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Global Roles of Ssn6 in Tup1- and Nrg1-dependent Gene Regulation in the Fungal Pathogen, *Candida albicans*

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In budding yeast, Tup1 and Ssn6/Cyc8 form a corepressor that regulates a large number of genes. This Tup1-Ssn6 corepressor appears to be conserved from yeast to man. In the pathogenic fungus *Candida albicans*, Tup1 regulates cellular morphogenesis, phenotypic switching, and metabolism, but the role of Ssn6 remains unclear. We show that there are clear differences in the morphological and invasive phenotypes of *C. albicans ssn6* and *tup1* mutants. Unlike Tup1, Ssn6 depletion promoted morphological events reminiscent of phenotypic switching rather than filamentous growth. Transcript profiling revealed minimal overlap between the Ssn6 and Tup1 regulons. Hypha-specific genes, which are repressed by Tup1 and Nrg1, were not derepressed in *ssn6* cells under the conditions studied. In contrast, the phase specific gene *WH11* was derepressed in *ssn6* cells, but not in *tup1* or *nrg1* cells. Hence Ssn6 and Tup1 play distinct roles in *C. albicans*. Nevertheless, both Ssn6 and Tup1 were required for the Nrg1-mediated repression of an artificial NRE promoter, and lexA-Nrg1 mediated repression in the *C. albicans* one-hybrid system. These observations are explained in models that are generally consistent with the Tup1-Ssn6 paradigm in budding yeast.

INTRODUCTION

Transcriptional repression plays a key role in controlling the growth and differentiation of eukaryotic cells. The fundamental importance of negative transcriptional regulators is reflected in their conservation during eukaryotic evolution. For example, orthologues of the Ssn6 (Cyc8) and Tup1 proteins, which act as global repressors in *Saccharomyces cerevisiae*, have been identified in humans, flies, worms, slime molds, and fungi (reviewed by Smith and Johnson, 2000).

Our understanding of the mechanisms of action of Ssn6 and Tup1 is based largely on studies in budding yeast. The *S. cerevisiae* paradigm suggests that Ssn6 and Tup1 interact physically to form a corepressor complex that actively represses the transcriptional machinery (Williams *et al.,* 1991; Redd *et al.,* 1997; Gounalaki *et al.,* 2000). The relevance of this paradigm to eukaryotes in general is emphasized by the observation that yeast Ssn6 can interact with human Tup1-like proteins to mediate transcriptional repression in human cells (Grbavec *et al.,* 1999).

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The Ssn6-Tup1 corepressor is thought to repress transcription in yeast by two main mechanisms. First, the corepressor is thought to remodel chromatin on yeast promoters by recruiting histone deacetylases to these promoters and by positioning nucleosomes via direct interactions with histone tails (Edmondson *et al.,* 1996; Davie *et al.,* 2002, 2003; Zhang and Reese, 2004a). Second, Tup1 is thought to interact directly with the transcriptional machinery to attenuate its activity (Carlson, 1997; Redd *et al.,* 1997; Gromoller and Lehming, 2000; Papamichos-Chronakis *et al.,* 2000). Recent experiments suggest that both mechanisms operate in yeast, but that they are utilized differentially to regulate distinct sets of yeast genes (Green and Johnson, 2004). However, both mechanisms appear to operate in a redundant manner to repress some yeast genes (Green and Johnson, 2004; Zhang and Reese, 2004b). In addition, Ssn6-Tup1 presumably regulates the expression of other genes indirectly by controlling the levels of transcriptional activators or repressors that act directly on these genes.

The Ssn6-Tup1 corepressor complex does not interact with DNA directly. Instead the complex is targeted to specific promoters through interactions with sequence-specific DNA-binding proteins (Keleher *et al.,* 1992; Smith and Johnson, 2000). For example, Mig1 and Nrg1 target Ssn6-Tup1 to glucose-repressed genes, whereas the a1/α2 heterodimer targets the corepressor complex to haploid-specific genes in *S. cerevisiae*. In contrast, Rox1 and Crt1 target Ssn6-Tup1 to hypoxia- and DNA damage–inducible genes in budding yeast. Therefore, Ssn6-Tup1 regulates the expression of functionally diverse sets of yeast genes via interactions with different DNA-binding proteins.

Ssn6-Tup1–mediated repression is generally controlled by regulating the levels or activities of these DNA-binding proteins (Smith and Johnson, 2000). For example, a1/α2 levels are controlled primarily at the transcriptional level in

S. cerevisiae: a1 and α2 are synthesized by the *MATa* and *MAT*α loci, respectively, and hence the a1/α2 heterodimer only forms in diploid cells. In contrast, the activity of Mig1 and Crt1 are regulated posttranscriptionally: the DNA-binding activity of Crt1 is inhibited by hyperphosphorylation, and the phosphorylation of Mig1 inhibits its interaction with Ssn6-Tup1 and leads to its export from the nucleus (Huang *et al.*, 1998; Ostling and Ronne, 1998; Papamichos-Chroakis *et al.*, 2004).

Ssn6 and Tup1 play different roles within the corepressor complex. Tzamarias and Struhl (1994) reported that the transcriptional repression mediated by a LexA-Ssn6 fusion in yeast was dependent on Tup1, whereas LexA-Tup1 repressed transcription even in the absence of Ssn6. Furthermore, the mating defect of yeast *tup1 ssn6* cells can be suppressed by Tup1 overexpression, but not by Ssn6 overexpression (Komanchi *et al.*, 1994). Hence, Tup1 is thought to mediate the transcriptional repression, whereas Ssn6 is thought to stabilize the interaction between Tup1 and the DNA-binding protein. As described above, a diverse set of Ssn6-Tup1–targeting proteins have been identified in *S. cerevisiae*. These diverse proteins appear to interact with different regions of the Ssn6-Tup1 corepressor complex (Komanchi *et al.*, 1994; Tzamarias and Struhl, 1995; Smith and Johnson, 2000). For example, Rox1 and Mig1 contact different regions of the Ssn6 protein within the Ssn6-Tup1 corepressor, whereas α2 is thought to contact Tup1 directly (Komanchi *et al.*, 1994; Tzamarias and Struhl, 1995).

Candida albicans is the major systemic fungal pathogen of humans (Odds, 1988; Calderone, 2002). This fungus causes oral and vaginal candidiasis and life-threatening bloodstream infections in immunocompromised patients. Braun and Johnson (1997) were the first to reveal the importance of transcriptional repression in the regulation of *C. albicans* virulence attributes by showing that Tup1 represses hyphal development in this fungus. Tup1 was subsequently shown to repress the transcription of hypha-specific genes (i.e., genes that are expressed specifically during the hyphal growth phase of *C. albicans*; Sharkey *et al.*, 1999; Braun and Johnson, 2000; Braun *et al.*, 2000, 2001; Murad *et al.*, 2001a; Zheng *et al.*, 2004). This was confirmed by a transcript profiling study that examined the effects of inactivating Tup1 or the Tup1-targeting protein, Nrg1, upon the expression of about one-third of *C. albicans* genes (Murad *et al.*, 2001a, 2001b). This study also revealed that both Tup1 and Nrg1 play broader roles by coordinating yeast-hypha morphogenesis with the expression of other virulence attributes in this pathogen. Not surprisingly, a *tup1* mutant displayed attenuated virulence in the mouse model of systemic candidiasis (Murad *et al.*, 2001a). Hence transcriptional repression, and Tup1 in particular, is an important regulator of *C. albicans* virulence.

On the basis of the *S. cerevisiae* Ssn6-Tup1 paradigm, we and others predicted that *C. albicans* Ssn6 would play an important role in Tup1-mediated transcriptional repression in this fungus (Smith and Johnson, 2000; Garcia and Brown, 2001). We have tested this prediction by examining the cellular and molecular phenotypes of *C. albicans ssn6* mutants. During the course of this study Hwang *et al.* (2003) reported in an elegant article that Ssn6 regulates yeast-hypha morphogenesis and virulence in *C. albicans*. They suggested that complex relationships exist between Ssn6 and the MAP kinase and cAMP-protein kinase A signaling pathways that activate morphogenesis in *C. albicans*. They also reported subtle differences in the morphological phenotypes of *ssn6* and *tup1* mutants, suggesting that Ssn6 might play distinct roles in *C. albicans* morphogenesis (Hwang *et al.*, 2003). We show that, although *ssn6* cells do display morphogenetic defects, these defects are distinct from those of *tup1* or *nrg1* cells and more reminiscent of phenotypic switching than yeast-hypha morphogenesis. We also show that although Ssn6 contributes to Tup1- and Nrg1-mediated repression at some promoters, Ssn6 is not essential for the repression of most Tup1- or Nrg1-regulated genes in *C. albicans*. The data suggest that Ssn6 executes roles that are independent of the Ssn6-Tup1 corepressor. These observations have important implications for the eukaryotic Ssn6-Tup1 paradigm.

MATERIALS AND METHODS

Strains and Growth Conditions

C. albicans strains (Table 1) were grown in YPD, SD, SC (Sherman, 1991, Kaiser *et al.*, 1994), YPDA (YPD containing 0.01% adenine) or YPDAU (with 0.02% adenine and 0.008% uridine). Hyphal development was stimulated using YPD containing 10% serum at 37°C. Cell and colony morphology were analyzed using Olympus BX50 (Lake Success, NY) and Zeiss stereo microscopes (Thornwood, NY) as described previously (Murad *et al.*, 2001a).

Complementation tests were performed using the *S. cerevisiae ssn6* strains MAP6, which was a generous gift from Markus Proft, and PHFT5 (Tzamarias and Struhl, 1994; Loubradou *et al.*, 2001). *C. albicans SSN6* was cloned as a 6-kb *Eco*RI fragment into YCp50, and the phenotypes of YCp50-SSN6 and YCp50-containing *S. cerevisiae* PHFT5 cells were compared.

Both alleles of the *SSN6* locus were sequentially disrupted in *C. albicans* CAI8 by Ura-blasting (Fonzi and Irwin, 1993) using a *ssn6::hisG-URA3-hisG* cassette that deleted codons 385–955 of the 1081 codon ORF (Table 1). To generate this cassette the *hisG-URA3-hisG* was cloned between the *Kpn*I and *Hin*dIII sites of a 6.2-kb *Pst*I fragment from the *SSN6* gene. The genotypes of homozygous *ssn6/ssn6* mutants were confirmed by Southern blotting and PCR analysis (unpublished data). Eight independent *ssn6/ssn6* mutants were generated, all with similar phenotypes (unpublished data). Uridine auxotrophic mutants were generated by selecting *ura3* segregants on YPD containing 1 mg/ml 5-fluoroorotic acid (Fonzi and Irwin, 1993). *SSN6* was also disrupted in *C. albicans* HLC54ura3 to generate the triple *ssn6/ssn6 cph1/cph1 efg1/efg1* mutant, SGC134 (Table 1). A conditional *ssn6* mutant, SGC179, was generated by inactivating one *SSN6* allele and placing the remaining *SSN6* allele under the control of the *C. albicans MET3* promoter (Care *et al.*, 1999). Again, the genotype of this *ssn6/MET3-SSN6* strain was confirmed by Southern blotting and PCR analysis (unpublished data). The expression of *MET3-SSN6* allele was repressed using 2.5 mM methionine and cysteine.

To create the NRE reporter, the sequence 5′-*GTCGAC*GGATCCGCTAGC-CCCCCTGACTGCTACCATCCCCCTAAATCGGATCCGCCCCCTTGCGAA-CAAGTCCCCCCTGCCTTGAACGAA*CTGCAG* (*Sal*I and *Pst*I sites in italics; four C_5T NRE elements underlined) was cloned between the *Sal*I and *Pst*I sites upstream in plac-basal. This plasmid contains the basal *ADH1* promoter region (Tripathi *et al.*, 2002) cloned upstream of the *Streptococcus thermophilus lacZ* reporter (Uhl and Johnson, 2001) in CIp10 (Murad *et al.*, 2000). A second NRE reporter was generated with two A_2C_3T and two C_5T elements. A single copy of each plasmid was integrated into the *Xba*I-plus allele of *RPS1* (Murad *et al.*, 2000). (*RPS1* was previously known as *RPS10* and *RP10*.)

To use the *C. albicans* one-hybrid system (Russell and Brown, 2005), an expression vector encoding a *Staphylococcus aureus* LexA-*C. albicans* Nrg1 protein fusion was constructed by PCR amplification of the *NRG1* ORF and inserting it into CIp-LexA, to create CIp-LexA-Nrg1. The 5′ PCR primers used introduced a $(Gly)_3$-Pro-$(Gly)_2$ linker between the amino-terminal LexA domain and the carboxy terminal domains of Nrg1. CIp-LexA and CIp-LexA-Nrg1 were introduced into *C. albicans* reporter strains that carried pCR-lacZ (no *lexA* operator upstream of basic *ADH1* promoter) or pCR-OplacZ (containing the lexA operator) integrated into the *ade2::hisG* locus (Doedt *et al.*, 2004). The one-hybrid system requires both *URA3* and *ADE2* markers for the CIp- and pCR-based plasmids, respectively. Hence the analyses were performed in derivatives of CAI8 (Table 1). The *nrg1* and *ssn6* mutants were MMC9 and SGC124. The *tup1* mutant, CRC004, was created in CAI8 by Ura-blasting (Fonzi and Irwin, 1993) using the same *tup1::hisG-URA3-hisG* cassette that was used to generate the original *C. albicans tup1* mutants in CAI4 (Braun and Johnson, 1997).

DNA, RNA, and Protein Analyses

Southern, Northern (Murad *et al.*, 2001a), and Western blotting (Cormack *et al.*, 1997) were performed as described previously. RT-PCR was performed using standard methods using the intron-containing *EFB1* product to control for loading and genomic DNA contamination (Schaller *et al.*, 1998). To measure *lacZ* reporter activity, X-gal overlay assays and liquid β-galactosidase assays were performed as described previously and expressed in Miller units (Rupp, 2002).

Table 1. *C. albicans* strains

Strain	Genotype	Scource
SC5314	Wild type	Gillum *et al.* (1984)
CAF2-1	*URA3/ura3::λ imm434*	Fonzi and Irwin (1993)
CAI4	*ura3::λ imm434/ura3::λ imm434,*	Fonzi and Irwin (1993)
CAI8	*ura3::λ imm434/ura3::λ imm434, ade2::hisG/ade2::hisG*	Fonzi and Irwin (1993)
BCA2-9	*ura3::λ imm434/ura3::λ imm434, tup1::hisG/tup1::hisG*	Braun and Johnson (1997)
MMC3	*ura3::λ imm434/ura3::λ imm434, nrg1::hisG-URA3-hisG/nrg1::hisG*	Murad *et al.* (2001a)
MMC4	*ura3::λ imm434/ura3::λ imm434, nrg1::hisG/nrg1::hisG*	Murad *et al.* (2001a)
MMC9	*ura3::λ imm434/ura3::λ imm434, ade2::hisG/ade2::hisG, nrg1::hisG/nrg1::hisG*	Murad *et al.* (2001a)
HLC54	*ura3::1 imm434/ura3::1 imm434, cph1::hisG/cph1::hisG, efg1::hisG/efg1::hisG-URA3-hisG*	Lo *et al.* (1997)
HLC54*ura3*	*ura3::1 imm434/ura3::1 imm434, cph1::hisG/cph1::hisG, efg1::hisG/efg1::hisG*	This study
SGC121	*ura3::λ imm434/ura3::λ imm434, ade2::hisG/ade2::hisG, SSN6/ssn6::hisG-URA3-hisG*	This study
SGC122	*ura3::λ imm434/ura3::λ imm434, ade2::hisG/ade2::hisG, SSN6/ssn6::hisG*	This study
SGC123	*ura3::λ imm434/ura3::λ imm434, ade2::hisG/ade2::hisG, ssn6::hisG-URA3-hisG/ssn6::hisG*	This study
SGC124	*ura3::λ imm434/ura3::λ imm434, ade2::hisG/ade2::hisG, ssn6::hisG/ssn6::hisG*	This study
SGC131	*ura3::λ imm434/ura3::λ imm434, cph1::hisG/cph1::hisG, efg1::hisG/efg1::hisG, SSN6/ssn6::hisG-URA3-hisG*	This study
SGC132	*ura3::λ imm434/ura3::λ imm434, cph1::hisG/cph1::hisG, efg1::hisG/efg1::hisG, SSN6/ssn6::hisG*	This study
SGC133	*ura3::λ imm434/ura3::λ imm434, cph1::hisG/cph1::hisG, efg1::hisG/efg1::hisG, ssn6::hisG-URA3-hisG/ssn6::hisG*	This study
SGC134	*ura3::λ imm434/ura3::λ imm434, cph1::hisG/cph1::hisG, efg1::hisG/efg1::hisG, ssn6::hisG/ssn6::hisG*	This study
SGC179	*ura3::λ imm434/ura3::λ imm434, ade2::hisG/ade2::hisG, ssn6::hisG/MET3-SSN6*	This study
CRC001	*ura3::λ imm434/ura3::λ imm434, ade2::hisG/ade2::hisG, TUP1/tup1::hisG-URA3-hisG*	This study
CRC002	*ura3::λ imm434/ura3::λ imm434, ade2::hisG/ade2::hisG, TUP1/tup1::hisG*	This study
CRC003	*ura3::λ imm434/ura3::λ imm434, ade2::hisG/ade2::hisG, tup1::hisG-URA3-hisG/tup1::hisG*	This study
CRC004	*ura3::λ imm434/ura3::λ imm434, ade2::hisG/ade2::hisG, tup1::hisG/tup1::hisG*	This study

Transcript Profiling

Transcript profiling was performed using congenic *C. albicans* strains: wild-type, CAI4 and CAI8; *nrg1,* MMC4; *tup1,* BCA2–9; *ssn6,* SGC124 (Table 1). For consistency, the *nrg1, tup1,* and *ssn6* strains were compared directly to CAI4. Then, to control for possible differences between CAI4 and CAI8, these strains were compared directly. Cells were grown to midexponential phase (OD_{600} = 0.6–0.8) in YPDAU at 30°C. The cells were then harvested, frozen rapidly in liquid N_2, sheared mechanically using a microdismembrator (Braun Melsungen, Germany) and RNA prepared by extraction with Trizol Reagent (Gibco-BRL), as described previously (Hauser *et al.,* 1998). Cy3- and Cy5-labeled cDNAs were prepared from total RNA, and the probes were hybridized with (nearly) whole genome *C. albicans* microarrays (Eurogentec, Seraing, Belgium). Slides were scanned using a ScanArray Lite scanner (PerkinElmer Life Sciences, Beaconsfield, United Kingdom) and quantified using QuantArray software (version 2.0). Data normalization and analysis were performed using GeneSpring (Silicon Genetics, Redwood City, CA), and statistical analysis was performed using SAM (Significance Analysis of Microarrays: Tusher *et al.,* 2001). Data from at least three independent biological replicates were used for each analysis, and the SAM False Discovery Rate was set at 10%. Expression ratios for each gene that displayed a reproducible and statistically significant change in expression in mutant cells relative to the wild-type control are available in the Supplementary Data and at the *Galar Fungail* website (http://www.pasteur.fr/recherche/unites/Galar_Fungail/; http://www.galarfungail.org/data.htm). *C. albicans* gene annotations were obtained from CandidaDB (http://genolist.pasteur.fr/CandidaDB: d'Enfert *et al.,* 2005). Functional categories for *C. albicans* genes were assigned mainly on the basis of the MIPS functional assignments for *S. cerevisiae* homologues (http://mips.gsf.de/proj/yeast/CYGD/db/index.html: Yin *et al.,* 2004). Genes were assigned to a new virulence category was based on the functional analysis of putative virulence attributes in *C. albicans.*

Chromatin Immunoprecipitation Assays

Polyclonal rabbit anti-peptide antibodies were raised against the carboxy-terminal region of *C. albicans* Ssn6 using the peptide CMRKIEEDENYDDE (Diagnostics Scotland, Carluke, United Kingdom). The specificity of the anti-Ssn6 antiserum was confirmed by Western blotting of protein extracts from *C. albicans* wild-type and *ssn6* cells (unpublished data). Chromatin immunoprecipitation (ChIPs) was performed in triplicate (Strahl-Bolsinger *et al.,* 1997) comparing preimmune and anti-Ssn6 antisera and comparing *C. albicans* wild-type and *ssn6* cells.

RESULTS

C. albicans Ssn6 Is Related to Ssn6-like Proteins in Other Eukaryotes

As reported by Hwang *et al.* (2003), *C. albicans* has a single locus with significant sequence similarity to *S. cerevisiae SSN6* (orf19.6798; IPF5957.1). This gene encodes a 1081 amino acid protein, which contains nine tetratricopeptide (TPR) repeats between amino acids 98 and 430. An additional degenerate TPR-like sequence lies between TPR repeats six and seven (Figure 1). This TPR region of the *C. albicans* Ssn6 protein is closely related to the corresponding regions of Ssn6-like proteins from other fungi, slime molds, flies, worms, and mammals (Figure 1). However, the sequence similarity between these Ssn6-like proteins is relatively low outside the TPR region. The TPR repeats in *S. cerevisiae* Ssn6 mediate protein-protein interactions with Tup1 and with Ssn6-Tup1 targeting proteins (Tzamarias and Struhl, 1995).

We tested whether the *C. albicans SSN6* gene encodes a functional homologue of *S. cerevisiae* Ssn6 (unpublished data). When transformed into *S. cerevisiae* using the single-copy vector YCp50, *C. albicans SSN6* suppressed the flocculation defects of the *ssn6* cells as well as their growth defects at 37°C and on glycerol. This was consistent with the observations of Hwang *et al.* (2003). We conclude that *C. albicans* Ssn6 is a functional homologue of *S. cerevisiae* Ssn6.

Phenotypes of C. albicans ssn6 Mutants

Tup1 inactivation derepresses morphogenesis in *C. albicans*, leading to the formation of characteristically wrinkly colonies (Braun and Johnson, 1997). If Ssn6 simply acts as a corepressor with Tup1 in *C. albicans*, one might predict that the inactivation of Ssn6 would generate a similar phenotype. However, Hwang *et al.* (2003) described subtle morpholog-

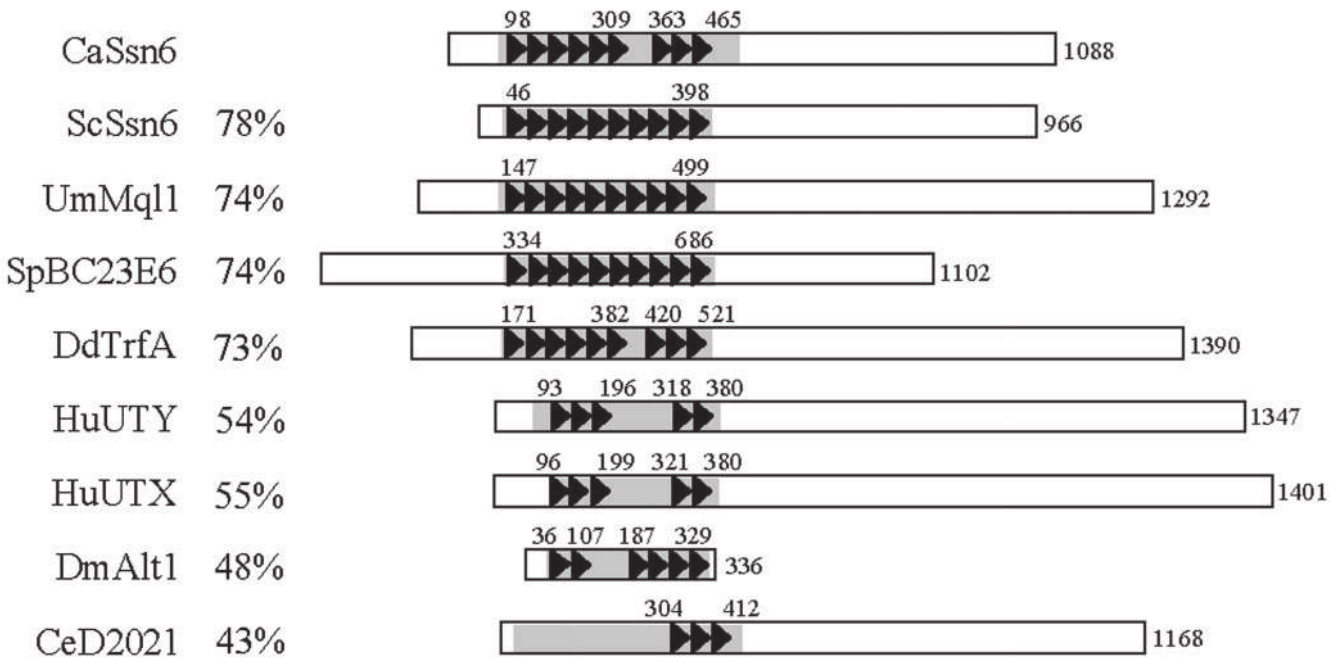


Figure 1. Ssn6-like proteins in eukaryotes. (A) *S. cerevisiae* paradigm of Ssn6-Tup1 function. (B) Arrangement of TPR repeats in Ssn6-like proteins from *C. albicans*, *S. cerevisiae*, *Ustilago maydis*, *Schizosaccharomyces pombe*, *Dictyostelium discoideum*, humans, *Drosophila melanogaster*, and *Caenorhabditis elegans*. Coordinates of TPR domains are shown, and percentage amino acid sequence similarities between the shaded regions of CaSsn6 and other Ssn6-like proteins are indicated.

ical differences between *C. albicans ssn6* and *tup1* mutants. To examine these differences in more detail, we generated independent homozygous *ssn6/ssn6* mutants in *C. albicans* CAI8 (*ura3, ade2*) and carefully examined their morphologies.

As expected, wild-type *SSN6/SSN6* cells and heterozygous *ssn6/SSN6* cells generated large smooth white colonies when streaked onto YPDA plates (Figure 2). However, when freshly generated homozygous *ssn6/ssn6* mutants were restreaked onto fresh YPDA, dramatic colony phenotypes were observed routinely. A range of colony phenotypes arose from a single *ssn6/ssn6* colony: small versus large, red versus white, and wrinkled versus smooth colonies (Figure 2). Cells from all of these colonies were *ade2/ade2 ssn6/ssn6* mutants as confirmed by diagnostic PCR. This colony variability was reproduced in eight independent mutants.

In addition to displaying colony variability, most *ssn6* colony types displayed phenotypic instability. For example, 5.9% of cells from large pink wrinkly colonies switched to different colony morphologies when restreaked onto fresh YPDA (Figure 3). *Ssn6* cells from large smooth white colonies were relatively stable, with only 0.1% of these cells switching colony morphologies. As a result, serial passaging of *ssn6* strains generally led to an accumulation of the large smooth white growth form. Large wrinkly white colonies also arose relatively frequently after serial passaging.

A conditional *ssn6/MET3-SSN6* mutant was constructed to confirm that these dramatic phenotypic effects were caused by Ssn6 depletion. In the absence of methionine and cysteine, when the *MET3-SSN6* allele was expressed, this mutant displayed a poppy-like colony morphology (Figure 3), presumably because of abnormal Ssn6 expression patterns from the *MET3* promoter. This poppy-like phenotype was stable. However, a range of different colony morphologies was observed when clonal *ssn6/MET3-SSN6* cells were plated onto medium containing methionine and cysteine, and these phenotypes were unstable. Most cells in these colonies (73%) switched colony morphologies when replated onto medium containing methionine and cysteine (Figure 3). In contrast they reverted to the poppy-like morphology when replated onto medium lacking methionine and cysteine (unpublished data). These data confirmed that Ssn6 depletion dramatically effects *C. albicans* colony morphology and enhances switching between different colony phenotypes.

We examined the phenotypes of cells from the different colony types (Figure 2). During growth in liquid YPD, *ssn6* cells from smaller colonies displayed longer lag periods and generated less biomass than *ssn6* cells from larger colonies. With the exception of the large smooth white colonies, all cell types grew on media containing ethanol, glycerol as the sole carbon source.

Colonies formed by wild-type and *SSN6/ssn6* strains on YPDA at 30°C contained the ovoid budding cells typical of the yeast growth form of *C. albicans* (Sudbery *et al.,* 2004). In contrast, homozygous *ssn6/ssn6* cells from some colonies resembled pseudohyphae with septa at mother-daughter junctions (Figure 2). However, there was significant population heterogeneity with respect to the morphology of these cells, and they did not divide synchronously like true *C. albicans* pseudohyphae. This suggested that these cells were displaying a pathological morphological phenotype caused by Ssn6 inactivation, rather than bone fide pseudohyphal development. *Ssn6/ssn6* cells from other colonies were small and stumpy, reminiscent of *C. albicans efg1* cells (Lo *et al.,* 1997). There was no clear correlation between the degree of filamentation and the wrinkliness of *ssn6* colonies.

These *ssn6* phenotypes were significantly different from those of *tup1* and *nrg1* cells, which display stable cellular and colonial phenotypes (Braun and Johnson, 1997; Braun *et*

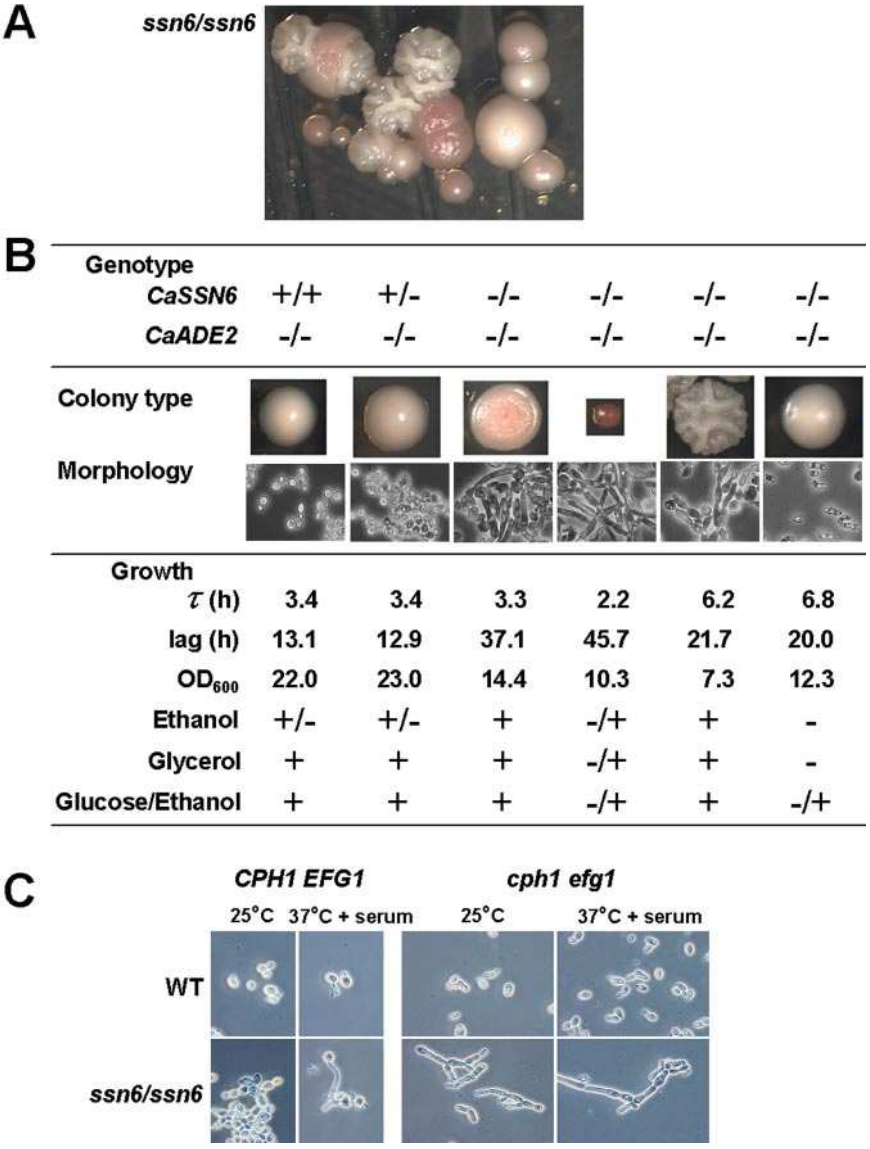


Figure 2. Phenotypes of *C. albicans ssn6* cells. (A) Mixed colony phenotypes of a freshly generated *ssn6/ssn6* mutant (SGC123) restreaked on YPDA. (B) Characterization the various *ssn6* colony types. Genotypes were rechecked by PCR diagnosis: +, wild-type allele; −, null allele (CAI8, SGC121 and SGC123: Table 1). For each colony type, cell morphology and growth in liquid medium in YPD at 30°C (length of lag phase, growth rate, and final OD_{600}) were determined. Growth on nonfermentable carbon sources as sole carbon source was examined on YNB plates at 30°C: +, robust growth; +/−, weak growth; −/+, very weak growth; −, no growth. (C) Morphogenetic responses of *ssn6* (SGC123) and *ssn6, cph1, efg1* cells (SGC133) after 3 h in YPD containing 10% serum.

al., 2001; Murad *et al.,* 2001a). A direct comparison of cells growing on YPDA at 30°C confirmed the contrasting cellular and colonial morphologies of wild-type, *nrg1, tup1,* and *ssn6* cells (Figure 4). The *ssn6* cells were more similar to the budding wild-type parent than the pseudohyphal *nrg1* and *tup1* cells. Furthermore, the wrinkly *ssn6* colonies were not invasive, unlike the *nrg1* and *tup1* colonies (Figure 4).

Hwang *et al.* (2003) examined the role of Ssn6 in morphogenesis by examining the epistatic relationships between Ssn6, Tup1, Cph1, and Efg1. Cph1 and Efg1 define the MAP kinase and cAMP-protein kinase A pathways, respectively, that activate hyphal development in *C. albicans* (Liu *et al.,* 1994; Bockmuhl and Ernst, 2001). Based on the complex phenotypes of the various mutants they examined, Hwang *et al.* (2003) suggested that Ssn6 inactivation induces pseudohyphal growth and that both Cph1 and Efg1 might activate this pseudohyphal growth. In our hands, Ssn6 inactivation did not lead to the formation of true pseudohyphae (Figures 2 and 4). We examined the effects of serum on *ssn6* mutants because serum is a strong inducer of hyphal development in *C. albicans*. In response to serum stimulation, *ssn6* cells appeared to retain the ability to form germ tubes, the progenitors of true hyphae (Figure 2C). Furthermore, unlike *tup1* cells (Braun and Johnson, 1997; Murad *et al.,* 2001), *ssn6* cells were still able to respond to serum after Cph1 and Efg1 inactivation, although the filaments formed by the *ssn6, efg1, cph1* triple mutants were not true hyphae. We suggest that although Cph1 and Efg1 can influence the morphology of *ssn6* cells, the morphological phenotypes caused by Ssn6 inactivation are not related to bone fide morphogenetic developmental programs.

Global Molecular Phenotypes of Wrinkly and Smooth C. albicans ssn6 Cells

The global molecular phenotypes of *C. albicans ssn6* cells from large white wrinkly and smooth colonies were compared by transcript profiling. The analysis of other growth forms was precluded by their phenotypic instability. About 1.5% of the ~6000 *C. albicans* genes displayed reproducible and statistically significant increases in expression of twofold or more in the wrinkly or smooth growth forms relative to wild-type cells (Figure 5A). Similar numbers of *C. albicans* genes displayed decreased expression. A direct comparison of the transcript profiles of *C. albicans* CAI4 and CAI8 (Sup-

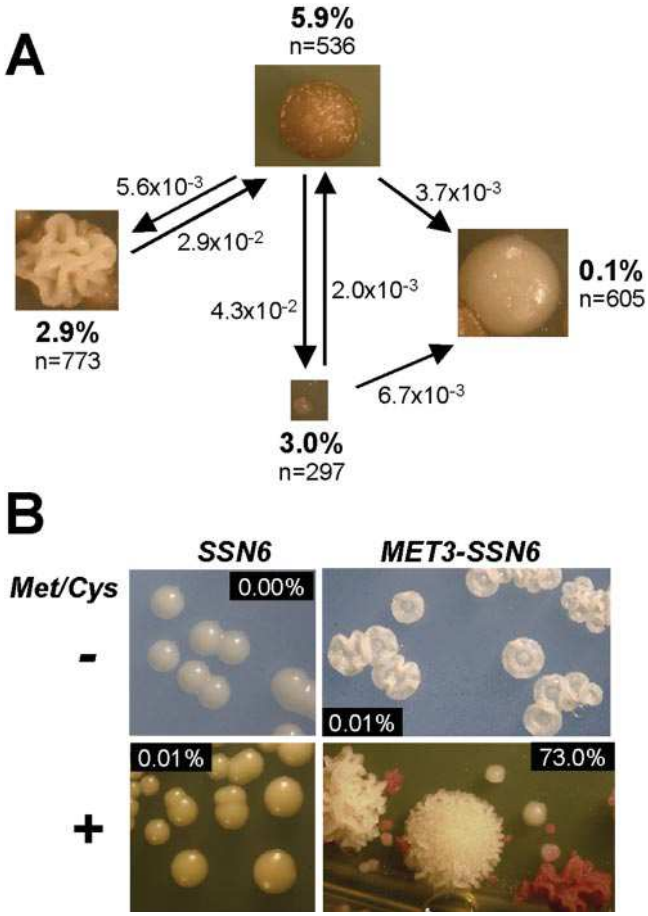


Figure 3. Phenotypic instability of *C. albicans ssn6* cells. (A) The phenotypic stability of each colony type was assayed by replating cells on fresh YPD and measuring the proportion of colonies that had switched to an alternative form. (B) Phenotypic instability could be induced in a conditional *ssn6/MET3-SSN6* strain following the addition of 2.5 mM methionine and cysteine to the medium: *SSN6*, CAI8; *MET3-SSN6*, SGC179 (Table 1).

plementary Data) confirmed that these differences were not due to differences between the parental strains use in this study (Table 1). The Ssn6 repressor might regulate these genes indirectly. Alternatively, Ssn6 might also be able to act as a transcriptional activator (Conlan *et al.*, 1999).

There was limited overlap between the transcript profiles of the wrinkly and smooth growth forms (Figure 5A), which might account for at least some of the phenotypic differences between these cells. A significant proportion (31%) of the genes that were up-regulated only in the wrinkly form encode functions involved in carbon metabolism (Table 2). In contrast, glycolytic genes were down-regulated in the smooth form. Ribosomal protein genes represented 28% of those that were up-regulated only in smooth cells. Iron assimilatory functions (*FTR1, HEM1, RBT2*) represented 9% of the genes that were up-regulated in both cell types. These observations were consistent with the differences in growth rates and carbon assimilation patterns of the various *ssn6* growth forms (Figure 2).

Global Comparison of the Ssn6, Tup1, and Nrg1 Regulons in C. albicans

Transcript profiling was also performed to compare the global roles of Ssn6, Tup1, and Nrg1 in *C. albicans*. Ssn6,

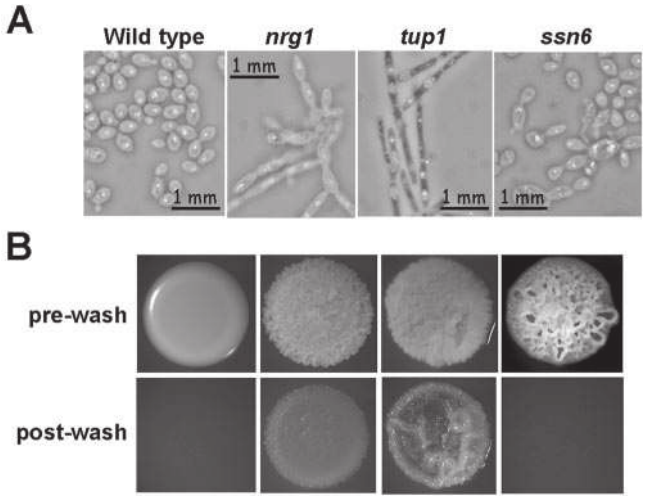


Figure 4. Phenotypic differences between wild-type, *nrg1, tup1,* and *ssn6* cells. (A) Morphology of cells growing exponentially in YPD at 30°C: wild-type, CAI8 containing CIp10; *nrg1,* MMC3; *tup1,* BCA2–9; *ssn6*, SGC123 (Table 1). (B) Colony morphology (prewash) and invasiveness (postwash) of the same strains growing on YPD at 30°C. To measure invasiveness, cells were washed off the surface of the plate into H_2O with a glass spreader.

Tup1, and Nrg1 regulons were defined operationally as those subsets of *C. albicans* genes whose expression was altered twofold or more in *ssn6, tup1,* and *nrg1* cells, respectively. If Ssn6 acts simply as a corepressor with Tup1, one might expect their regulons to overlap. Nrg1 is thought to be one of a number of proteins that targets the Tup1-Ssn6 complex to specific *C. albicans* promoters. On this basis one might predict that the Nrg1 regulon would be a subset

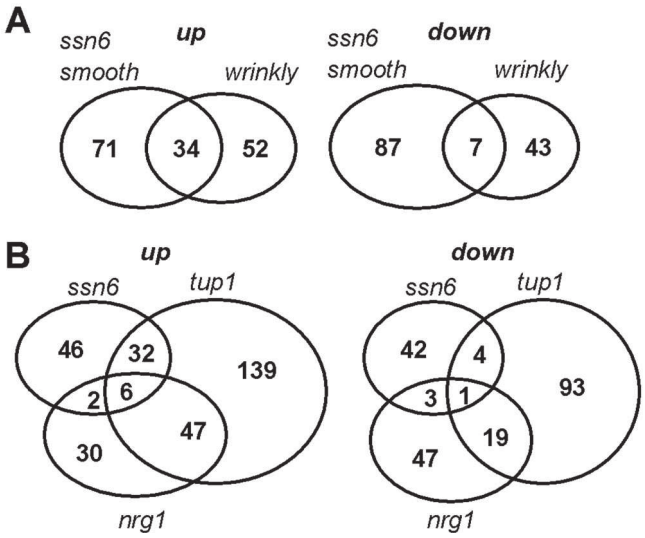


Figure 5. (A) Numbers of *C. albicans* genes that were up- or down-regulated twofold or more in smooth and wrinkly *ssn6* cells (SGC123), relative to the wild-type control (CAF2–1). (B) Numbers of *C. albicans* genes that were significantly up- or down-regulated in wrinkly *ssn6* (SGC123), *tup1* (BCA2–9), and *nrg1* cells (MMC3), relative to the wild-type control (CAF2–1). Significant changes of twofold or more were identified using SAM (FDR set at 10%) using data from three independent replicates for each cell type.

Table 2. *C. albicans* genes that are up-regulated in smooth and/or wrinkly *ssn6* cells

Smooth and wrinkly	Smooth only	Wrinkly only
ADH2, ALD5, CRD2, CTA1, FTR1, FRP6, GRP2, HEM1, HGT12, HSP12, INO1, MAM33, MET15, NTF2, OSM2, PCK1, PLB4.5, PLB4.3, PRY2, RBT2, RBT4, SAP7, IPF1580, IPF3277, IPF3282, IPF3282.3, IPF3964, IPF4181, IPF4182, IPF13493, IPF14109, IPF15839, IPF17186	ATP7, ATP20, CAN2, CAR1, CAR1.3, CCC1, CIT1, CYB5, CYS3, DLD3, FRE32, FRP1, FTR2, GAP2, GAP5, HGT11, HHF21, HHF22, HNM1, IDP2, IFD7, LYS9, LYS21, MRPL10, MRPL37, OPT1, PLC3, QCR8, QCR9, RIM8, RPL11, RPL25, RPL29, RPL30, RPL34B, RPL35, RPL38, RPL39, RPL42, RPL43A, RPS18, RPS21B, RPS22A, RPS22, RPS24, RPS26A, RPS28B, RPS30, SOD1, STL1, TOM22, UBI4, IPF277, IPF643, IPF1617, IPF2277, IPF2690.5, IPF2690.3, IPF3485, IPF3912, IPF4553, IPF6629, IPF7109, IPF12942, IPF14452, IPF14850, IPF14895, IPF15679, IPF17190, IPF17237	AAH1, ACS1, ADH1, ARC35, AYR2, CDR4, CRD1, CYB3, ECM41, ECM331, FBP1, GLC3, GLK1, GPD2, GSY1, HXK2, HXT5, HXT61, HXT62, MAE1, MLS1, PFK2, RHR2, RNR22, RPL23B, SNG3, SSA4, TFS1, TPS3, IPF1531, IPF4724, IPF5185, IPF6881, IPF7715, IPF8321, IPF8369, IPF10533, IPF10558, IPF12101, IPF13121.3, IPF13607, IPF13836, IPF15301, IPF15870, IPF15957, IPF16565, IPF17840, IPF19066, IPF19640, IPF19953, IPF20008

Genes that were up-regulated twofold or more in a reproducible and statistically significant manner are shown. Further details are provided in the Supplementary Data.

within the Ssn6-Tup1 regulon. However, neither of these simplistic predictions was supported by our data (Figure 5B), indicating that the roles of these factors are more complex.

C. albicans genes that are repressed by Tup1 or Ssn6 are expected to be overexpressed in *tup1* and *ssn6* cells, respectively. However, there was limited overlap between the Ssn6 and Tup1 regulons (Figure 5B). Most genes that were up-regulated in *tup1* cells were not significantly up-regulated in *ssn6* cells (186 of 224 genes). This suggests that Tup1 represses the expression of these genes, but that Ssn6 is not essential for their repression.

A significant number of *C. albicans* genes were down-regulated in *tup1* cells (Figure 5B). Tup1 might regulate these genes indirectly. Significantly, most genes that were down-regulated in *tup1* cells were not down-regulated in *ssn6* cells (112 of 117 genes). Again this suggests that Ssn6 is not essential for the Tup1-mediated regulation of these genes.

Of the 85 *C. albicans* genes that were up-regulated in *nrg1* cells, 32 were not up-regulated in *tup1* cells, and most were not up-regulated in *ssn6* cells (Figure 5B). This suggested that Tup1 is not essential for the repression of some genes by Nrg1 and that Ssn6 is not essential for the repression of most Nrg1-repressed genes. Hence Nrg1 appears to act independently of Tup1 and Ssn6 to repress some *C. albicans* genes.

We examined the functions of the genes in the Nrg1, Tup1, and Ssn6 regulons in *C. albicans*. These genes and their functions are listed in the Supplementary Data. Previously, we reported that hypha-specific genes are regulated both by Nrg1 and Tup1 (Murad *et al.,* 2001a). This observation, which was made using macroarrays containing about one-third of *C. albicans* genes, was confirmed in this study, which used (nearly) whole genome *C. albicans* microarrays. The hypha-specific genes *HWP1, ECE1, RBT1,* and *RBT5* were among the most strongly regulated genes in the subset of genes regulated by Tup1 and Nrg1 (Table 3). These data are consistent with those of Kadosh and Johnson (2005) in the accompanying paper. The hypha-specific genes, *ALS3* and *ALS8,* were absent from the subset of Tup1- and Nrg1-regulated genes because they were not on the microarray. However, we have shown previously that *ALS3* is regulated both by Nrg1 and Tup1 (Murad *et al.,* 2001a). (*ALS8* is now known to be an allele of *ALS3*: Zhao *et al.,* 2004.) The expression of *HYR1,* which encodes a hypha-specific cell wall glycoprotein (Bailey *et al.,* 1996), was elevated 2.3-fold in *tup1* cells, but was not elevated in *nrg1* or *ssn6* cells. The expression of *HGC1/CLN21*, which encodes a hypha-specific cyclin (Zheng *et al.,* 2004), was elevated 4.0-fold in *tup1* cells, 1.6-fold in *nrg1* cells, and 1.0-fold in *ssn6* cells. Therefore, whereas *HGC1/CLN21* formally lies outside the Nrg1-Tup1 regulon, it does appear to be regulated by both of these factors.

Interestingly, our transcript profiling indicated that hypha-specific genes were not derepressed in *ssn6* cells grown at 30°C and was confirmed by northern blotting (Figure 6A). The *HWP1* mRNA was derepressed in *nrg1* and *tup1* cells, but not in the *ssn6* mutant. Hwang *et al.* (2003) reported that *HWP1* and *ECE1* were derepressed in *ssn6* cells. However, this derepression was partial, only being observed at 37°C and not at 30°C. We conclude that Nrg1-Tup1 mediates the repression of most hypha-specific genes in *C. albicans*, but that Ssn6 is not essential for this repression, at least under the conditions examined in this study.

A comparison of the global cellular roles of Ssn6 and Tup1 was performed by examining whether specific functional categories were enriched in the subsets of genes whose expression levels were altered in *ssn6* and *tup1* cells (Table 4). This analysis reinforced the view that Ssn6 does not contribute to the regulation of hypha-specific genes under these growth conditions. The analysis also highlighted the important roles of Tup1 and Ssn6 in repressing genes involved in carbon metabolism. Most notably, the gluconeogenic genes *PCK1* and *FBP1* and the glyoxylate cycle gene *MLS1* were derepressed in both *ssn6* and *tup1* cells (Table 4; Figure 6A). We note that phenotypic switching is associated with changes in the expression of genes involved in central carbon metabolism (Lan *et al.,* 2002). Tup1 and Ssn6 also appear to play roles in the regulation of genes involved in amino acid metabolism. Further details are provided in the Supplementary Data.

Table 3. *C. albicans* genes that are up-regulated in *nrg1* and *tup1* cells, but not in *ssn6* cells

Systematic name	Common name	Fold regulation			Function[a]
		nrg1	*tup1*	*ssn6*	
CA2830	RBT1	9.6	27.9	1.0	Repressed by TUP1 protein 1
CA4336	DDR48	13.6	22.1	1.0	Stress protein (by homology)
CA1402	ECE1	8.7	20.0	1.0	Cell elongation protein
CA2825	HWP1	11.8	15.4	1.0	Hyphal wall protein
CA2558	RBT5	2.7	13.3	1.2	Repressed by TUP1 protein 5
CA5112	IPF1341	5.9	10.0	1.7	Similarity to mucin proteins (by homology)
CA2405	IPF3844	3.4	7.4	1.1	Unknown function
CA4381	IPF20169	2.8	6.0	1.1	Unknown function
CA0386	IPF4065	2.1	5.4	1.1	Unknown function
CA0448	ALS10	8.5	4.7	1.1	Agglutinin-like protein
CA4174	IPF4119.5	2.4	4.6	1.1	Unknown function, 5′ end
CA3154	FUN34.5	2.1	4.4	1.8	Unknown function, 5′ end
CA5641	GAC1	2.7	4.3	1.0	ser/thr phosphoprotein phosphatase 1, regulatory chain (by homology)
CA0074	IFD7	2.5	4.3	1.3	Putative aryl-alcohol dehydrogenase (by homology)
CA4857	PHR1	3.2	4.2	1.1	GPI-anchored pH responsive glycosyl transferase
CA3813	FRP1	2.6	4.2	1.0	Related to *Y. lipolytica* glyoxylate pathway regulator Gpr1p
CA4985	IPF2050	3.6	3.9	1.1	Similar to *S. cerevisiae* Kip1p kinesin-related protein (by homology)
CA1238	IPF15781	3.9	3.8	1.1	Unknown function
CA0722	ERK1	2.3	3.8	1.2	Mitogen-activated protein kinase (FUS3 homolog)
CA4120	SOD1.3	3.9	3.7	1.0	Cu,Zn-superoxide dismutase, 3-prime end
CA0671	GRP4	2.2	3.6	1.0	Putative reductase (by homology)
CA0316	ALS1.3eoc	3.4	3.5	0.8	Agglutinin-like protein, 3-prime end
CA0840	IFD1	2.1	3.5	1.1	Putative aryl-alcohol dehydrogenase (by homology)
CA3827	IPF10662	6.3	3.3	1.2	Unknown function
CA3260	IPF7968	2.3	3.3	1.0	Unknown function
CA5650	IPF7109	2.1	3.3	1.8	Unknown function
CA3842	YKE2.3	2.1	3.3	1.4	gim complex component, 3-prime end (by homology)
CA4189	IPF7527	3.0	3.0	1.0	Unknown function
CA3173	IPF14145	5.9	2.9	1.0	Unknown function
CA4127	IPF6629	2.6	2.9	1.6	Unknown function
CA2302	IPF6518	2.3	2.8	1.4	Unknown function
CA2391	ADH5	2.7	2.7	1.2	Probable alcohol dehydrogenase (by homology)
CA5039	GAP2	4.9	2.6	1.4	General amino acid permease (by homology)
CA2291	IPF9740	2.5	2.6	1.7	Oligo-1,4 -1,4-glucantransferase/amylo-1,6-glucosidase (by homology)
CA1188	RPL30.3	2.0	2.6	1.7	RNA binding, 3′ end (by homology)
CA2937	RPS21B.3	2.0	2.6	1.4	Ribosomal protein S21, 3′ end
CA5339	IPF885	2.8	2.5	1.2	Glucan 1,3-beta-glucosidase (by homology)
CA5225	ACB1.exon2	2.2	2.5	1.5	Acyl-coenzyme-A-binding protein, exon 2 (by homology)
CA5953	IPF3506	2.2	2.5	1.0	Unknown function
CA0559	GPX1	2.0	2.5	1.3	Glutathione peroxidase (by homology)
CA2589	KRE1	2.4	2.4	0.9	Secretory pathway protein
CA3766	IPF18298.3	2.6	2.3	1.1	Unknown function, 3-prime end
CA4534	RPS26A	2.1	2.3	1.7	Ribosomal protein S26.e.A, cytosolic (by homology)
CA3897	PFY1	2.5	2.2	1.1	Binds to actin
CA4113	CHO1	2.3	2.2	1.1	Phosphatidylserine synthase
CA0716	DOG2	3.0	2.1	1.1	2-deoxyglucose-6-phosphate phosphatase (by homology)
CA2947	IPF6298	2.4	2.0	1.2	Unknown function

Genes were considered to be up-regulated if their expression ratio in mutant vs. wild type cells was twofold or more in a reproducible and statistically significant manner. Further details are provided in the Supplementary Data.

[a]Information on gene function was taken from CandidaDB (http://genolist.pasteur.fr/CandidaDB)

Role of Tup1 and Ssn6 in NRE-mediated Repression

Nrg1 represses the hypha-specific gene expression via Nrg1 Response Elements (NREs: Murad *et al.*, 2001a). Therefore, we tested whether Ssn6 and Tup1 are required for NRE-mediated repression in *C. albicans*. An oligonucleotide containing four NRE elements of the type C_5T was cloned upstream of a basal *lacZ* reporter and transformed into *C. albicans*. The expression levels of the NRE-*lacZ* and basal-*lacZ* reporters were then compared in wild-type, *nrg1*, *tup1*, and *ssn6* cells (Figure 7A). The introduction of the NRE elements repressed expression in wild-type cells, and this repression was released in *nrg1* cells. This confirmed that Nrg1 was required for the repression imposed by the NRE element. Repression was also released in *tup1* and *ssn6* cells. This indicated that both Ssn6 and Tup1 were required for Nrg1-NRE–mediated repression in *C. albicans*. Similar find-

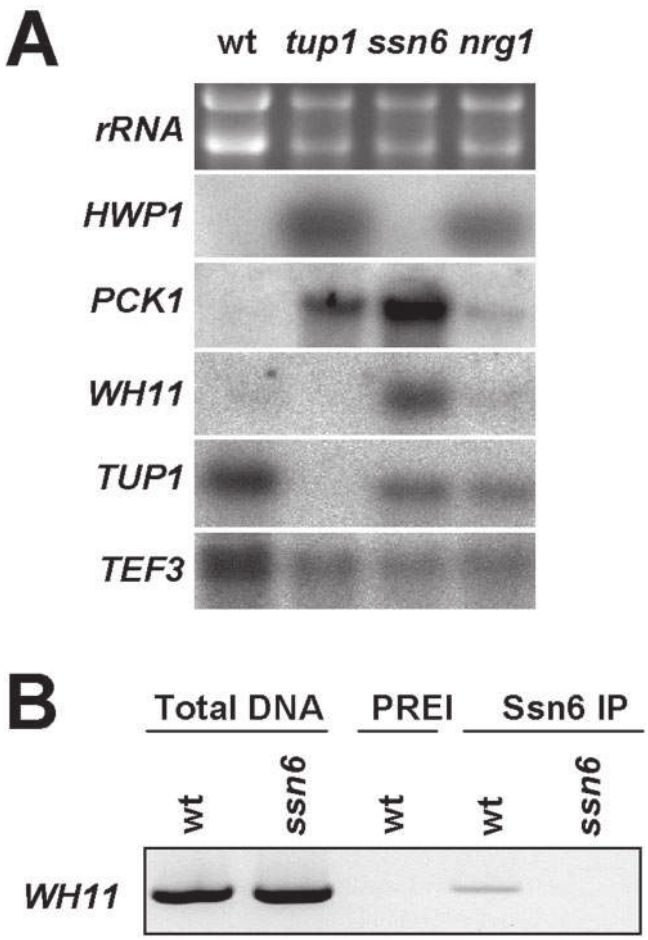


Figure 6. (A) Northern analysis of *C. albicans* mRNAs in wild-type (CAI8), *tup1* (BCA2–9), *ssn6* (SGC123), and *nrg1* cells (MMC3). (B) Chromatin immunoprecipitation revealing association of Ssn6 with the *WH11* promoter: wild-type cells (CAI8), *ssn6* cells (SGC123); PCR with genomic DNA template, Total DNA; reaction with preimmune serum, PREI; reactions with anti-Ssn6 serum.

ings were made using a second NRE reporter containing the A_2C_3T and C_5T elements seen in the *ALS3* promoter (unpublished data).

The roles of Ssn6 and Tup1 in Nrg1-mediated repression were analyzed further using the *C. albicans* one-hybrid system (Doedt *et al.*, 2004; Russell and Brown, 2005). Briefly, we tested the ability of a lexA-Nrg1 fusion to impose repression on a *lacZ* reporter that carries a lexA operator. The expression levels of the LexOP-*lacZ* reporter were analyzed in wild-type, *nrg1*, *tup1*, and *ssn6* cells expressing either the lexA-Nrg1 fusion or the lexA protein alone (Figure 7B). The presence of Nrg1 in the lexA-fusion caused nearly threefold repression in wild-type and *nrg1* cells. This repression was significantly attenuated in *ssn6* and *tup1* cells, indicating that both Ssn6 and Tup1 were required for repression by the lexA-Nrg1 fusion in *C. albicans*.

Regulation of WH11 by Ssn6

The phenotypic instability induced by Ssn6 depletion (Figure 3) was reminiscent of the phenotypic switching described by Soll and colleagues (Slutsky *et al.*, 1985; Soll, 2002). They have reported that misexpression of *TUP1* or the white-phase-specific gene *WH11* can induce phenotypic switching in the *C. albicans* strain WO1 (Kvaal *et al.*, 1997; Zhao *et al.*, 2002). Therefore, we examined whether *WH11* expression was affected in *C. albicans ssn6* cells. *WH11* mRNA levels were significantly elevated in *ssn6* cells, but not in *tup1* or *nrg1* cells (Figure 6A). Then we tested whether Ssn6 is associated with the *WH11* promoter by chromatin immunoprecipitation. *WH11* promoter sequences were significantly enriched in wild-type cells, but not in *ssn6* cells after chromatin immunoprecipitation with an anti-Ssn6 antibody (Figure 6B). This enrichment was not observed with preimmune sera. Therefore, Ssn6 appears to associate with the *WH11* promoter in vivo.

These observations lend weight to the idea that Ssn6 can act independently of Tup1 and Nrg1 to regulate the expression of some *C. albicans* genes (Figure 5B). Furthermore, they provide a possible mechanism by which Ssn6 depletion can promote phenotypic instability. Ssn6 depletion appears to cause *WH11* misexpression, which is known to promote phenotypic switching in *C. albicans* (Kvaal *et al.*, 1997).

DISCUSSION

The prevailing view is that Ssn6 interacts with Tup1 to form a corepressor complex that regulates a large number of genes in budding yeast and that this corepressor complex is

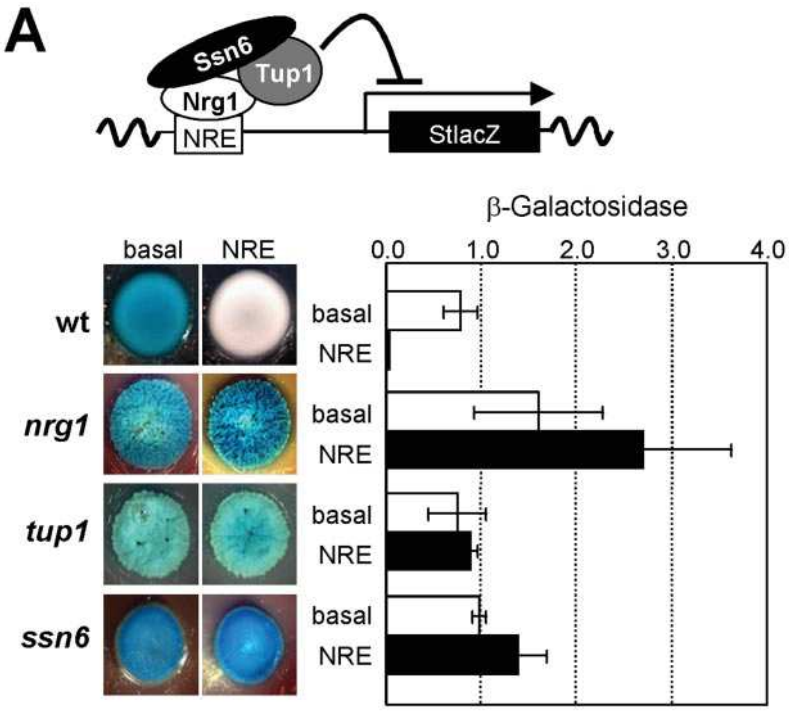
Table 4. Enrichment of functional categories in subsets of genes whose expression was significantly altered in *ssn6* or *tup1* cells

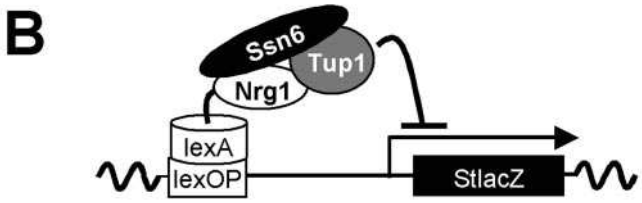
Realtive enrichment of functional category[a]

	Up			Down		
Functional category	*tup1*	*ssn6*	*tup1 and ssn6*	*tup1*	*ssn6*[b]	*tup1 and ssn6*
Amino acid metabolism	1.16	5.00	1.39	2.01	6.25	3.57
Carbon metabolism	2.88	1.67	5.56	0.43	0.00	2.38
Cell rescue and defense	0.63	2.50	1.39	0.46	2.08	0.00
Hypha-specific genes	16.88	0.00	0.00	0.00	0.00	0.00
Unknown function	1.76	0.45	1.52	0.88	0.00	0.16

[a]The Relative Enrichment of Functional Category was calculated by determining the proportion of genes in the subset of Ssn6- or Tup1-regulated genes that belong to these functional categories and then dividing this by the proportion of all *C. albicans* genes that belong to the categories (Yin *et al.*, 2004).

[b]The number of genes in this subset was too low to generate significant data on the enrichment of Functional Category.





β-Gal^{ase}	lexA	lexA-Nrg1	Fold Repression
wt	181.4 +/- 23.2	63.7 +/- 2.5	2.8
nrg1	69.2 +/- 6.6	25.4 +/- 6.1	2.7
tup1	175.4 +/- 64.0	115.7 +/- 66.9	1.5
ssn6	117.0 +/- 31.2	82.3 +/- 6.8	1.4

Figure 7. (A) An NRE imposes repression on a *lacZ* reporter in *C. albicans* in an Nrg1-, Ssn6-, and Tup1-dependent manner: wild-type, CAI4; *nrg1*, MMC4; *tup1*, BCA2–9; *ssn6*, SGC124 (Table 1); basal reporter lacking NRE, basal; NRE [$(C_5T)_4$] containing reporter. (B) In a *C. albicans* one-hybrid assay, a lexA-Nrg1 fusion imposes repression on a Lex Operator-*lacZ* reporter in an Ssn6- and Tup1-dependent manner: wild-type, CAI8; *nrg1*, MMC9; *tup1*, CRC004; *ssn6*, SGC124. Fold repression was measured by comparing β-galactosidase levels (Miller Units) for *C. albicans* cells expressing LexA with those expressing the LexA-Nrg1 fusion. Means and standard deviations from triplicate assays on three independent transformants are shown.

conserved from yeast to humans (reviewed by Smith and Johnson, 2000). Based on this *S. cerevisiae* paradigm, it was predicted that Ssn6 and Tup1 might execute equivalent functions in *C. albicans*. Consistent with this, Johnson and coworkers have shown that Tup1 plays a critical role in the repression of filamentous growth and the expression of hypha-specific genes in *C. albicans* (Braun and Johnson, 1997, 2000; Braun *et al.*, 2000, 2001; Kadosh and Johnson, 2005). Others have added to this by confirming that Tup1 regulates hypha-specific genes and by identifying other putative Tup1 gene targets in *C. albicans* (Sharkey *et al.*, 1999; Murad *et al.*, 2001b).

The role of Ssn6 in *C. albicans* remained less clear. In their elegant article, Hwang *et al.* (2003) showed that *C. albicans SSN6* is a functional homologue of *S. cerevisiae SSN6*. They also examined the morphological phenotypes of *C. albicans ssn6* mutants and the epistatic relationships between Ssn6, Tup1, Cph1, and Efg1, which were complex. They suggested that Ssn6 plays an important role in regulating yeast-(pseudo)hypha morphogenesis, pointing out that Ssn6 might play some distinct roles in this process. However, the relationship between Ssn6 and Tup1 in *C. albicans* remained obscure. Hence we have examined this issue in some detail. As a result we are able to draw a number of important conclusions.

First, *C. albicans* Ssn6 is member of a conserved family of eukaryotic Ssn6-like proteins that display a relatively high level of sequence similarity in their TPR domains (Figure 1). This is significant because these domains mediate interactions with Tup1-like proteins (Tzamarias and Struhl, 1995). On this basis, *C. albicans* Ssn6 would be expected to form a corepressor complex with Tup1.

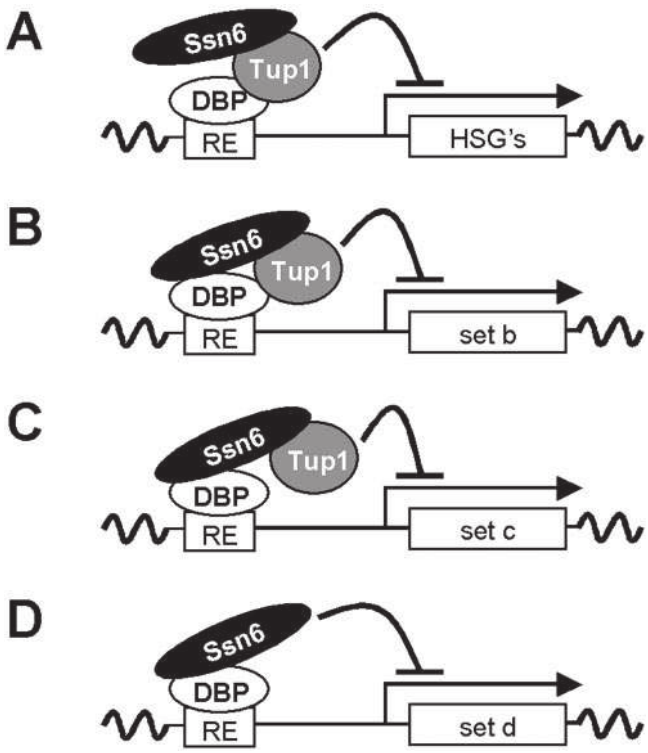


Figure 8. Models illustrating Ssn6 function in *C. albicans*. (A) Ssn6 is not required for Tup1-mediated repression at some promoters, suggesting that Tup1 might interact directly with the DNA-binding protein (DBP). (B) Ssn6 is partially required for Tup1-mediated repression at some *C. albicans* promoters, suggesting that Ssn6 stabilizes Tup1 interactions with the DNA-binding protein. (C) Ssn6 is essential for Tup1-mediated repression at other promoters, suggesting that Tup1 depends on Ssn6 for its interaction with the DNA-binding protein. The DNA-binding protein might differ between these promoters, or alternatively differences in the sequence context of the Response Element (RE) might affect the Ssn6 dependency. (D) The repression of some *C. albicans* promoters by Ssn6 is not dependent on Tup1 (see text).

Second, Ssn6 and Tup1 do not execute equivalent cellular roles in *C. albicans*. This conclusion is based on several complementary observations. *C. albicans ssn6* and *tup1* mutants display dramatically different phenotypes. *C. albicans tup1* cells display stable phenotypes, growing constitutively as pseudohyphae that form highly wrinkled and invasive colonies (Braun and Johnson, 1997). In contrast, independent *C. albicans ssn6* mutants and conditional *MET3-ssn6* mutants displayed unstable phenotypes, switching at high frequencies between different growth forms (strains SGC123 and SGC179: Table 1, Figures 2 and 3). Some *ssn6* growth forms generated pseudohyphal-like cells. However, these cells did not divide synchronously, they showed a high degree of population heterogeneity, and they were not invasive (Figure 4). We also observed that *ssn6* cells were able to form filaments in response to serum, even in the absence of Cph1 and Efg1 (Figure 2C). This indicated that the filamentous structures generated by *ssn6* cells were not dependent on the MAP kinase and cAMP-protein kinase A signaling pathways that trigger hyphal development in response to serum (Lo *et al.,* 1997; Ernst, 2000; Brown 2002). Furthermore, the inactivation of Ssn6 led to the derepression of the phase-specific gene *WH11,* but did not affect the expression of hypha-specific genes (Figures 5 and 6). These data are consistent with the idea that Ssn6 depletion does affect cell morphology (Hwang *et al.,* 2003). However, we conclude that, under the conditions analyzed in this study, Ssn6 depletion promotes phenotypic switching rather than morphogenesis.

Third, Ssn6 is not essential for Tup1-mediated repression of many genes in *C. albicans.* If Ssn6 were essential for Tup1-mediated repression, transcript profiling would have revealed a high degree of concordance between the Ssn6 and Tup1 regulons, as was the case in *S. cerevisiae* (Hughes *et al.,* 2000). In general, *S. cerevisiae ssn6* and *tup1* cells display only quantitative differences in expression levels. However, this was not the case in *C. albicans*. The repression of most Tup1-regulated genes was not dependent on Ssn6 (Figure 5). Hence Ssn6 does not simply act as a corepressor with Tup1 in *C. albicans*.

Fourth, Ssn6 can act independently of Tup1 to repress some *C. albicans* genes. The repression of a significant number of Ssn6-regulated genes was not dependent on Tup1 (Figure 5). Furthermore, although the repression of the *HWP1* gene was dependent on Tup1 but not Ssn6, the repression of *WH11* was dependent on Ssn6 but not Tup1 (Figure 6).

Fifth, Ssn6 and Tup1 are required to differing extents for Nrg1-mediated repression at specific *C. albicans* promoters. Nrg1 represses the transcription of some *C. albicans* promoters in a Tup1-dependent manner via the NRE element (Braun *et al.,* 2001; Murad *et al.,* 2001a). This knowledge facilitated the functional analysis of Ssn6 in *C. albicans* by allowing us to investigate the role of this protein at specific promoters. Under the conditions analyzed in this study, it is clear that Ssn6 is not essential for the Nrg1-mediated repression of *C. albicans* genes, including hypha-specific genes (Figures 5 and 6). However, both Ssn6 and Tup1 are essential for Nrg1-mediated repression at two artificial promoters (Figure 7).

Many of these observations can be explained in the context of the *S. cerevisiae* Ssn6-Tup1 paradigm (Tzamarias and Struhl, 1995; Smith and Johnson, 2000). According to this paradigm, Ssn6 stabilizes the interaction between Tup1 and the Tup1 targeting protein. We suggest that the extent to which Ssn6 is required to stabilize this interaction depends on the *C. albicans* promoter (Figure 8). Ssn6 might essentially be redundant at some *C. albicans* promoters, but this does not exclude the possibility that Ssn6 remains bound to Tup1 at these promoters (Figure 8A). At other promoters, Ssn6 might promote the interaction between Tup1 and the Tup1-targeting protein. Under some conditions the role of Ssn6 might be minimal, but this does not exclude the possibility that under other conditions, Ssn6 might become more important to maintain the stability of the complex (Figure 8B). Finally at other promoters, Ssn6 might be essential for the integrity of the complex (Figure 8C). In reality, Tup1-regulated *C. albicans* promoters probably represent a continuum between the extremes shown in Figure 8.

These models predict that different Tup1 targeting proteins will depend to differing extents on Ssn6 for their interaction with Tup1 (Tzamarias and Struhl, 1995; Smith and Johnson, 2000). However, if one assumes that the sequence context of the NRE can influence Nrg1 conformation, then these models can also explain why the Nrg1-mediated repression of some promoters, but not others, depends on Ssn6 (Figures 5 and 7). Furthermore, these models also account for the observation that Ssn6 is not required for the repression of hypha-specific promoters at 30°C (Figures 5 and 6), but is apparently essential at 37°C (Hwang *et al.,* 2003). However, an additional model is required to account for the

observation that the repression of some promoters, such as *WH11*, is dependent on Ssn6, but not Tup1 (Figures 5 and 6). Clearly, Ssn6 can repress some *C. albicans* genes independently of Tup1 (Figure 8D). It remains to be established whether Ssn6 can act in concert with an alternative corepressor at some *C. albicans* promoters.

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