$ gene, which encodes one subunit of a bipartite iron transporter, is partially derepressed in *tup12*Δ mutants but not in *tup11*Δ mutants. *fio1*$^+$ was not detected with statistical significance in our array data but showed a high degree of Tup12 dependency in one experiment. The coregulated gene encoding its dimerization partner, *fip1*$^+$, was however reproducibly identified in our study. In our microarray and RT-PCR data, the *cta3*$^+$ gene encoding a cation transporter is also heavily dependent on Tup12 for its repression. However, derepression in a *tup12*$^-$ strain was not seen in the previous study mentioned above (12). The identification of *hsp16*$^+$ as a specific target of Tup12-dependent repression is interesting in the light of previous results. Tup12 has been shown to interact with Hsp16 in the two-hybrid system (34), suggesting the possibility that Hsp16 could participate in feedback regulation of its own expression or in the expression of other Tup12-repressed stress genes. Furthermore, together with SPAC19C7.04c, *hsp16*$^+$ has been shown to be the target of RNAi-mediated repression (29). Thus, Tup12-specific attributes could represent evolutionary adaptations that integrate Tup12-mediated repression with RNAi-mediated silencing mechanisms. Recently, it has also been suggested that ATF/CRE-regulated genes are involved in heterochromatization by an alternative silencing mechanism involving components of the stress signaling pathway (17, 18). It is possible that Tup11/12-Ssn6 corepressors could be involved in such a mechanism.

In budding yeast there are several different signal transduction pathways that mediate the response to different types of environmental stress. In fission yeast a large number of different stresses appear to elicit responses via a common signaling pathway that is dependent on the Sty1 protein. This has led to the question of how different stress conditions elicit different responses at the level of gene regulation (5). In the light of our results, it is possible that the different Tup proteins give specificity to gene targets associated with different environmental stresses. However, many of the genes we identified that differentially require Tup11 and Tup12 for their regulation are overrepresented in genes that are regulated by most of the stresses for which microarray data are available (H_2O_2, Cd, heat, sorbitol, and MMS) (5). Thus, the Tup12-specific mechanism is probably a component of the general stress response. Indeed, the set of genes we identify here also shows an overlap with the CESR genes. Importantly though, the majority of the Tup12-specific genes are induced by a more restricted range of stress conditions and are not CESR genes. In such cases, Tup12 and Ssn6 might contribute to specificity of the stress response. One example is *cta3*$^+$ where Tup corepressors have recently been reported to play a role in stress response specificity by creating an appropriate chromatin structure in the promoter region (13). It would be interesting to determine whether these effects are mediated specifically by Tup12.

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