

Regulation of G1 phase and differentiation in
Candida albicans by the cyclin Cln3p and MBF transcription factor complex

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ABSTRACT

Regulation of G1 phase and differentiation in

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Bahira Hussein

The G1/S transition represents the stage in the cell cycle when cells either commit to mitosis and continue to proliferate, or embark on developmental pathways in response to environmental and internal cues. In the model yeast *S. cerevisiae*, the CDK Cdc28p and G1 cyclin Cln3p control passage through the G1/S transition by activating the transcription factor complex SBF/MBF, which is composed of the regulatory subunit Swi6p and the DNA-binding elements Swi4p (SBF) or Mbp1p (MBF). SBF/MBF in turn activates transcription of numerous genes to initiate cell proliferation. In the multi-morphic fungal pathogen *C. albicans*, the G1/S regulatory circuit and control of basic cell proliferation are poorly understood. Previously work demonstrated that the cyclin Cln3p was essential for growth of yeast cells but also linked to development of hyphae. To gain more insight on the circuitry governing the G1/S transition and identify potential mediators of Cln3p function, we obtained transcription profiles of cells depleted of Cln3p and characterized orthologues of Swi6p, Swi4p and Mbp1p. Our results confirmed that cells depleted of Cln3p were arrested in G1 phase, and provide the first picture of factors associated with the G1/S transition in white phase yeast cells. The data demonstrate that the emerging G1/S circuit contains unique features compared to those in other fungi, including the fact that Cln3p activity is mediated only in part by MBF, that Mbp1p does not play a significant role in regulating yeast cell proliferation, and that novel, fungal-

specific factors may be associated with growth control. The data also identify potential factors involved in linking Cln3p and MBF activity with development of hyphae and possibly the opaque yeast state, through novel means. Overall, our results have laid the groundwork for constructing a framework of the G1/S regulatory circuit in *C. albicans* yeast cells, which will lead to a more comprehensive understanding of how basic cell proliferation in the pathogen is regulated and potentially linked with development, an important virulence-determining trait.

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Abbreviations

5-FOA	5-Fluoroorotic acid
BCIP	5-Bromo-4-Chloro-3'-Indolylphosphate p-Toluidine Salt
bp	base pair(s)
BSA	Bovine Serum Albumin
cAMP	Cyclic adenosine monophosphate
CDC	Cell division cycle
CDK	Cyclin-dependent kinase
DAPI	4', 6' diamidino-2-phenylindole dihydrochloride
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dCTP	Deoxycytidine triphosphate
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal Calf Serum
gDNA	Genomic DNA
hr	Hour(s)
IMDM	Iscoe's Modified Dulbecco's Medium
kb	kilo base pair(s)
L	Litre(s)
LiAc	Lithium acetate
MAP	Mitogen-activated protein
MBF	<i>MluI</i> binding factor
-MC	SD medium lacking methionine and cysteine.
+MC	SD medium supplemented with 2.5mM methionine and 0.5mM cysteine.
min	Minute(s)
ml	Milliliter(s)
MTL	Mating Type-like Locus
NBT	Nitro-Blue Tetrazolium Chloride
nt	nucleotides
O.D.	Optical Density
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
PEG	Polyethylene glycol
Rcf	Relative Centrifugal Force
RNA	Ribonucleic acid
rpm	Rotations per minute
SBF	Swi4-Swi6 cell cycle box binding factor
SD	0.67% yeast nitrogen base without amino acids, 2% glucose
SDS	Sodium Dodecyl Sulfate
sec	Second(s)
SSC	Saline-Sodium Citrate
ssDNA	Salmon Sperm DNA
TRIS	Tris(hydroxymethyl)aminomethane
YPD	1% yeast extract, 2% peptone, 2% dextrose

1. Introduction

Candida albicans is one of the most medically important fungal pathogens, which lives as a commensal in the gastrointestinal or genitourinary tracts of healthy humans with no harmful effects. As an opportunistic pathogen it can cause both mucosal and systemic infections in immune-compromised people (1). Systemic fungal infections have emerged as an important cause of mortality in immune-compromised patients (2). Current treatments for *C. albicans* infection involve azole-based drugs, including amphotericin B and fluconazole, but these can have harsh side-effects in patients since *C. albicans* is also a eukaryote (3, 4). In addition, use of these azole-based drugs results in increased drug resistance (5). Thus, there is a strong need to find new drug targets. In order to fully understand the mechanisms involved in fungal infection and the potential actions of therapeutic drugs, a comprehensive understanding of the biology of the pathogen is required. However, we presently have very little knowledge of the many regulatory networks that govern basic growth as well as virulence in the organism.

1.1 *Candida albicans* morphology

The morphological diversity of *C. albicans* promotes its survival, growth, and dissemination in the host, and the ability to switch between yeast, hyphal, and pseudohyphal growth forms is essential for virulence and pathogenicity. Mutants locked in one cell form are avirulent (6-9). *C. albicans* can grow in a variety of forms, including unicellular white or opaque budding yeast, pseudohyphae, true hyphae, or chlamydospores. Yeast cells are characterized by growth via polar followed by isometric

expansion of buds, which then separate from the mother cell. In contrast, pseudohyphal cells produce buds that remain attached to the parent cell after septum formation, resulting in filaments with constrictions where the septa are formed (1). True hyphae form when an unbudded yeast cell extends a germ tube with parallel side-walls under certain environmental stimuli. The nucleus divides within the elongating germ tube, after which one migrates back into the mother cell, and the second moves further into the elongating germ tube. Unlike yeast and pseudohyphal cells, hyphae do not have a constricted septum at the junction with the mother cell; the first hyphal septum forms well within the germ tube past the bud neck. Subsequent unconstricted septa compartmentalize the filament, and enclose a single nucleus per compartment (1, 2, 10). Chlamydospores are less characterized but consist of thick-walled round cells that occasionally form at the ends of pseudohyphae and hyphae in response to stress (2, 10). While the previously described yeast cells are considered “white” phase cells, due to their white colony appearance, another yeast form exists known as “opaque” cells. Opaque cells are rectangular-shaped, elongated cells with pits in the cell wall, that show a darker appearance on plates in the colony form, and demonstrate distinct gene expression patterns (1, 11). Although *C. albicans* was initially thought to be an asexual diploid, a mating type-like locus (*MTL*) was discovered and the opaque cell type was found to be the mating-competent form of the organism (12). In contrast, *Saccharomyces cerevisiae* exists as budding yeast and pseudohyphae under certain conditions, and reproduces sexually through the standard yeast form.

Differences in shape, surface components, and virulence factors associated with the various cell types of *C. albicans* are thought to be beneficial in the diverse host

environments. For instance, both hyphae and pseudohyphae are invasive, and one opinion is that this promotes tissue penetration during infection (2). In addition, filamentous growth is believed to provide increased resistance to phagocytosis, where phagocytized yeast cells can extend hyphal protrusions that puncture and kill the offending macrophages (7). In comparison, yeast cells are thought to be more suitable for dissemination in the circulatory system, although the yeast form is able to pass through the gastrointestinal wall of mice (13). Yeast cells also promote pathogenesis by secreting aspartyl proteases that have been associated with virulence (14). Thus, cell differentiation, or the ability to switch between cell fates, is essential for virulence, but the regulatory mechanisms are not fully understood.

1.2 Regulation of differentiation:

Environmental conditions and signal transduction pathways

Several environmental factors can induce cell differentiation; this probably reflects the various host environments in which *C. albicans* must survive. A lower temperature of 30°C, a low pH (4.0), and the absence of serum induce yeast growth. On the other hand, a higher temperature of 37°C, high pH (7.0), and the presence of serum, for example, induce hyphal growth. A pH of 6.0 and nitrogen-limiting conditions on solid medium can induce pseudohyphal growth (2).

The environmental cues are mediated by a number of signal transduction pathways (15) (Fig. 1). For example, the yeast to hyphal switch can be activated by a conserved Ras-cAMP signaling pathway that responds to nutrient deprivation or serum,

of which the transcription factor Efg1p is an important constituent. Ras1p mediates the environmental cues with adenyl cyclase Cdc35p (2, 16).

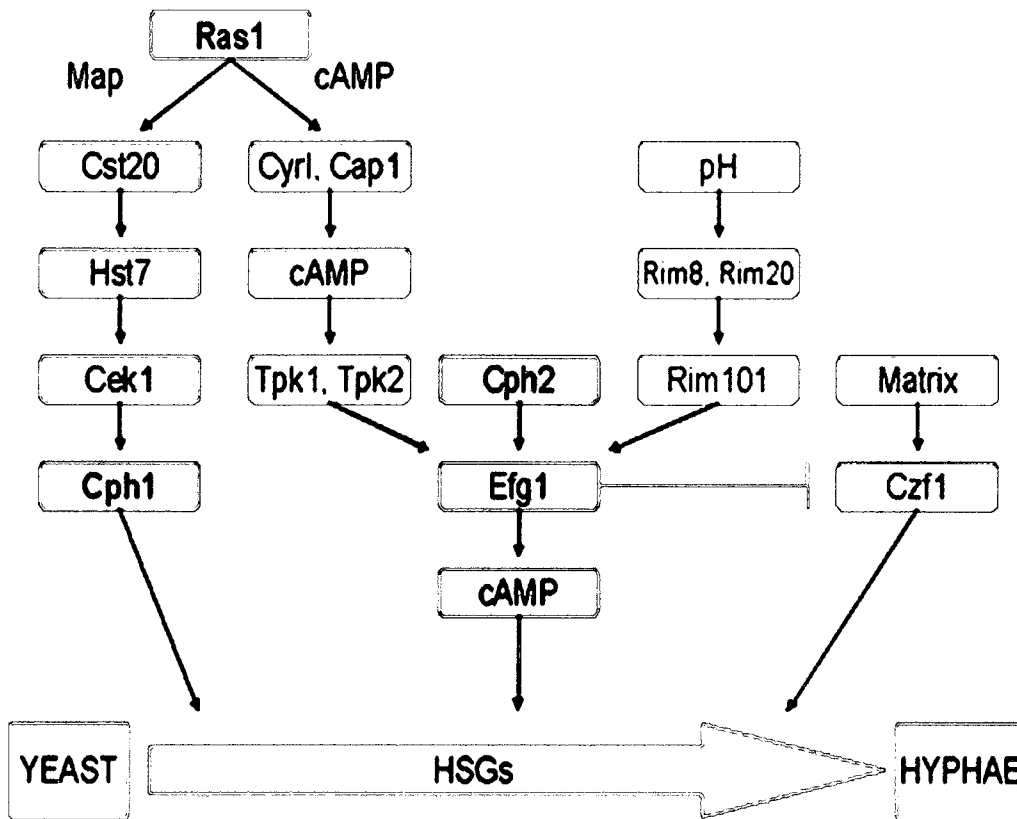


Figure 1. Signal transduction pathways that regulate hyphal morphogenesis.

A conserved Ras-mitogen-activated protein (MAP) kinase pathway also promotes hyphal development in response to nutrient deprivation, which involves the Cph1p transcription factor (2, 10). Environmental pH influences hyphal development through the induction of Rim101 induction, which is activated by alkaline pH. The transcription factor Efg1p is also downstream of this pathway (2). A fourth pathway involves the transcription factor Czf1p, which stimulates hyphal formation in response to growth in solid matrix.

Repressors of the yeast to hyphal switch include *NRG1*, *TUP1*, *RPG1*, and *RBF1* (2). While these pathways control hyphal-specific gene (HSG) expression and hyphal development, they are not fully characterized. In addition, there are factors and emerging pathways that affect hyphal differentiation in *C. albicans* that do not appear to be directly involved with the aforementioned signaling systems (Fig. 1).

Regulation of opaque cell differentiation is not well understood, but involves major alterations in chromosome structure to induce homozygosity at the Mating Type-Like (*MTL*) locus (12). *C. albicans* contains a single *MTL* locus on chromosome 5, unlike the 3 mating loci on chromosome 3 in *S. cerevisiae*. Standard white cells are heterozygous at this locus where one allele encodes *Mtl α 1* and *Mtl α 2*, and the second allele encodes *Mtl α 1* and *Mtl α 2*. α 1 and α 2 products form a heterodimer that blocks mating (17), which explains why heterozygous white cells are unable to mate. Chromosome changes, such as the loss of either *MTL α* or *MTL α* , followed by gene duplication or mitotic recombination are required to produce a homozygous *MTL* locus, which in turn is a prerequisite for white cells to differentiate into opaque cells (18-20). Homozygosity of *MTL* results in homozygous white cells which can then switch to the opaque form through stochastic elevations in expression of *WOR1*, the master regulator of the opaque state (21, 22). *WOR1* in turn is necessary for opaque-specific gene expression, including *CDR3*, *OP4*, and *SAP1* (23). The opaque phase cells in turn can express mating genes in response to α -pheromone, and undergo another morphological change where they extend conjugation tubes known as “shmoos”. The shmoos of opposite mating types fuse together to allow karyogamy and the creation of an intermediate tetraploid state ($a/a/\alpha/\alpha$), which can be reduced back to diploid progeny.

Meiosis and sporulation have not been identified in *C. albicans* (24), but the tetraploids are believed to undergo mitotic recombination followed by chromosome loss to produce recombined diploid progeny (11, 24). Environmental signals including anaerobic conditions and low temperatures can enhance low frequency, spontaneous switching from white to opaque cells under homozygous *MTL* conditions (25-27). The detailed mechanisms underlying opaque cell differentiation are not clear, but a picture is starting to emerge.

1.3 Regulation of differentiation: Cell cycle control

There is mounting evidence that the cell cycle plays a prominent role in regulating differentiation in *C. albicans*, but the mechanisms are not clear. In most organisms, the G1/S transition of the cell cycle is a key control point, where cells either commit to mitosis and proliferate, or arrest and differentiate through different developmental pathways. In mammals, the G1/S transition is restrained by the Retinoblastoma protein (Rb). Rb is negatively regulated by the cyclin-dependent kinase (CDK) Cdc2 (CDK4/6) associated with a G1 cyclin (Cyclin D). Repression of Rb allows the activation of the E2F transcription factor, which consequently controls a transcription cascade required for the G1/S transition and subsequent cell proliferation (28, 29). In *S. cerevisiae*, a similar circuit exists, where the CDK Cdc28p associates with the G1 cyclin Cln3p, which in turn negatively regulates the functional equivalent of Rb, known as Whi5p. Repression of Whi5p allows the activation of the transcription factor complexes SBF (Swi4-Swi6 cell cycle box binding factor) and MBF (*MluI* binding factor). Swi6p is the regulatory subunit for both SBF and MBF, whilst Swi4p and Mbp1p are the DNA-binding subunits

of the SBF and MBF complexes, respectively. Activation of SBF/MBF mediates Cln3p function in regulating G1/S by inducing a battery of G1/S genes, including other G1 cyclins such as *CLN1* and *CLN2*, and several transcription factors such as *TOS4*, *TOS8*, *HCM1*, *YOX1*, and *PLM2* (Fig. 2). This expression pathway is required for bud morphogenesis, DNA replication, spindle pole body duplication, and passage through START (30-32). Bck2p is specific to *S. cerevisiae*, and functions with Cdc28p/Cln3p to activate SBF. SBF/MBF are essential for START in *S. cerevisiae*, since absence of *SWI4* and *SWI6*, or *SWI4* and *MBP1* is lethal and results in a G1 phase arrest (33). The proteins comprising SBF/MBF contain ankyrin repeats, which are specific to fungi. A conserved role in fungal growth control is suggested by the fact that *Schizosaccharomyces pombe* contains MBF equivalents and deletion of the regulatory subunit Cdc10p results in a G1 phase block, while deletion of the DNA-binding subunits Res1p and Res2p is lethal (34, 35). G1/S regulation has not been investigated in other fungi to date, but SBF/MBF orthologues are present in most species that have been sequenced.

Under conditions that stimulate development, the G1/S transition is blocked. For example, in *S. cerevisiae*, nutrient limitation and presence of pheromone lead to down-regulation of *CLN3* activity, cell cycle arrest in G1 phase, and activation of development, resulting in conjugate tube formation and eventual sporulation (36-38). Blocking G1 phase alone, however, does not induce development in *S. cerevisiae*. In mammals, blocking G1 phase can be sufficient for inducing development, in some but not all cases (39, 40), indicating that development is not a universal default state upon G1 phase arrest.

In *C. albicans*, a relationship between the cell cycle and differentiation is emerging, but the mechanisms are not clear, due in part to the fact that the cell cycle is

relatively unexplored and a comprehensive understanding of the basic G1/S regulatory circuit is lacking. However, the current framework for the G1/S transition possesses unique features compared to *S. cerevisiae*, because *C. albicans* lacks functional homologues of Whi5p, Bck2p, and some of the downstream transcription factor targets of SBF/MBF (Fig. 2). In addition, putative G1 cyclin homologues, such as Ccn1p and Hgc1p, show different functions than their counterparts in *S. cerevisiae*. While Ccn1p may contribute somewhat to G1 progression, as cells lacking the gene grow slightly slower, Hgc1p has no known cell cycle role (41, 42).

The molecular link between differentiation and the cell cycle in *C. albicans* is also unclear due to controversy over whether a specific cell cycle stage correlates with hyphal growth. One study suggests that hyphae can be induced at any stage of the yeast cell cycle (43), while others suggest that hyphal emergence is restricted to G1 phase (44, 45). In support of the latter, putative G1 phase-associated factors have been shown to influence hyphal growth. For example, the G1 cyclin homologue Hgc1p is essential for hyphal growth, and is only expressed in hyphae (42, 44). Secondly, the G1 cyclin Ccn1p is required for the maintenance of hyphal growth but not initiation (41, 44). Finally, Cdc4p, a homologue of the *S. cerevisiae* F-box protein component of the SCF^{CDC4} ubiquitin ligase that controls progression through G1 phase, has some role in negatively regulating hyphal development, as *C. albicans* cells lacking the gene grew constitutively as pseudohyphae, that switched into hyphae (46). Although, suggestive of a link between G1 phase and hyphal development, none of these factors showed a direct influence on G1 phase itself.

The strongest evidence for a relationship between G1 phase and hyphal development in *C. albicans* was obtained from studies on the homologue of the G1 cyclin Cln3p. Inactivation of the Cln3p homologue in *C. albicans* caused an arrest in G1 phase, followed by a dramatic increase in cell size, and a switch in morphology to hyphal and pseudohyphal cells in the absence of hyphal-inducing conditions (47). Intriguingly, these differentiated cells then resumed their cell cycles, despite the absence of Cln3p, suggesting that Cln3p may differentially regulate the cell cycle and growth in yeast versus hyphal cells. The effects were Ras1p-dependent, as a *cln3/ras1* double mutant strain did not filament, and cells died much faster, suggesting synthetic lethal effects (47, 48). This link between the cell cycle and hyphal development was specific to G1 phase, since blocking cells in S or M phase did not lead to hyphal growth, but other types of polarized cells (44, 45, 47, 49, 50). In *S. cerevisiae*, neither arresting in G1 phase, nor the absence of *CLN3*, lead to differentiation; depletion resulted in a short G1 phase delay, slight enlargement of the yeast cells, then resumption of the cell cycle and budding (51-53). Thus, G1 phase is important for development in *C. albicans*, but the molecular basis is not clear, and cannot be extrapolated from related organisms.

1.4 Objectives

Taken together, this work demonstrates two important points. Firstly, the G1/S circuit in *C. albicans* involves novel regulatory features compared to *S. cerevisiae* and mammals, which has important implications for control of basic growth. Secondly, G1 phase is specifically linked to hyphal development, but it is not clear whether Cln3p is a direct negative regulator of hyphal and pseudohyphal growth, or if development was a

response to some other aspect of G1 phase arrest. The main objectives of this thesis were to characterize the G1/S regulatory circuit in yeast cells of *C. albicans* and to screen for effectors of Cln3p that may be important for cell division and possibly differentiation, through obtaining transcription profiles of cells depleted of Cln3p, and exploring the function of orthologues of *SWI6*, *SWI4*, and *MBP1*. Our results have shed significant light on the regulation of cell proliferation and identified new modes of controlling differentiation; both of which are critical for virulence in this important pathogen.

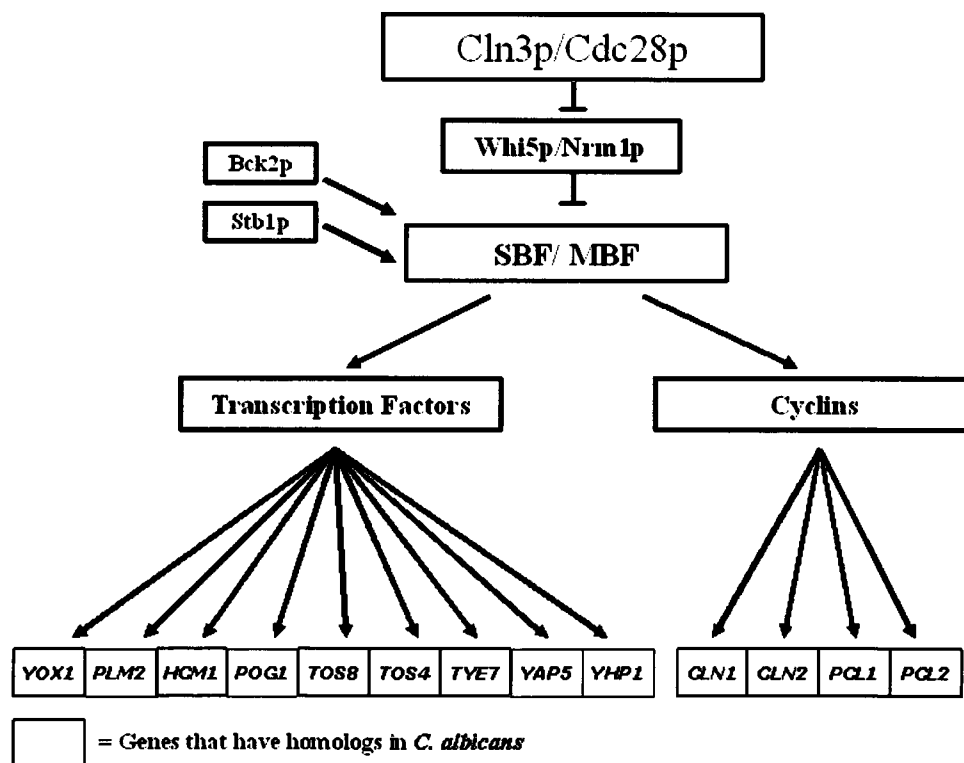


Figure 2. Network of selected factors activated by the SBF/MBF complexes during the G1/S transition in *S. cerevisiae*, compared to *C. albicans*. The downstream transcription factors that currently have known homologs in *C. albicans* are highlighted in red.

2. Materials and Methods

2.1 Strains, Oligonucleotides, Plasmids, and Media

C. albicans strains used are shown in Table 1. Oligonucleotides are included in Table 2, and plasmids are listed in Table 3. For analysis of cell phenotype, cells were cultured at 30°C in either liquid YPD medium (1% yeast extract, 2% peptone, 2% dextrose) or minimal synthetic defined medium SD (0.67% yeast nitrogen base without amino acids, 2% glucose), supplemented with uridine, histidine, or arginine, as required. For strains carrying genes under control of the *Candida MET3* promoter, cells were grown in SD inducing (-MC) or repressing (+MC) medium with or without methionine (2.5 mM) and cysteine (0.5 mM), respectively (54). In addition, plasmid-bearing strains of *Escherichia coli* (DH5α) were grown on 2YT (1% yeast extract, 1.6% tryptone, 0.5% NaCl) supplemented with 100 µg/ml Ampicillin (Fisher) for selection.

Table 1: *Candida albicans* strains used in this study

Strain	Genotype	Parent/Source
BWP17	<i>ura3Δ::imM434/ura3Δ::imm434</i> <i>his1Δ::hisG/his1Δ::hisG</i> <i>arg4Δ::hisG/arg4Δ::hisG</i>	CAI4 Fonzi <i>et al.</i> 1993
CAI4	<i>ura3Δ::imm434/ura3Δ::imm434</i>	Fonzi <i>et al.</i> 1993
BH420	BWP17 (pRM100, pBS-CaARG4)	BWP17
BH440	BWP17 (pBS-CaHIS1, pBS-CaURA3)	BWP17
BWPUH	BWP17 (pRM100)	BWP17
CB488	<i>cln3Δ::hisG/MET3::CLN3-URA3</i>	CAI4 Bachewich <i>et al.</i> 2005
CB498	<i>cln3Δ::hisG/MET3::CLN3-URA3</i> <i>ras1Δ::hisG/ras1Δ::hisG</i>	Bachewich <i>et al.</i> 2005
CB504	CAI4(pCaDIS)	Bachewich <i>et al.</i> 2005
CB547	<i>swi4Δ::URA3/MET3::SWI4-ARG4</i>	BWP17
CB548	<i>swi4Δ::URA3/MET3::SWI4-ARG4</i>	BWP17
CB552	<i>swi6Δ::URA3/MET3::SWI6-HIS1</i>	BWP17
CB557	<i>swi6Δ::URA3/MET3::SWI6-HIS1</i>	BWP17
CB600	<i>SWI6/MET3 ::SWI6-HIS1</i>	BWP17
KMCa4a	<i>mbp1Δ::URA3/MET3::MBP1-ARG4</i>	BWP17 (K. Mogilevsky)
BH101	<i>swi6Δ::HIS1/SWI6</i>	BWP17
BH104	<i>swi4Δ::hisG-URA3-hisG/SWI4</i>	BWP17
BH113	<i>swi4Δ::hisG/SWI4</i>	BWP17
BH115	<i>swi4Δ::hisG/SWI4</i>	BWP17
BH120	<i>swi6Δ::HIS1/swi6Δ::URA3</i>	BWP17
BH137	<i>mbp1Δ::HIS1/MBP1</i>	BWP17
BH140	<i>swi4Δ::hisG/SWI4</i> <i>swi6Δ::HIS1/SWI6</i>	BWP17
BH150	<i>swi4Δ::hisG/MET3::SWI4-ARG4</i>	BWP17
BH160	<i>swi4Δ::hisG/MET3::SWI4-ARG4</i> <i>swi6Δ::HIS1/SWI6</i>	BWP17
BH180	<i>swi4Δ::HIS1/SWI4</i>	BWP17
BH185	<i>swi4Δ::URA3/swi4Δ::HIS1</i>	BWP17
BH190	<i>swi4Δ::hisG/MET3::SWI4-ARG4</i> , <i>swi6Δ::HIS1/swi6Δ::URA3</i>	BWP17

BH261	<i>mbp1Δ::HIS1/mbp1Δ::URA3</i>	BWP17
BH270	<i>swi4Δ::hisG/MET3::SWI4-ARG4,</i> <i>mbp1Δ::HIS1/MBP1</i>	BWP17
BH276	<i>swi4Δ::hisG/MET3::SWI4-ARG4,</i> <i>mbp1Δ::HIS1/mbp1Δ::URA3</i>	BWP17
BH339	<i>swi4Δ::hisG/swi4Δ::URA3</i>	BWP17
BH341	<i>swi4Δ::hisG/swi4Δ::URA3 mbp1Δ::HIS1/MBP1</i>	BWP17
BH348	<i>swi4Δ::hisG/swi4Δ::URA3,</i> <i>mbp1Δ::HIS1/mbp1Δ::ARG4</i>	BWP17
BH352	<i>swi4Δ::hisG/swi4Δ::URA3,</i> <i>mbp1Δ::HIS1/mbp1Δ::ARG4</i>	BWP17

Table 2: Oligonucleotides used in this study

Oligo	Sequence 5'-3'
BH2F	GAAGATTCATTGATATGTGTGGTAAGGCAC
BH2R	CCAGCGTTTATAATGATAACGTTTCAGCTTC
BH3F	GAAGCTGAACGTTATCATTATAAACGCTGGTATAGGGCGAATTGGAGCTC
BH3R	CACGGGGAATTAGAAAGTATACATGTGTTCGGACGGTATCGATAAGCTTGA
BH4F	CGAACACATGTATACTTCTAATCCCCGTG
BH4R	TTCCACATCCATACTAAATCTTACTACAGC
BH6F	CATGGACTTGGTTGTCTTGA
BH6R	ACGCTTACTTCTATGGAGCT
BH7F	GTAACAATACCTTTATCAGAGGATTCACCC
BH7R	GATGTGATGGGTTGATAAATGAAATGAGCG
BH8F	CGCTCATTTCATTTATCAACCCATCACATCTATAGGGCGAATTGGAGCTC
BH8R	GGATGCTGGTAAAGTAGTAGAGTATGATAAGACGGTATCGATAAGCTTGA
BH9F	TTATCATACTCTACTACTTTACCAGCATCC
BH9R	GTTCTATTATCTGTTGCTTGTGTTGCCCA
BH10F	AGCAGTATCTACATGAGAATTAATCAGATG
BH10R	TTGGTATAACATACATTTGAGTAGGTAGCT
BH11F	AGCTACCTACTCAAATGTATGTTATACCAAGGATCCCCCCTTTAGTAAGA
BH11R	ACACCTTCTCATAATTGAGGACTCGTTCATCATGTTTTCTGGGGAGGGTA
BH12F	ATGAACGAGTCCTCAATTATGAGAAGGTGT
BH12R	CGATCAATGTATTTCCATTGTCAATCGGTT
BH13F	AGCTACCTACTCAAATGTATGTTATACCAATATAGGGCGAATTGGAGCTC
BH13R	TTGTTCCGATTTAATTTCCCCCATCTATCGGACGGTATCGATAAGCTTGA

BH14F	CGATAGATGGGGGAAATTAAATCGGAACAA
BH14R	AATATTTGTGTTGGCCACATTTGAGTCTGA
BH32F	ATGTACCACCAAGAGATTGT
BH32R	ACTAATATGGTACAGACTCA
SWI6F	GGACACGACACTTACACTTC
SWI6R	GGTGGATATTTGATACGAGT
SWI4F	TCAGACCGATACCTACCTGT
SWI4R	TTACGCCGCCGCAGATTGCA
MBP1F	CACATCACTGGTAGCATATA
MBP1R	TCGTTTGACAAACCCAGATT
CB115F	CCAAATGGGATATATATGAAGATTCATTGATATGTGTGGTAAGGCACAACCTT ACACTCTAGCATACCCAAATGGTTTCGGTATAGGGCGAATTGGAGCTC
CB115R	CAAACCTCCTGAAGCTAGAGTAGATAGATATTTCCAGTTGTTGGGCAAAGACA AGAATACCGACAAATTAGATTTGAATGAGACGGTATCGATAAGCTTGA
CB117F	GAGAACTCAACTGATGCTCT
CB117R	CCATTGTGAGGGCTACTTAT
CB119F	ACGCGTCGACTCTAATTGACATGGATACGA
CB119R	ACGCTAGAGCTCTCTGGATTAGTCACATCTTC
CB120F	ACGCTAGGATCCTCGTACTGGCAATGTATAACT
CB120R	ACGCTATAGATCTCATCGGTCTAGATTGTAATAT
CB123F	CTACTACATAATGTCTGAACCTCCCCAAGTATTTTCGAGCTACCTACTCAAATG TATGTTATACCAATGTTTCTTAACCTCGGATCCCCCTTTAGTAAGA
CB123R	GGAAAATTACAACATTTCAAATCTGTGTAGCATTGACCCAGTCATCTTTACA CCTTCTCATAATTGAGGACTCGTTCATCATGTTTTCTGGGGAGGGTA
CB126F	ACAGAGAGACAGATAGAGCG
CB126R	CACAGTTATTACTGAAGGCG
CB127F	CCAAATGGGATATATATGAAGATTCATTGATATGTGTGGTAAGGCACAACCTA CACTCTAGCATACCCAAATGGTTTCGGGGATCCTGGAGGATGAGGAG
CB127R	ATTGTTGTTATTTATATGGGTCTCGAATAATTTTTGTTGAATTGACTGTGTTGT CAAGTCACCAATGTGTATAGGAGAATCCATGTTTTCTGGGGAGGGT
CB129F	CGTTCAGCTTCCTTTCAATGAAATAAGTAT
CB129R	TCAGCAGACACAACAAGATACTGATACTTG
CB130F	GAACACATGTATACTTCTAATTCCCCGTGT
CB130R	ATTTGAGGCAGCTTCGACAGGCCACGTATT
CB131F	ATACTTATTTTATTGAAAGGAAGCTGAACGTATAGGGCGAATTGGAGCTC
CB131R	ACACGGGGAATTAGAAGTATACATGTGTTTCGACGGTATCGATAAGCTTGA

CaURA3F	GGTAATACCGTGAAGAAACA
CaURA3R	TTCAAATAAGCATTCCAACC
CaHIS1F	CCTGCAGCTGATATCCCAGT
CaHIS1R	ACTGGGATATCAGCTGCAGG

Table 3: Plasmids used in this study

Plasmid	Description	Parent/Source
pBS-Ca <i>URA3</i>	pBluescript Ca <i>URA3</i>	A.J.P. Brown
pBS-Ca <i>HIS1</i>	pBluescript Ca <i>HIS1</i>	C. Bachewich
pBS-Ca <i>ARG4</i>	pBluescript Ca <i>ARG4</i>	H. Huang
pFA-MET3-Ca <i>URA3</i>	<i>MET3</i> promoter – Ca <i>URA3</i>	Gola <i>et al.</i> 2003
pFA-MET3-Ca <i>HIS1</i>	<i>MET3</i> promoter – Ca <i>HIS1</i>	Gola <i>et al.</i> 2003
pFA-MET3-Ca <i>ARG4</i>	<i>MET3</i> promoter – Ca <i>ARG4</i>	Gola <i>et al.</i> 2003
p5921	pUC18- <i>hisG-URA3-hisG</i>	W.A. Fonzi
pRM100	pUC19 <i>URA3,HIS1</i>	J. Pla
pCB180	pUC18- <i>SWI4</i>	This study
pCB181	pUC18- <i>hisG-URA3-hisG</i> with <i>SWI4</i> flanks	This study

2.2 Growth conditions

For analysis of cell phenotypes, cells were grown overnight in SD medium to stationary phase, diluted the following day to an O.D._{600nm} of 0.1 – 0.2 in fresh medium, incubated at 30°C for a defined time period, and subsequently collected for observation. Cells carrying a gene under control of the *MET3* promoter, were diluted into SD inducing (-MC) or repressing (+MC) medium. Strains with gene deletions were also incubated in SD repressing (+MC) medium in order to make quantitative comparisons of phenotypes with the *MET3* conditional strains. Deletion strains produced a similar range in phenotypes despite growth in inducing, repressing, or YPD medium. For microarray analysis, cells were incubated overnight at 30°C in 2 ml of SD inducing medium (-MC), then diluted to an O.D._{600nm} of 0.1 in SD repressing medium (+MC) for 1, 3, 6, or 7 hr, depending on the experiment. Cell pellets were then quickly collected by centrifugation, and stored at -80°C until use.

For transcription profiling of cells lacking *CLN3* and *RAS1*, strains CB488, CB498 and the prototrophic control strain CB504, strains were inoculated in 2 ml of SD inducing medium lacking uridine, and grown overnight at 30°C. Cells were then diluted to an O.D._{600nm} of 0.2 in SD repressing medium (+MC), incubated at 30°C for 1, 3 or 6 hr, and centrifuged for 10 min at 2095 rcf (Beckman-Coulter Allegra X-12R Centrifuge with a SX4750 rotor). The medium was removed and the cell pellets were transferred to Eppendorf tubes, which were further centrifuged at 16 rcf (Eppendorf 5415D) to remove traces of medium. The pellets were stored immediately at -80°C. 25 ml was collected at 1 hr, while 12 ml and 10 ml were collected for the 3 and 6 hr time points, respectively.

For transcription profiling of strains lacking *SWI4* and *SWI6*, strains BH190 and the prototrophic control strain BH420 were inoculated in 2 ml of SD inducing medium lacking uridine, histidine, and arginine, and grown overnight at 30°C. Cells were diluted to an O.D._{600nm} of 0.2 in 10 ml of SD repressing medium. After 7 hr at 30°C, the cells were centrifuged for 10 min at 2095 rcf. The medium was removed and the cell pellets were transferred to Eppendorf tubes, which were further centrifuged at 16 rcf to remove traces of medium. The pellets were stored immediately at -80°C, until RNA extraction.

2.3 *Escherichia coli* transformation

Subcloning Efficiency DH5α Chemically Competent cells (F- ϕ 80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17*(rk-, mk+) *phoA supE44 thi-1 gyrA96 relA1* λ-; Invitrogen) were stored at -80°C. 50 μl were mixed gently with 1 to 5 μl (1 – 10 ng) of DNA in an Eppendorf tube. The cells were incubated for 30 min on ice, heat shocked for 30 sec in a 37°C water bath, then placed on ice for an additional 2 min. 950 μl of prewarmed 2YT medium was immediately added, and the cells were incubated at 37°C for 1 hr with shaking at 225 rpm. The transformed cells were then spun down, 900 μl of the media was removed, and the remaining 100 μl was plated on 2YT agar plates containing 100 μg/ml of Ampicillin.

2.4 *Candida albicans* transformation

Cells were transformed using a lithium acetate method (adapted from (55-58)). Cells were inoculated into 2 ml of YPD and incubated for 18 to 24 hr at 30°C with shaking (250 rpm), allowing the yeast cells to reach stationary phase of growth. 300 μl

of the stationary culture was transferred to an Eppendorf tube, and centrifuged for 2 min at 16 rcf. The medium was removed, and 100 - 200 µl of ONE-STEP buffer [0.2M Lithium acetate; 40% Polyethylene glycol (PEG) 3350, pH 5.0; 100 mM Dithiothreitol (DTT); 0.25 mg/ml of single-stranded carrier DNA (Salmon sperm DNA; Invitrogen)] was combined with the cell pellet. The contents were vortexed for 1 min, after which 5 to 10 µg of DNA was added. The mixture was vortexed for 1 min and incubated overnight at 30°C. The cells were then heat shocked at 43°C for 15 – 60 min, plated directly onto selective medium, and incubated at 30°C for 2 – 4 days.

2.5 Genomic DNA extraction

Genomic DNA (gDNA) was extracted according to Rose *et al.*, (1990) (59). Strains were inoculated into 5 – 10 ml of YPD or SD medium, grown overnight at 30°C, and subsequently centrifuged in an Eppendorf tube, to which 0.3g of acid-washed glass beads (Sigma), 200 µl lysis buffer (10 mM TRIS pH8.0, 1 mM EDTA, 100 mM NaCl, 1% SDS, 2% Triton-X), and 200 µl of a 1:1 mixture of phenol and chloroform was added. The mixture was vortexed for 5 min, and then 200 µl of TE buffer (1 mM EDTA, 10 mM TRIS pH8.0) was added. After vortexing, the suspension was spun down at 16 rcf for 10 min. The upper phase was transferred to a new Eppendorf tube, 500 µl of 1:1 phenol:chloroform was added, the solution was vortexed for 5 min, and then spun down at 16 rcf for 10 min. The resulting upper phase was transferred to a new Eppendorf tube, and 1 ml of ice-cold 95% ethanol was added and mixed gently to precipitate the DNA. The pellet was collected by centrifuging at 4°C, 16 rcf for 10 min. The resulting pellet was washed with cold 70% ethanol and allowed to air-dry for 15 min. The gDNA was

resuspended in 50 μ l of TE buffer containing 50 μ g/ml RNaseA (Fermentas), and incubated at 37°C for 30 min. The gDNA was stored at 4°C.

An alternative approach (60-62) for extracting gDNA was used, which resulted in higher quality and quantity of gDNA. Strains were inoculated into 10 – 25 ml of YPD or SD medium, and grown overnight at 30°C, with shaking at 250 rpm. The overnight culture was centrifuged at 2095 rcf for 5 min, supernatant was discarded, and the cell pellet was resuspended and transferred to a microcentrifuge tube. The suspension was centrifuged at 16 rcf for 2 min, the media was removed, and the pellet was washed once with 500 μ l of sterile water. The pellet was then completely resuspended by vortexing in 1 ml of sorbitol buffer [1M Sorbitol, 0.1M EDTA], 100U of Lyticase (Sigma), and 8 mM of Dithiothreitol (DTT)], and incubated at 37°C for 1 – 2 hr. The mixture was spun down for 1 min at 16 rcf, and the pellet was resuspended completely with 200 μ l of Tris-EDTA solution [50 mM Tris pH8.0, 20 mM EDTA]. 40 μ l of 10% SDS was added and the suspension was incubated at 65°C for 30 min. Subsequently, 100 μ l of 5M Potassium acetate (KAc) was added and mixed gently so as not to shear the DNA, and incubated on ice for 30 – 60 min. The mixture was then centrifuged for 10 min at 16 rcf at 4°C, and the supernatant was transferred to a new microcentrifuge tube. After adding an equal volume of ice cold isopropanol, the tube was mixed by inversion for 1 min, and centrifuged for 10 min at 16 rcf, 4°C. The resulting DNA pellet was washed once with 70% ethanol, and then incubated at 37°C for 30 min in 150 μ l of TE buffer (10 mM Tris, 1 mM EDTA) and 2 μ l of RNaseA (10 mg/ml). The gDNA was stored at 4°C. gDNA was quantified with a fluorometer (Hoefer DQ300) using Hoechst Dye (Invitrogen).

2.6 RNA extraction and quantification

Cell pellets were collected as described above, and total RNA was extracted from cells using the MasterPure™ Yeast RNA Purification Kit purchased from Epicentre Biotechnologies (InterScience, ON). RNA quantification was carried out by measuring absorbance at 260nm (Ultraspec 2100 pro). The quality of select RNA samples was assessed using the Agilent 2100 Bioanalyzer.

2.7 Construction of Strains

2.7.1 *SWI4*

A deletion mutant was created by replacing both alleles of *SWI4* in strain BWP17 with the *URA3* and *HIS1* markers, using 2-step PCR fusion constructs. To start, a 759 bp fragment corresponding to the 5' flank of *SWI4*, located 684 bp upstream of the Start codon, was Polymerase Chain Reaction (PCR) amplified from gDNA with oligonucleotides BH10F and BH10R, using the following thermocycling conditions: 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 46°C for 30 sec, 68°C for 45 sec, and a final elongation at 68°C for 7 min. The reaction mix was composed of a final concentration of 0.6 µM of oligonucleotides, 0.4 mM dNTPs, 100 ng of gDNA as template, 3.75U of Expand Long Template Polymerase (Roche), and 1X Buffer 3. A 762 bp fragment corresponding to the 3' flank of *SWI4*, located 4 bp after the stop codon, was similarly amplified using oligonucleotides BH14F and BH14R and the following thermocycling conditions: 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 48°C for 30 sec, 68°C for 46 sec, and a final elongation at 68°C for 7 min. The reaction mix composition was similar to that used for the 5' flank. To amplify the 1755 bp *HIS1*

cassette fragment from plasmid pBS-Ca*HIS1*, oligonucleotides BH13F and BH13R were used, which contain homology to the plasmid plus an additional 30 bp sequence that is the reverse complement of oligonucleotides BH10R and BH14F, respectively. The following thermocycling conditions were used: 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 42°C for 30 sec, 68°C for 1 min 45 sec, and a final elongation at 68°C for 7 min. The reaction mix was composed of a final concentration of 0.6 µM of oligonucleotides, 0.4 mM dNTPs, 100 ng of pBS-Ca*HIS1* as template, 3.75U of Expand Long Template Polymerase, and 1X Buffer 3. In order to create the final construct for transformation, oligonucleotides BH10F and BH14R were used at a concentration of 0.45 µM with a 1:3:1 (50ng:150ng:50ng) amount of the three PCR fragments, in a reaction including 0.5 mM of dNTPs, 3.75U of Expand Long Template Polymerase, and 1X Buffer 3. The following thermocycling conditions were used: 95°C for 2 min, followed by 10 cycles of 95°C for 10 sec, 47°C for 30 sec, and 68°C for 2 min 55 sec, followed by 15 cycles of 95°C for 10 sec, 47°C for 30 sec, 69°C for 2 min 55 sec with a 20 sec auto-segment extension, and a final elongation at 69°C for 7 min. The final 2916 bp PCR product was cleaned using a PCR purification kit (QIAGEN), and 10 µg was used to transform strain BWP17, resulting in strain BH180 (*swi4*Δ::*HIS1/SWI4*).

Colonies of transformants were screened directly using PCR with 2 µl of spheroplasted yeast cells, 0.9 µM oligonucleotides BH6F and BH6R, 0.2 mM dNTPs, 3.75U Expand Long Template DNA, and 1X Buffer 1. Thermocycling conditions included an initial denaturation at 94°C for 3 min, followed by 25-30 cycles of 94°C for 1 min, 40°C for 1 min, 68°C for 5 min, and a final elongation at 68°C for 7 min. The *SWI4* wildtype band was 5357 bp, while the *swi4*Δ::*HIS1* deletion produced a 3552 bp band.

To delete the second copy of *SWI4*, a PCR fusion construct containing the same 759 bp 5' flank and 762 bp 3' flanks were used as described above. A 1765 bp *URA3* cassette fragment was amplified from plasmid pBS-Ca*URA3*, with oligonucleotides BH13F and BH13R. The following thermocycling conditions were used: 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 42°C for 30 sec, 68°C for 1 min 45 sec, and a final elongation at 68°C for 7 min. The reaction mix was composed of a final concentration of 0.6 µM of oligonucleotides, 0.4 mM dNTPs, 100 ng of pBS-Ca*URA3* as template, 3.75U of Expand Long Template Polymerase, and 1X Buffer 3. In order to create the final construct to be used for transformation, oligonucleotides BH10F and BH14R were used at a concentration of 0.45 µM with a 1:3:1 (50ng:150ng:50ng) ratio of the three fragments, in a reaction including 0.5 mM of dNTPs, 3.75U of Expand Long Template Polymerase, and 1X Buffer 3. The following thermocycling conditions were used: 95°C for 2 min, followed by 10 cycles of 95°C for 10 sec, 47°C for 30 sec, and 68°C for 2 min 55 sec, followed by 15 cycles of 95°C for 10 sec, 47°C for 30 sec, 69°C for 2 min 55 sec with a 20 sec auto-segment extension, and a final elongation at 69°C for 7 min. The final 2926 bp PCR product was cleaned using QIAGEN spin columns, and 10 µg was transformed into strain BH180, resulting in strain BH185 (*swi4Δ::HIS1/swi4Δ::URA3*). Transformants were screened by PCR, using oligonucleotides BH6F and BH6R, as described before. A 3562 bp band represented *swi4Δ::URA3*.

To create a prototrophic control strain, strain BWP17 was transformed sequentially with 5 µg of plasmid pBS-Ca*HIS1*, then pBS-Ca*URA3*, producing strain

BH440. An alternative control strain BWPUH was created by transforming strain BWP17 with the plasmid pRM100, which contained both the *URA3* and *HIS1* markers.

In order to confirm that the phenotype of BH185 was due to the deletion of *SWI4*, a conditional strain carrying a single copy of *SWI4* under the control of the *MET3* promoter was created (63). To first delete a single copy of *SWI4* from strain BWP17, a 3 kb fragment containing the *SWI4* open reading frame and approximately 1 kb of 3' and 5' flanking sequence was amplified with oligonucleotides CB119F and CB119R, and cloned into *Sall/SacI* sites of pUC18 creating plasmid pCB180. Primers CB120F and CB120R were then used to amplify the flanking and vector sequences from pCB180, into which the *BamHI/BglIII* hisG-*URA3*-hisG cassette (p5921; (64)) was cloned, replacing the *SWI4* open reading frame and resulting in plasmid pCB181. The *SWI4* deletion construct was liberated using *Sall* and *SacI* restriction enzymes and transformed into strain BWP17. Transformants were screened by PCR, as previously described, to confirm the deletion of one copy of *SWI4*. The resulting strain BH104 was grown overnight in YPD medium, then plated onto 5-Fluoroorotic acid (5-FOA) to select for strains that looped out *URA3*. The *URA3*- strains were screened by PCR, and strain BH115 (*swi4Δ::hisG /SWI4*) was isolated. The second copy of *SWI4* was placed under the control of the *MET3* promoter using a promoter replacement construct made through fusion PCR. A 759 bp fragment corresponding to the 5' flank of *SWI4* was amplified from strain BWP17 gDNA using oligonucleotides BH10F and BH10R, using the following thermocycling conditions: 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 46°C for 30 sec, 68°C for 45 sec, and a final elongation at 68°C for 7 min. The reaction mix was composed of a final concentration of 0.6 μM of oligonucleotides, 0.4 mM dNTPs, 100 ng of BWP17 gDNA

as template, 3.75U of Expand Long Template Polymerase, and 1X Buffer 3. A 732 bp fragment corresponding to the region immediately downstream of the *SWI4* start codon was amplified from BWP17 gDNA using oligonucleotides BH12F and BH12R and the following thermocycling conditions: 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 48°C for 30 sec, 68°C for 44 sec, and a final elongation at 68°C for 7 min. The reaction mix was composed of a final concentration of 0.6 μ M of oligonucleotides, 0.4 mM dNTPs, 100 ng of BWP17 gDNA as template, 3.75U of Expand Long Template Polymerase, and 1X Buffer 3. To amplify the *MET3* promoter cassette from plasmid pFA-*MET3*-Ca*ARG4*, oligonucleotides BH11F and BH11R were used, which contain 20 bp homology to the plasmid plus an additional 30 bp reverse complement sequence of oligonucleotides BH10R and BH12F, respectively. The following thermocycling conditions were used: 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 42°C for 30 sec, 68°C for 3 min 27 sec, and a final elongation at 68°C for 7 min. The reaction mix was composed of a final concentration of 0.6 μ M of oligonucleotides, 0.4 mM dNTPs, 100 ng of pFA-*MET3*-Ca*ARG4* as template, 3.75U of Expand Long Template Polymerase, and 1X Buffer 3. In order to create the final construct for transformation, oligonucleotides BH10F and BH12R were used at a concentration of 0.45 μ M with a 1:3:1 (50ng:150ng:50ng) ratio amount of the three fragments, in a reaction including 0.5 mM of dNTPs, 3.75U of Expand Long Template Polymerase, and 1X Buffer 3. The following thermocycling conditions were used: 95°C for 2 min, followed by 10 cycles of 95°C for 10 sec, 46°C for 30 sec, and 68°C for 4 min 30 sec, followed by 15 cycles of 95°C for 10 sec, 46°C for 30 sec, 69°C for 4 min 30 sec with a 20 sec auto-segment extension, and a final elongation at 69°C for 7 min. The final product was cleaned, and

5.4 µg of the 4895 bp product was used to transform strain BH115, resulting in strain BH150 (*swi4Δ::hisG/MET3::SWI4-ARG4*).

BH150 was initially screened by PCR, as described, using oligonucleotides BH6F and BH6R. A 3.4 kb band represented *swi4Δ::hisG*, and a 8.6 kb band represented *MET3::SWI4*. Strains BH150, BH185, BH113, BH115, BH180, and BWP17 were also screened by Southern Blot Analysis. 60 units of the restriction enzyme *NdeI* (NEB) was used to digest 4 µg of gDNA, in a 100 µl reaction mix containing 1X Buffer 4 (NEB), and incubated at 37°C overnight. The 1 kb probe was made using the oligonucleotides SWI4F and SWI4R, with the following PCR conditions: 0.2 mM dNTPs, 0.3 µM oligonucleotides, 1X PCR Buffer, 100 ng of BWP17 gDNA, and 3.75U of Short EXPAND DNA Polymerase (Roche), in a total volume of 50 µl. Thermocycling conditions involved 94°C for 2 min, 10 cycles at 94°C for 10 sec, 44°C for 30 sec, and 72°C for 50 sec, followed by another 15 cycles at 94°C for 10 sec, 44°C for 30 sec, and 72°C for 50 sec, with a 5 sec auto-segment extension, and a final 7 min extension at 72°C. The probe was then cleaned using a PCR column, and quantified. The DIG-labeled DNA probe was made from the PCR product using random primed labeling, as described later.

2.7.2 MBP1

In order to delete one copy of *MBP1*, a 790 bp fragment corresponding to the 5' flank of *MBP1*, starting 812 bp upstream of the Start site, was amplified from gDNA as described with *SWI4*, but using oligonucleotides BH7F and BH7R, and the following thermocycling conditions: 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec,

49°C for 30 sec, 68°C for 47 sec, and a final elongation at 68°C for 7 min. A 799 bp fragment corresponding to the 3' flank of *MBP1*, starting 161 bp downstream of the stop site, was then amplified using oligonucleotides BH9F and BH9R and the following thermocycling conditions: 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 49°C for 30 sec, 68°C for 47 sec, and a final elongation at 68°C for 7 min. A 1441 bp *HIS1* cassette fragment was then amplified from plasmid pBS-*CaHIS1* using oligonucleotides BH8F and BH8R, which contained homology to the plasmid plus 30 bp reverse complement sequence of oligonucleotides BH7R and BH9F, respectively, to allow for PCR fusion. The following thermocycling conditions were used: 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 41°C for 30 sec, 68°C for 1 min 26 sec, and a final elongation at 68°C for 7 min. The reaction mix was composed of a final concentration of 0.6 µM of oligonucleotides, 0.4 mM dNTPs, 100 ng of pBS-*CaHIS1* as template, 3.75U of Expand Long Template Polymerase, and 1X Buffer 3. In order to create the final construct for transformation, oligonucleotides BH7F and BH9R were used at a concentration of 0.45 µM with a 1:3:1 (50ng:150ng:50ng) ratio of the three fragments, in a reaction including 0.5 mM of dNTPs, 3.75U of Expand Long Template Polymerase, and 1X Buffer 3. The following thermocycling conditions were used: 95°C for 2 min, followed by 10 cycles of 95°C for 10 sec, 49°C for 30 sec, and 68°C for 3 min, followed by 15 cycles of 95°C for 10 sec, 49°C for 30 sec, 69°C for 3 min with a 20 sec auto-segment extension, and a final elongation at 69°C for 7 min. 10 µg of the final 3006 bp fusion PCR product was used to transform strain BWP17, resulting in strain BH137 (*mbp1Δ::HIS1/MBP1*).

Strain BH137 was screened by PCR from whole yeast cells, as previously described, using oligonucleotides CB126F and CB126R, and thermocycling conditions including an initial denaturation at 94°C for 3 min, followed by 25-30 cycles of 94°C for 1 min, 42°C for 1 min, 68°C for 3 min, and a final elongation at 68°C for 7 min.

The second copy of *MBP1* was deleted by a similar strategy, using the same 790 bp 5' flank and 799 bp 3' flanks as above. A 1765 bp *URA3* cassette fragment was amplified from plasmid pBS-*CaURA3* with oligonucleotides BH8F and BH8R, using the following thermocycling conditions: 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 42°C for 30 sec, 68°C for 1 min 45 sec, and a final elongation at 68°C for 7 min. The reaction mix was composed of a final concentration of 0.6 µM of oligonucleotides, 0.4 mM dNTPs, 100 ng of pBS-*CaURA3* as template, 3.75U of Expand Long Template Polymerase, and 1X Buffer 3. In order to create the final fusion construct for transformation, oligonucleotides BH7F and BH9R were used at a concentration of 0.45 µM with a 1:3:1 (50ng:150ng:50ng) ratio amount of the three fragments, in a reaction including 0.5 mM of dNTPs, 3.75U of Expand Long Template Polymerase, and 1X of Buffer 3. The following thermocycling conditions were used: 95°C for 2 min, followed by 10 cycles of 95°C for 10 sec, 49°C for 30 sec, and 68°C for 3 min, followed by 15 cycles of 95°C for 10 sec, 49°C for 30 sec, 69°C for 3 min with a 20 sec auto-segment extension, and a final elongation at 69°C for 7 min. 10 µg of the final 3016 bp PCR product was used to transform strain BH137, resulting in strain BH261 (*mbp1*Δ::*HIS1*/*mbp1*Δ::*URA3*).

Strain BH261 was screened by PCR, as described above, using oligonucleotides BH32F and BH32R. Thermocycling conditions included an initial denaturation at 94°C

for 3 min, followed by 25-30 cycles of 94°C for 1 min, 37°C for 1 min, 68°C for 3 min 6 sec, and a final elongation at 68°C for 7 min. The *mbp1Δ::HIS1* and *mbp1Δ::URA3* products produced a band at 3.2 kb. Alternatively, some strains were screened using nested oligos. Thermocycling conditions to screen for *mbp1Δ::HIS1*, with CaHIS1F and BH32R, included an initial denaturation at 94°C for 3 min, followed by 25-30 cycles of 94°C for 30 sec, 37°C for 30 sec, 68°C for 1 min 32 sec, and a final elongation at 68°C for 7 min. Conditions for *mbp1Δ::URA3*, with CaURA3F and BH32R, included an initial denaturation at 94°C for 3 min, followed by 25-30 cycles of 94°C for 30 sec, 40°C for 30 sec, 68°C for 1 min 40 sec, and a final elongation at 68°C for 7 min. *mbp1Δ::HIS1* produced a band at 1669 bp, and the *mbp1Δ::URA3* product produced a band at 1526 bp.

Strains BH137, BH261, and BWP17 were also screened by Southern Blot Analysis. 40 units of the restriction enzyme *SpeI* (NEB) were used to cut 4 µg of gDNA in a 100 µl reaction mix containing 1X Buffer 4 (NEB), 1X Bovine Serum Albumin (BSA) (NEB), and incubated at 37°C overnight. The 1 kb probe was made using the oligonucleotides MBP1F and MBP1R, with the following PCR conditions: 0.2 mM dNTPs, 0.3 µM oligonucleotides, 1X PCR Buffer, 100 ng of BWP17 gDNA, and 3.75U of Short EXPAND DNA Polymerase, in a total volume of 50 µl. Thermocycling conditions involved 94°C for 2 min, 10 cycles at 94°C for 10 sec, 38°C for 30 sec, 72°C for 50 sec, followed by another 15 cycles at 94°C for 10 sec, 38°C for 30 sec, 72°C for 50 sec with a 5 sec auto-segment extension, and a final 7 min extension at 72°C. The probe was then cleaned using a PCR column, and quantified.

2.7.3 *SWI6*

In order to create a *SWI6* deletion strain, one copy was replaced with the *HIS1* marker using a construct created with oligonucleotides CB115F and CB115R, which contained 80 bp complementary to the 5' and 3' flanks of *SWI6*, respectively, and 20 bp homology to pBS-*CaHIS1*. CB115F was located 680 bp upstream of the *SWI6* Start site, and CB115R was located 226 bp downstream of the stop codon. The following thermocycling conditions were used: 94°C for 4 min, followed by 25 cycles of 94°C for 1 min, 41°C for 1 min, and 68°C for 1 min 34 sec, with a final elongation at 68°C for 7 min. The reaction mix was composed of a final concentration of 0.6 µM of oligonucleotides, 0.4 mM dNTPs, 100 ng of pBS-*CaHIS1* as template, 3.75U of Expand Long Template Polymerase, and 1X of Buffer. 10 µg of the final 1508 bp PCR product was used to transform strain BWP17, resulting in strain BH101 (*swi6Δ::HIS1/ SWI6*). BH101 was initially screened by PCR, as described for other genes, using oligonucleotides CB117F and CB117R. Thermocycling conditions included an initial denaturation at 94°C for 3 min, followed by 25-30 cycles of 94°C for 1 min, 40°C for 1 min, 68°C for 1 min 42 sec, and a final elongation at 68°C for 7 min. *SWI6* produced a wildtype band at 3320 bp, and the *swi6Δ::HIS1* product produced a band at 1702 bp.

The second copy of *SWI6* was deleted using a PCR-fusion construct. A 5', 642 bp sequence located 663 bp upstream of the Start site, was amplified from gDNA with BH2F and BH2R oligonucleotides, using the following thermocycling conditions: 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 49°C for 30 sec, and 68°C for 39 sec, with a final elongation at 68°C for 7 min. The reaction mix was composed of a final concentration of 0.6 µM of oligonucleotides, 0.4 mM dNTPs, 100 ng of BWP17 gDNA

as template, 3.75U of Expand Long Template Polymerase, and 1X of Buffer 3. A 3', 658 bp flanking sequence of *SWI6*, located 107 bp downstream of the stop codon, was similarly amplified with BH4F and BH4R oligonucleotides using the following thermocycling conditions: 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 49°C for 30 sec, and 68°C for 40 sec, with a final elongation at 68°C for 7 min. The *URA3* cassette from the pBS-*CaURA3* plasmid was then amplified with BH3F and BH3R oligonucleotides, containing homology to the plasmid and an additional 30 bp reverse complement sequence of BH2R and BH4F oligonucleotides, respectively. The following thermocycling conditions were used: 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 41°C for 30 sec, and 68°C for 1 min 30 sec, with a final elongation at 68°C for 7 min. The reaction mix was composed of a final concentration of 0.6 μ M of oligonucleotides, 0.4 mM dNTPs, 100 ng of pBS-*CaURA3* as template, 3.75U of Expand Long Template Polymerase, and 1X of Buffer 3. In order to create the final construct, the reaction mix included 0.45 mM BH2F and BH4R, a 1:3:1 (50ng:150ng:50ng) ratio amount of the three fragments, 0.5 mM of dNTPs, 3.75U of Expand Long Template Polymerase, and 1X Buffer 3. The following thermocycling conditions were used: 95°C for 2 min, followed by 10 cycles of 95°C for 10 sec, 48°C for 30 sec, and 68°C for 2 min 39 sec, followed by 15 cycles of 95°C for 10 sec, 48°C for 30 sec, and 69°C for 2 min 39 sec with auto-extend by an additional 20 sec each cycle, and a final elongation at 69°C for 7 min. Approximately 4 μ g of the final 2721 bp PCR fusion product was transformed into strain BH101, resulting in strain BH120 (*swi6 Δ ::HIS1/swi6 Δ ::URA3*).

BH120 was initially screened by PCR, as described, using oligonucleotides CB117F and CB117R. *swi6 Δ ::HIS1* produced a band at 1.7 kb, and the *swi6 Δ ::URA3*

product produced a band at 2.5 kb. Strains BH101, BH120, and BWP17 were also screened by Southern Blot Analysis. 40 units of the restriction enzyme *PvuI* (NEB) was used to cut 4 µg of gDNA, in a 70 µl reaction mix containing 1X Buffer 3 (NEB), 1X BSA, and incubated at 37°C overnight. The 1031 bp probe was made using the oligonucleotides SWI6F and SWI6R, with the following PCR conditions: 0.2 mM dNTPs, 0.3 µM oligonucleotides, 1X PCR Buffer, 100 ng of BWP17 gDNA, 3.75U of Short EXPAND DNA Polymerase, in a total volume of 50 µl. Thermocycling conditions involved 94°C for 2 min, 10 cycles at 94°C for 10 sec, 40°C for 30 sec, 72°C for 50 sec, followed by another 15 cycles at 94°C for 10 sec, 40°C for 30 sec, 72°C for 50 sec with a 5 sec auto-segment extension, and a final 7 min extension at 72°C. The probe was then cleaned using a PCR column, and quantified.

To create a strain containing a conditional copy of *SWI6*, one allele of *SWI6* was placed under control of the *MET3* promoter. The construct was PCR amplified using oligonucleotides CB127F and CB127R, which contained 80 bp complementary to the 5' region immediately up and downstream of the Start codon, respectively, and 20 nt homologous to plasmid pFA-*MET3*-Ca*HIS1* (63). The following thermocycling conditions were used: 94°C for 4 min, followed by 25 cycles of 94°C for 1 min, 41°C for 1 min, and 68°C for 3 min, with a final elongation at 68°C for 7 min. The reaction mix was composed of a final concentration of 0.6 µM of oligonucleotides, 0.4 mM dNTPs, 100 ng of pFA-*MET3*-Ca*HIS1* as template, 3.75U of Expand Long Template Polymerase, and 1X Buffer 3. 10 µg of the final product was cleaned and transformed into strain BWP17, resulting in strain CB600. To delete the second allele of *SWI6*, a 2-step PCR fusion construct was created. A 5' 1043bp flanking region of *SWI6*, located 40 bp

upstream of the Start site, was amplified from strain BWP17 gDNA with CB129F and CB129R oligonucleotides, using the following thermocycling conditions: 94°C for 2 min, followed by 25 cycles of 94°C for 10 sec, 46°C for 30 sec, and 68°C for 55 sec, with a final elongation at 68°C for 7 min. The reaction mix was composed of a final concentration of 0.6 μ M of oligonucleotides, 0.4 mM dNTPs, 100 ng of BWP17 gDNA as template, 3.75U of Expand Long Template Polymerase, and 1X of Buffer 3. A 3' 590 bp flank of *SWI6*, starting 107 nt downstream of the stop codon, was then similarly amplified from strain BWP17 gDNA with CB130F and CB130R oligonucleotides, using the following thermocycling conditions: 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 49°C for 30 sec, and 68°C for 45 sec, with a final elongation at 68°C for 7 min. The *URA3* cassette from the pBS-Ca*URA3* plasmid was then amplified with CB131F and CB131R oligonucleotides, which additionally contain 30 bp reverse complement of CB129R and CB130F oligonucleotides, respectively. The following thermocycling conditions were used: 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 40°C for 30 sec, and 68°C for 1 min 30 sec, with a final elongation at 68°C for 7 min. The reaction mix was composed of a final concentration of 0.6 μ M of oligonucleotides, 0.4 mM dNTPs, 100 ng of pBS-Ca*URA3* as template, 3.75U of Expand Long Template Polymerase, and 1X of Buffer 3. In order to create the final construct, the reaction mix included 0.45 mM CB129F and CB130R, a 1:3:1 (50ng:150ng:50ng) ratio amount of the three fragments, 0.5 mM of dNTPs, 3.75U of Expand Long Template Polymerase, and 1X Buffer 3. The following thermocycling conditions were used: 95°C for 2 min, followed by 10 cycles of 95°C for 10 sec, 49°C for 30 sec, and 68°C for 3 min followed by 15 cycles of 95°C for 10 sec, 49°C for 30 sec, and 68°C for 3 min with auto-

extend by an additional 20 sec each cycle, and a final elongation at 69°C for 7 min. Approximately 4 µg of the final 3.0 kb PCR fusion product was transformed into strain CB600, resulting in strain CB552 (*swi6Δ::URA3/MET3::SWI6-HIS1*).

CB552 was initially screened by PCR, using CB117F and a nested oligonucleotide. Thermocycling conditions to screen for *swi6Δ::URA3*, with CB117F and CaURA3R, included an initial denaturation at 94°C for 3 min, followed by 25-30 cycles of 94°C for 30 sec, 40°C for 30 sec, 68°C for 1 min 50 sec, and a final elongation at 68°C for 7 min. Conditions for *MET3::SWI6-HIS1*, with CB117F and CaHIS1R, included an initial denaturation at 94°C for 3 min, followed by 25-30 cycles of 94°C for 30 sec, 42°C for 30 sec, 68°C for 50 sec, and a final elongation at 68°C for 7 min. *MET3::SWI6* produced a band at 0.7 kb, and the *swi6Δ::URA3* product produced a band at 2.0 kb. Strain CB552 was also screened by Southern Blot Analysis, using 40 units of the restriction enzyme *PvuI* (NEB) and the 1031 bp probe was made using the oligonucleotides SWI6F and SWI6R, as described above.

2.7.4 *SWI4/SWI6* strains

In order to create a strain lacking both *SWI4* and *SWI6*, the first copy of *SWI6* was deleted from strain BH115 (*swi4Δ::hisG/SWI4*) using a PCR construct created with oligonucleotides CB115F and CB115R, as described above. The final 1508 bp PCR product was transformed into strain BH115, resulting in strain BH140 (*swi4Δ::hisG/SWI4 swi6Δ::HIS1/SWI6*). The second copy of *SWI4* was then placed under control of the *MET3* promoter using oligonucleotides BH10F,R BH11F,R and BH12F,R as described previously to create a PCR fusion construct. The final 4895 bp product was

transformed into strain BH140 resulting in strain BH160 (*swi4Δ::hisG/MET3::SWI4-ARG4*, *swi6Δ::HIS1/SWI6*). The second copy of *SWI6* was subsequently deleted using a PCR-fusion construct created from oligonucleotides BH2F,R BH3F,R and BH4F,R as described. 10 µg of the final 2721 bp PCR product was transformed into strain BH160 resulting in strain BH190 (*swi4Δ::hisG/MET3::SWI4-ARG4*, *swi6Δ::HIS1/swi6Δ::URA3*). All strains were screened by PCR, and by Southern Blot Analysis, as described previously for the single *SWI4* or *SWI6* manipulations.

In order to create a prototrophic control strain, strain BWP17 was transformed sequentially with 5 µg of plasmid pRM100, which contains both the *URA3* and *HIS1* markers, then pBS-Ca*ARG4*, resulting in strain BH420.

2.7.5 *SWI4/MBP1* strains

In order to create a strain that lacked *SWI4* and *MBP1*, the first copy of *MBP1* was deleted from strain BH150 (*swi4Δ::hisG/MET3::SWI4-ARG4*) using a PCR-fusion mediated construct with oligonucleotides BH7F,R BH8F,R and BH9F,R, gDNA and pBS-Ca*HIS1* as templates, as previously described. The final 3006 bp product was transformed into strain BH150, resulting in strain BH270 (*swi4Δ::hisG/MET3::SWI4-ARG4*, *mbp1Δ::HIS1/MBP1*). Finally, the second copy of *MBP1* was deleted as previously described, utilizing a fusion PCR construct obtained with oligonucleotides BH7F,R BH8F,R and BH9F,R, and BWP17 gDNA and pBS-Ca*URA3* as templates. The final 3016 bp construct was transformed into strain BH270 to create strain BH276 (*swi4Δ::hisG/MET3::SWI4-ARG4*, *mbp1Δ::URA3/mbp1Δ::HIS1*).

To confirm the phenotype of strain BH276 under repressing conditions, a strain deleted for both *SWI4* and *MBP1* alleles was created. Strain BH113 (*swi4Δ::hisG /SWI4*) was transformed with a fusion PCR construct to replace the second copy of *SWI4* with a *URA3* marker. Briefly, a 5' 759 bp flanking sequence of *SWI4* was amplified from gDNA using oligonucleotides BH10F and BH10R with the following thermocycling conditions: 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 46°C for 30 sec, and 68°C for 45 sec, with a final elongation at 68°C for 7 min. The reaction mix was composed of a final concentration of 0.6 μM of oligonucleotides, 0.4 mM dNTPs, 100 ng of BWP17 gDNA as template, 3.75U of Expand Long Template Polymerase, and 1X Buffer 3. The 3' 762 bp flanking sequence of *SWI4* was amplified using oligonucleotides BH14F and BH14R with the following thermocycling conditions: 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 48°C for 30 sec, and 68°C for 46 sec, with a final elongation at 68°C for 7 min. The *URA3* cassette was amplified from the pBS-Ca*URA3* plasmid using oligonucleotides BH13F and BH13R, and the following thermocycling conditions: 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 42°C for 30 sec, and 68°C for 1 min 45 sec, with a final elongation at 68°C for 7 min. The reaction mix was composed of a final concentration of 0.6 μM of oligonucleotides, 0.4 mM dNTPs, 100 ng of pBS-Ca*URA3* as template, 3.75U of Expand Long Template Polymerase, and 1X Buffer 3. The final fusion PCR reaction mix included 0.5 mM of dNTPs, 0.45 μM of oligonucleotides BH10F and BH14R, 3.75U of Expand Long Template Polymerase, 1X Buffer 3, and a 1:3:1 (50ng:150ng:50ng) ratio amount of the three fragments. The following thermocycling conditions were used: 95°C for 2 min, 10 cycles of 95°C for 10 sec, 47°C for 30 sec, and 68°C for 2 min 55 sec, then 15 cycles of

95°C for 10 sec, 47°C for 30 sec, and 69°C for 2 min 55 sec with a 20 sec auto-extension each cycle, and a final elongation at 69°C for 7 min. The final 2937 bp PCR product was transformed into strain BH113 resulting in strain BH339 (*swi4Δ::hisG/swi4Δ::URA3*). A copy of *MBP1* was then deleted from strain BH339, using a *HIS1* replacement construct as described previously, resulting in strain BH341 (*swi4Δ::hisG/swi4Δ::URA3 mbp1Δ::HIS1/MBP1*). To replace the second copy of *MBP1* with *CaARG4*, the 5' 790 bp and 3' 799 bp flanks of *MBP1* were amplified from BWP17 gDNA using oligonucleotides BH7F and BH7R, and BH9F and BH9R, respectively, as described above. Oligonucleotides BH8F and BH8R were used with pBS-*CaARG4* to amplify the *ARG4* cassette. The following thermocycling conditions were used: 94°C for 3min, followed by 25 cycles of 94°C for 30 sec, 42°C for 30 sec, and 68°C for 2 min 15 sec, with a final elongation at 68°C for 7 min. The reaction mix was composed of 0.6 μM of oligonucleotides, 0.4 mM dNTPs, 100 ng of pBS-*CaARG4* as template, 3.75U of Expand Long Template Polymerase, and 1X Buffer 3. The final fusion PCR reaction mix included 0.5 mM of dNTPs, 0.45 μM of oligonucleotides BH7F and BH9R, 3.75U of Expand Long Template Polymerase, 1X Buffer 3, and a 1:3:1 (50:150:50ng) ratio amount of the three fragments. The following thermocycling conditions were used: 95°C for 2 min, 10 cycles of 95°C for 10 sec, 49°C for 30 sec, and 68°C for 3 min 30 sec, then 15 cycles of 95°C for 10 sec, 49°C for 30 sec, and 69°C for 3 min 30 sec with a 20 sec autoextension, and a final elongation at 69°C for 7 min. 5 μg of the final 3785 bp PCR product was transformed into strain BH341 resulting in the strains BH348 and BH352 (*swi4Δ::hisG/swi4Δ::URA3, mbp1Δ::HIS1/mbp1Δ::ARG4*). All strains were screened by

PCR and by Southern Blot Analysis, as described previously for the single *SWI4* or *MBP1* manipulations.

2.8 Colony Screening

Strains were initially screened by Direct PCR screening of whole yeast cells (65, 66). Briefly, an average-sized yeast colony (0.5-2 mm) was incubated in 10 μ l Zymolyase Solution (2.5mg/ml Zymolyase (Fisher), 1.2M Sorbitol, 0.1M Sodium Phosphate pH7.4) for 1 hr at 37°C. 2 μ l of spheroplasted yeast cells were used in the 50 μ l PCR reactions as described previously for each gene.

2.9 Southern Blot Analysis

Southern Blot Analysis on *Candida albicans* was carried out using the DIG Hybridization System Kit (Roche).

2.9.1 Probe Construction

A probe of approximately 1 kb was made by PCR, using the conditions described previously for each gene. The product was cleaned using a PCR column, quantified, and 500 ng was boiled for 10 min, followed by a snap-cool on ice for 5 min. The DNA was added to a 100 μ l mixture containing 1X hexanucleotide mix, 1X dNTP labeling reaction mix, and 25U Klenow (DIG Hybridization System; Roche). The solution was mixed gently and incubated overnight at 37°C. To stop the reaction, 4 μ l of 0.5M EDTA was added. To precipitate the DNA, 1 μ l of 20 mg/ml glycogen, 0.1X volume of 5M LiCl, and 3 volumes of cold 95% ethanol were added. After mixing gently, the reaction was left at -20°C overnight. The following day the tube was centrifuged at 4°C for 10 min at

16 rcf. The pellet was washed with 70% ethanol, and resuspended in 50 μ l TE Buffer (1 mM EDTA, 10 mM TRIS pH8.0). The probe was stored at -20°C .

2.9.2 Probe Quantification

Serial dilutions of DIG labeled control DNA (5 ng/ μ l) and DIG-labeled DNA probe were made in Solution 1 (0.1M Maleic Acid, 0.15M NaCl, NaOH, pH7.5). The dilutions were spotted on a positively-charged Nylon membrane (Roche). The DNA was UV-crosslinked to the membrane using a Stratagene UV Linker, set at 1200 Joules (J). The membrane was incubated with 1X Blocking Solution in Solution 1 for 30 min, washed, then incubated in 1:5000 anti-DIG antibody coupled to alkaline phosphatase in Solution 1 for 30 min. After washing, the membrane was incubated in a 1:50 dilution of NBT/BCIP (Roche) in Solution 3 (0.1M NaCl and 0.1M Tris-HCl pH 9.5) in the dark for 30 min. The reaction was stopped by incubating in Solution 4 (0.01M TRIS pH 9.5, 5M NaCl).

2.9.3 Preparation of gDNA

4 μ g of gDNA was cut with the restriction enzyme determined to suit each gene's cutting scheme, as described previously. Digested gDNA was precipitated by added 4 μ l of 5M NaCl and 400 μ l 95% ethanol, mixed gently and left at -20°C for 6 hr to overnight. The following day the precipitated gDNA was centrifuged at 4°C , for 10 min at 16 rcf, then washed once with 70% ethanol. The gDNA pellet was air-dried for 20 min, then resuspended in 20 μ l of TE Buffer, loaded on a 0.7-1% agarose gel, containing 40 μ g/ml Ethidium Bromide, and run overnight at 30-40V. The gel was then washed twice for 15

min in Denaturizing solution (1.5M NaCl, 0.5M NaOH), rinsed in water, then washed twice for 15 min in Neutralizing Solution (3M NaCl, 0.5M TRIS pH 8.0). A positively charged Nylon membrane (Roche) was positioned on top of the gel and the apparatus was set up for capillary action to transfer the DNA to the membrane, for 5 – 6 hr. The DNA was UV-crosslinked to the membrane using the Stratagene UV Linker set at 1200 J.

The membrane was then incubated with preheated prehybridization solution (5X SSC, 1% blocking agent, 0.1% Sodium Lauryl Sarcosinate, 0.02% SDS) for 1 hr, in a hybridization oven at 65°C. To prepare the hybridization solution, 50 ng of DIG-labeled DNA probe was brought to a volume of 10 µl in TE buffer, boiled for 10 min, then snap-cooled on ice for 5 min. The probe was then added to the same preheated DIG Hybridization solution and incubated in a 65°C water bath until ready to add to the membrane. After prehybridization, the membrane was then incubated overnight with the Hybridization solution at 65°C. The following day the membrane was washed twice for 5 min at room temperature in 2X SSC, 0.1% SDS, then washed twice for 15 min at 65°C in 0.1X SSC, 0.1% SDS.

For chemiluminescence detection, the membrane was equilibrated for 3 min in Solution 1, and then incubated in 1X Blocking Solution, for 60 min with agitation. The membrane was then transferred to Blocking Solution containing 1:10000 dilution of Anti-DIG-Alkaline Phosphatase coupled antibody for 30 min. After washing twice for 15 min with 1:33 Tween-20 in Solution 1 with agitation, the membrane was equilibrated for 5 min in Solution 3. The membrane was then placed in a hybridization bag and incubated in 1:100 dilution of CSPD (Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan} phenyl phosphate; Roche) in Solution 3, for 5 min at

room temperature. The bag was then sealed and incubated for 15 min at 37°C to activate the chemiluminescence reaction. The film was placed into a cassette with development film, and exposed for 15 min – 1 hr.

2.10 Transcription Profiling

2.10.1 mRNA Labeling

Target sample preparation and hybridization were performed according to Nantel *et al.*, (2002) (67). Briefly, to label mRNA with dCTP linked to either Cy3 or Cy5, 30 µg of mRNA, 1.5 µl oligo(dT)₂₁ (100 pmol/µl), and water were combined in a total volume of either 18.5 (Cy3) or 19.5 µl (Cy5), incubated at 70°C for 10 min, and cooled to room temperature for 10 min. 3 µl dNTPs (excluding dCTP) (6.67 mM each), 1 µl dCTP (2 mM), 4 µl DTT (100 mM), and 8 µl 5X First Strand Buffer (Invitrogen) were then added on ice. 2 µl of cyanine 3-dCTP (1 mM) or 1 µl of cyanine 5-dCTP (1 mM) and 2 µl of SuperScript II Reverse Transcriptase (Invitrogen) were added and the reaction proceeded at 42°C. After 2 hr, an additional 1 µl of Superscript II was added, and incubation continued at 42°C for 1 hr. The reaction was stopped and RNA degraded by addition of 1 µl each of RNase A (10 mg/ml) and RNaseH (0.05 U/µl). The reaction took place at 37°C for 20 min, then was neutralized with 2.7 µl of NaOAc (3M, pH5.2). Purification was performed with Qiagen PCR cleaning columns.

2.10.2 Hybridization

Prehybridization solution consisted of DIG EASY HYB Buffer (Roche), with 0.45 mg/ml yeast tRNA and 0.45 mg/ml ssDNA. The solution was filter sterilized with a

0.22 μ m syringe filter. Half of this solution was kept at 4°C until hybridization, and the remaining half was heat-denatured at 95°C for 3 min, then placed in a 42°C water bath. 50 μ l was applied to the DNA microarray slide for prehybridization, which occurred for at least 1 hr at 42°C. After prehybridization, the DNA microarray slide was washed 10 times in 3 separate dishes containing filtered, double-distilled water at room temperature. The slides were centrifuged at 931 rcf for 2 min in 50 ml conical tubes containing tissue paper (Kimwipes) in order to dry. The target sample was made by concentrating the Cy3 and Cy5 labeled cDNA to a final volume of 3 – 4 μ l. The cDNA targets were combined together, and mixed with the remaining hybridization buffer, to a volume of 30 μ l. This hybridization solution was heat denatured at 95°C for 3 min, placed in a 42°C water bath, and then applied to the DNA microarray slide for overnight incubation at 42°C. The next day, slides were immersed in 250 ml of preheated 1X SSC (0.15 M Sodium chloride, 0.015 M Sodium citrate), and 0.2% Sodium Dodecyl Sulfate (SDS) in a slide dish to remove the 24x60 mM glass coverslip (Fisher Scientific). The microarray slide was then washed in 1X SSC, 0.2% SDS for 10 min at 42°C, twice at 37°C in 0.1X SSC, 0.2% SDS, and once in room temperature 0.1X SSC for 5 min. The slides were subsequently washed 5 times at room temperature in 0.1X SSC. Slides were dried as described and stored in the dark until scanning.

2.10.3 Data Analysis

The DNA microarray slides were scanned with an Axon GenePix Pro 4.0 scanner at a 10 μ M resolution, using 532nm and 635nm. The resulting files were quantified with GenePix software. The data was represented as an expression ratio (experiment/control).

The values were transformed by taking the \log_2 ratio, and normalized with Lowess. Statistical analysis and visualization were performed with GeneSpring software (Silicon Genetics, Redwood City, CA). Significant genes were selected based on a 1.5-fold up or down regulation, with a *t*-test function using $p < 0.05$ confidence.

2.11 Microscopy

Nuclei and septa were visualized by fixing cells in 70% ethanol for at least 1 hr, followed by incubation in 1 $\mu\text{g/ml}$ 4',6'diamidino-2-phenylindole dihydrochloride (DAPI, Sigma) for 20 min. After rinsing with ddH₂O, cells were incubated in 1 $\mu\text{g/ml}$ Calcofluor white (Sigma) for 10 min. After a final spin and rinse with ddH₂O, cells were placed on a microscope slide, covered with a coverslip and sealed with nail polish. Cells were examined on a Leica microscope (DM6000B) using 63X, or 100X objectives with Nomarski differential interference contrast (DIC) or fluorescence optics, using the appropriate filter sets (DAPI 460 nm).

3. RESULTS

3.1 Transcription profiling of Cln3p-depleted cells supports a G1 phase arrest and uncovers novel aspects of the G1/S circuitry in *C. albicans*.

We previously demonstrated that depletion of the cyclin Cln3p in yeast cells of *C. albicans* resulted in an apparent G1 phase arrest, followed by production of hyphae and pseudohyphae with active cell cycles (Fig. 3A). In order to characterize the G1/S regulatory circuit in yeast cells and screen for potential Cln3p effectors, we obtained time course transcription profiles of cells depleted of Cln3p. Cells of strain CB488 (*cln3/MET3::CLN3*) were incubated in repressing medium for 1, 3 or 6 h to identify the expression patterns associated with an early G1 phase arrest (1 h), an intermediate stage preceding initiation of hyphal development (3 h), and a later stage associated with hyphal growth (6 h), respectively. The prototrophic control strain CB504 (*CLN3/CLN3*) was incubated in repressing medium for the same time periods. The data were analyzed with Genespring software, and significantly modulated genes were identified based on a 1.5 fold cut-off and t-test ($p < 0.05$) function.

Hierarchical cluster analysis (Fig. 3B) of significantly modulated genes demonstrated several similar expression patterns over the time course, many of which were consistent with a G1 phase arrest as seen in *S. cerevisiae* (Tables 4, 5, Appendix Tables S1-S6; Fig. 4) (68, 69). For example, the large subunit of ribonucleotide reductase, *RNR1*, was repressed, as well as the putative G1 phase cyclin *PCL2*, the S phase and mitotic cyclins *CLB4* and *CLB2*, respectively, and putative targets of the

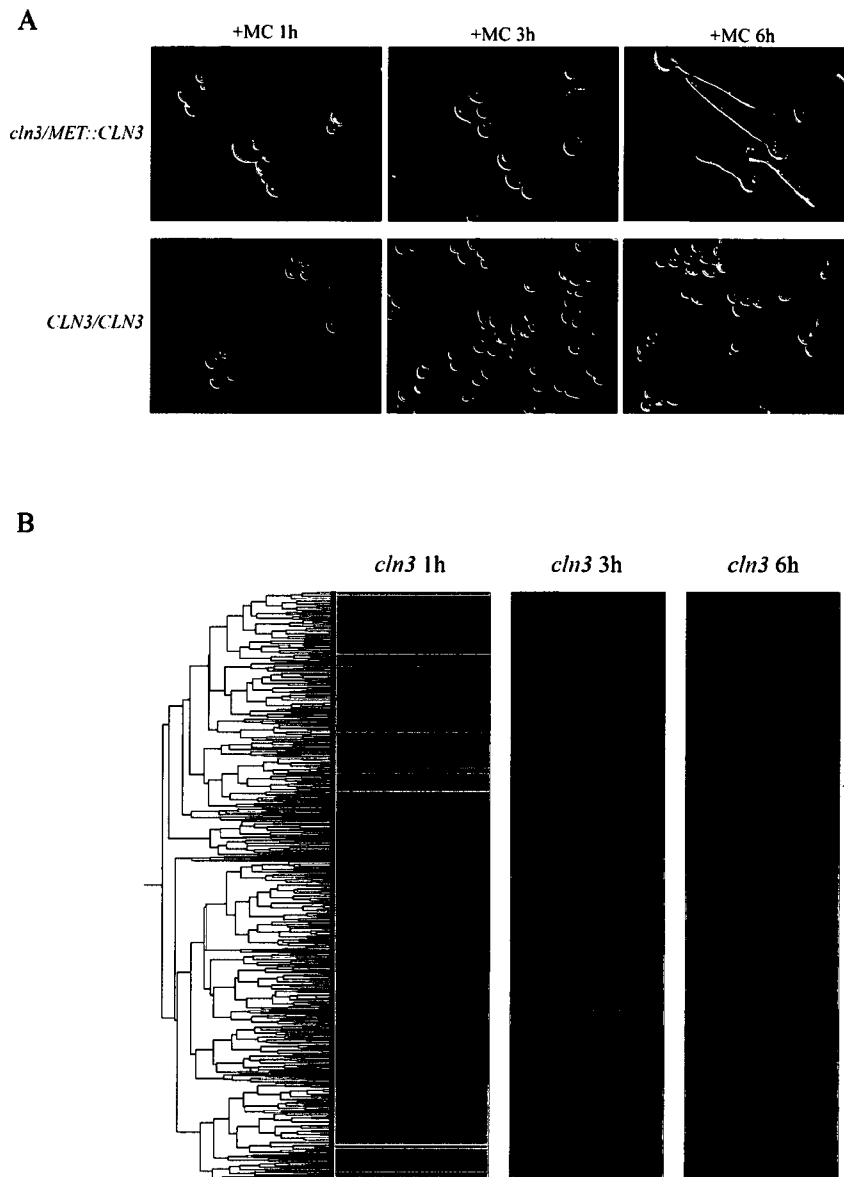


Figure 3. Depletion of Cln3p in yeast cells results in a G1 phase cell cycle arrest and cell enlargement, followed by the development of hyphae and pseudohyphae.

(A) Strains CB488 (*cln3/MET3::CLN3*) and isogenic control strain CB504 (*CLN3/CLN3*) were grown in inducing liquid medium (-MC) overnight, then diluted into repressing medium (2.5 mM Methionine, 0.5 mM Cysteine, +MC) at 30°C for the indicated times. Bar: 10 μ m. (B) Hierarchical cluster analysis of significantly modulated genes, from cells depleted of Cln3p for 1, 3, or 6h. Strains CB488 and CB504, were grown in inducing liquid medium overnight, then diluted into repressing medium (+MC) at 30°C for the different time points. RNA was extracted, and samples were labeled for DNA Microarray analysis. Results are based on 5-6 chips for each time point, from independent samples. Significant genes were selected based upon a 1.5 fold cut-off, and t-test function with $p < 0.05$. Colour change indicates fold variation, where down-regulation is green and up-regulation is red.

SBF/MBF transcription factor complex, including *HCM1*, *TOS4* and *TOS1* (Tables 4, S1, S3, S5). Surprisingly, SBF/MBF components, including *SWI6* and *MBP1*, were also repressed. In contrast, orthologues in *S. cerevisiae* were not repressed during a G1 phase arrest as they were not transcriptionally regulated in a cell cycle-dependent manner (70, 71). A G1 phase arrest was also supported by repression of genes associated with histones, DNA replication and chromatin remodeling (Tables 4, S1, S3, S5; Fig. 4). While some genes within the categories showed variability in expression across the time course, an overall trend in repression was observed for most genes within the groups, supporting the notion that a G1 phase arrest was taking place. In the absence of genome-wide expression data of white phase cells passing through the cell cycle in *C. albicans*, due to issues with cell synchronization (44), our results provide the first picture of factors potentially associated with the G1/S circuit in white phase cells of *C. albicans*.

The profiling data also demonstrated modulation of few genes associated with hyphal growth, albeit in a time-point specific manner. For example, *NGT1*, a GlcNAc transporter with a role in GlcNAc-induced hyphal growth (72), *SSA2*, a *HSP70* chaperone present only on the surface of hyphal cells (73), and *HSP90*, which plays a critical role in mediating the temperature requirement for hyphal growth (74), were up-regulated at 1h of Cln3p depletion (Tables 5, S2). However, none of these factors were regulated at other time points, suggesting that these expression patterns could be related to other functions. At 3 h, when many G1 phase-arrested cells were just initiating polarized growth (Fig. 3A),

Table 4: Select genes down-regulated during CLN3 repression.¹

Functional Category	Common Gene Name	Time Point (h)
Cyclins	<i>CCN1, CLN3, PCL2, CLB2, CLB4</i>	1, 3, 6
SBF/MBF-Associated	<i>SWI6, MBP1, SWI4, HCM1, TOS4, TOS1</i>	1, 3, 6
Cell Cycle (Other)	<i>HSL1, FKH2, SWE1, CDC6, CDC5, MCM1, CDC14, GIN4, ACE2</i>	1, 3, 6
CDKs	<i>CDC28, CRK1</i>	1, 6
Histones	<i>HHT1, HHO1, HTA3, HTA2, HTA1, HTB1</i>	1, 3, 6
DNA Repair/ Replication	<i>RNR1, RAD51, RAD2, RAD3, MSH6, MCM2, MCM16, SMC1, SMC2, SMC5, RFA1, RFA2, DUN1, POL1, POL2, POL30</i>	1, 3, 6
Chromatin Remodeling	<i>HDA1, SNF2, TAF14, TAF60, YAF9, ASH2</i>	1, 3, 6
Ribosome Biogenesis/ Translation	<i>RPL29, RPL38, RPL39, RSP17, RSP19A, RSP22A, RPP1B, RPP1A</i>	6
Hyphal-associated	<i>EFG1, CPH1, CST20, FLO8, YAK1, RBT4</i>	3, 6
Opaque-associated	<i>NDT80, ADAEC</i>	3, 6
	<i>WOR2, CZF1</i>	6

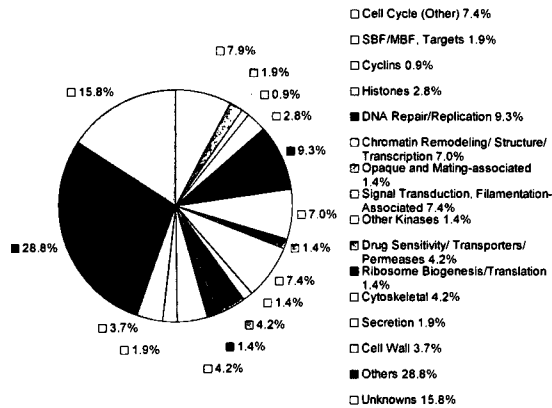
¹Experimental (*cln3/MET3::CLN3*) and prototrophic control cells (*CLN3/CLN3*) were incubated in repressing medium (+MC) for 1, 3, or 6 h. Fold change is based on 5-6 microarray chips for each time point representing independent samples. Significant genes were selected based upon a 1.5 fold cut-off, and t-test function with $p < 0.05$. For full gene list with fold changes, see Appendix Tables S1, S3, and S5

Table 5: Select genes up-regulated during CLN3 repression.¹

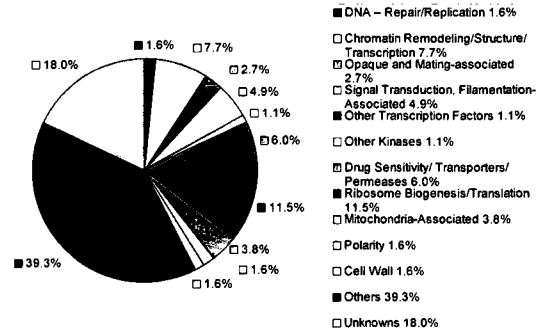
Functional Category	Common Gene Name	Time Point (h)
Hyphal -associated	<i>HSP90, NGT1, NOP15</i>	1
	<i>RBR1, MKC1</i>	3, 6
	<i>HWP1, RBT1, UME6, CEK1, ECE1, HYR1, IHD1, IHD2, ALS1</i>	6
DNA Repair/ Replication	<i>RNR3</i>	1
Oxidative Stress response, biofilm	<i>CAT1, TSA1B, TSA1</i>	3, 6
Opaque-associated	<i>MSC1, IFA12, orf19.5876</i>	3, 6
	<i>MPT5, WH11, ALD6, OBPA</i>	6

¹Experimental (*cln3/MET3::CLN3*) and prototrophic control cells (*CLN3/CLN3*) were incubated in repressing medium (+MC) for 1, 3, or 6 h. Fold change is based on 5-6 microarray chips for each time point representing independent samples. Significant genes were selected based upon a 1.5 fold cut-off, and t-test function with $p < 0.05$. For full gene list with fold changes, see Appendix Tables S2, S4, and S6.

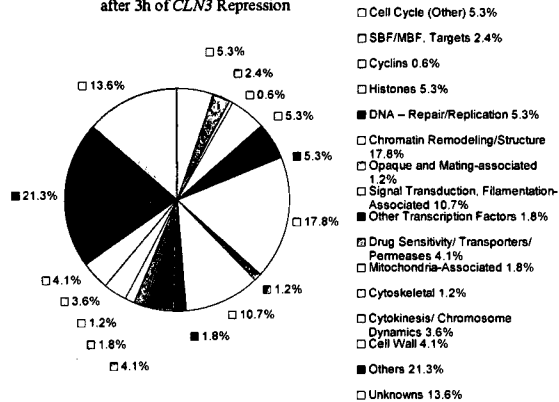
Distribution of Down-Regulated Genes after 1h of *CLN3* Repression



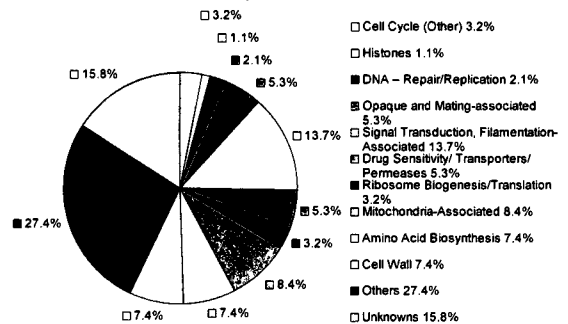
Distribution of Up-Regulated Genes after 1h of *CLN3* Repression



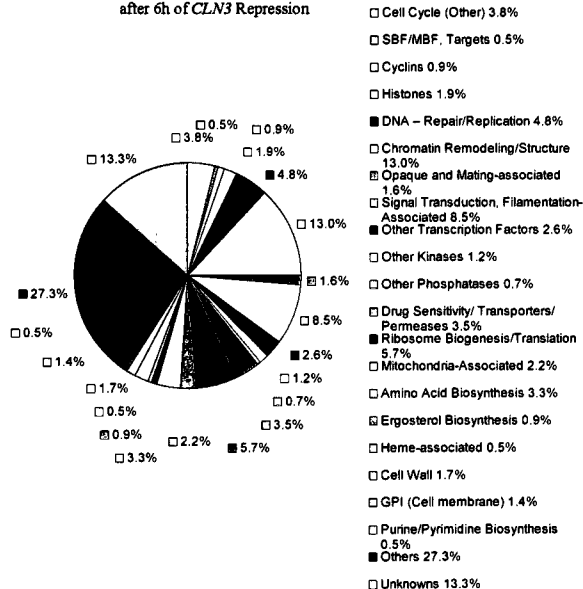
Distribution of Down-Regulated Genes after 3h of *CLN3* Repression



Distribution of Up-Regulated Genes after 3h of *CLN3* Repression



Distribution of Down-Regulated Genes after 6h of *CLN3* Repression



Distribution of Up-Regulated Genes after 6h of *CLN3* Repression

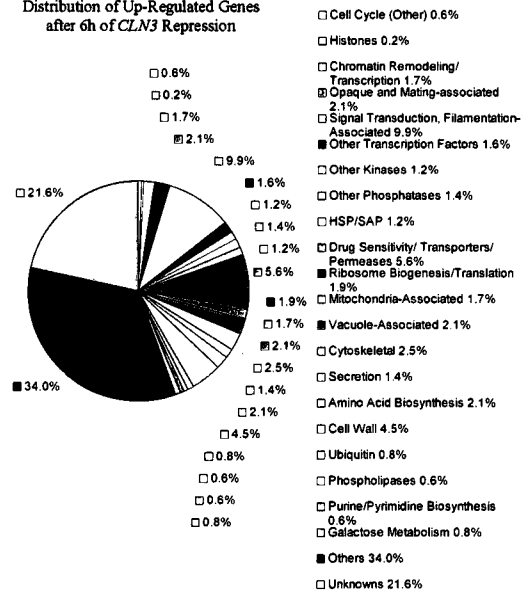


Figure 4. Pie charts of the functional categories of the significantly modulated genes, comprising major cellular processes, during different time points of *CLN3* repression. Gene names and function were identified through Genespring analysis, and manually verified with the *Candida* Genome Database (CGD) at <http://candidagenome.org/>. Different colours represent different categories, as indicated. Genes were categorized according to a single function, although some genes may have several functions. Significantly modulated genes were based on a 1.5 fold cut-off (t-test, $p < 0.05$).

additional genes associated with hyphal development were up-regulated, including the hyphal-specific cell wall protein *RBR1* (75), while others, including *RBT4* (76), were surprisingly down-regulated (Tables 4, S4). At 6 h, when hyphae were actively growing, many classic hyphal-associated virulence genes were strongly induced, including *HWPI1*, *ECE1*, *RBT1*, and *HYR1* (Tables 5, S6) (2, 76). Of the major regulators of the hyphal state, *UME6* (77) was induced, while others, including *FLO8*, *CPH1*, *CST20*, *YAK1* and *EFG1* (2) were repressed (Tables 4, S3, S5). Thus, the transcription profiles support the concept that true hyphae can form upon repression of *CLN3*, and also uncover potential signaling factors that may provide a link between Cln3p activity and hyphal development, albeit in a complex manner. With respect to hyphal development, the profiles also show that cell cycle-associated genes including *CDC5*, *FKH2*, *HSL1*, and *GIN4* were repressed during Cln3p depletion (Tables 4, S1, S3, S5). Since deletion of these individual factors leads to filamentous growth (44), it is possible that these expression patterns also contribute to the hyphal growth response upon repression of *CLN3*.

Intriguingly, several genes associated with the opaque cell fate (23, 78) were modulated at 3 and 6 h, including *ALD6*, *MPT5*, and *OBPA*, for example (Tables 5, S6). In addition, the white phase cell-specific gene *WH11* was highly up-regulated, a behavior that may occur when opaque cells switch back to the white phase (79). This expression pattern suggests that Cln3p and G1 phase may be linked to additional developmental states in *C. albicans*, specifically opaque cell formation.

The hierarchical cluster analysis highlighted several time point-specific gene expression patterns, many of which validated the data, while others were unexpected.

For example, genes that were down-regulated at 1 h but subsequently up-regulated at later time points (Fig. 3B) included those associated with the cytoskeleton, cell wall, polarized growth, and secretion, which are consistent with the fact that cells were undergoing an initial G1 phase arrest, followed by initiation and maintenance of hyphal growth (Tables 4, 5). Glucose transporters, such as *HGT6*, followed a similar expression pattern. While some genes associated with RNA processing were repressed at 1 h, as expected with a G1 phase arrest, many more associated with translation and ribosome biogenesis were repressed at 6 h, despite the fact that hyphae were growing at this stage (Tables 4, S1-3, S5).

A large group of genes modulated at specific time points or throughout the time course were those of unknown function (Fig. 4). Genes in this category have important implications for future drug target discovery, if they are found to be required for the G1/S transition and control of cell proliferation.

In an attempt to help distinguish which of the expression patterns during Cln3p depletion were functionally important for the G1/S transition vs. hyphal development, and were not an indirect consequence of either state, we exploited the fact that cells depleted of Cln3p formed hyphae in a Ras1p-dependent manner (47). Transcription profiles of cells depleted of Cln3p for 3 or 6 h in the absence of *RAS* were obtained and compared that of Cln3p-depleted cells containing *RAS*. Hierarchical cluster analysis demonstrated some similar patterns of gene expression (Fig. 5A). Such genes were modulated regardless of Ras1p, and thus represent Cln3p- dependent, Ras1p-independent factors. These genes are not likely to be modulated as a consequence of filamentous growth, for example, since Cln3p-depleted cells did not form hyphae without Ras1p.

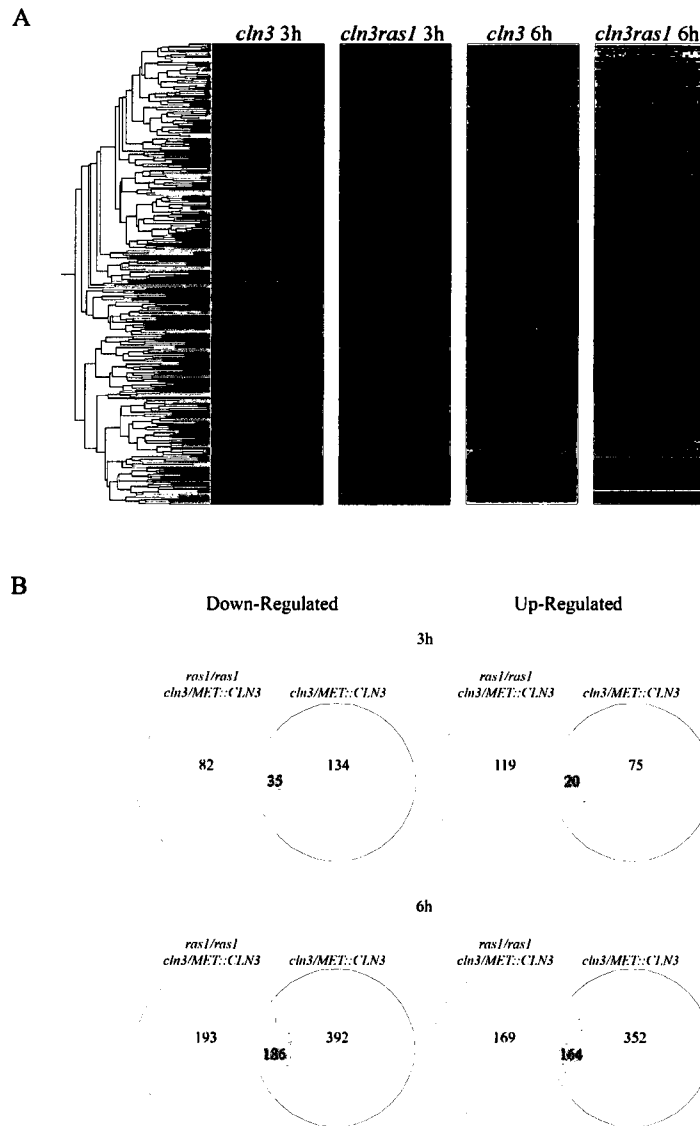


Figure 5. Analyses of significantly modulated genes, from cells depleted of Cln3p and Ras1p or Cln3p alone, at 3h and 6h.

(A) Hierarchical cluster analysis of the significantly modulated genes. Strains CB498 (*ras1/ras1*; *cln3/MET3::CLN3*) and the control CB504 (*CLN3/CLN3*), were grown in inducing liquid medium overnight, then diluted into repressing medium (+MC) at 30°C for the different time points. Samples were processed for DNA Microarray analysis. Results for the *ras1/cln3* strain are based on 4 chips for each time point, from independent samples, compared to those described previously for Cln3p-deleted cells. Significant genes were based on a 1.5 fold cut-off, and t-test function with $p < 0.05$. Colour variation indicates fold changes: green represents down-regulation and red represents up-regulation. **(B)** VENN diagrams comparing significantly modulated genes after 3 or 6 h of *CLN3* repression. Genelists were compared using Genespring. Genes modulated under both conditions were considered to be independent of Ras1p, whilst those modulated only during *CLN3*-repression were considered Ras1p-dependent.

Venn diagrams (Fig. 5B) demonstrated that this group was relatively small, and included some hyphal regulatory factors such as *CST20*, *CPH1*, *FLO8*, *YAK1*, and *EFG1* (2, 80, 81). Significant modulation of these genes in the absence of *RAS* could thus potentially reflect a role in mediating Cln3p function in differentiation. Efg1p acts either as a positive or negative regulator of hyphal growth, depending on the environmental condition (82, 83), while the remaining factors are inducers of hyphae (2, 80, 81). That the latter genes were repressed during Cln3p depletion suggests a more complex role in regulating Cln3p-dependent hyphal growth, if in fact they participate in the process.

Other *RAS*-independent expression patterns included genes involved in different aspects of the cell cycle, including DNA repair and replication, for example. However, no single functional category was completely Ras1p-independent, as seen with the histones, for example. Since cells were blocked in G1 phase, regardless of Ras1p, all histones were expected to be repressed and thus Ras1p-independent. The inconsistency could be due to technical issues associated with sensitivity and variability in data points on all chips meeting the requirements for significance. Thus, caution must be used in designating genes as being Ras1p-dependent, since their lack of modulation in Cln3p-depleted, *ras1/ras1* cells could be due to technical issues. The expression pattern of any single gene in the Ras1p-dependent category would have to be validated further to gain any insight on potential functional significance.

Overall, the transcription profiles support the notion that depletion of Cln3p results in a G1 phase arrest in yeast cells, provide the first picture of factors associated with the G1/S transition in white phase cells of *C. albicans*, and contribute to the construction of a putative framework for the G1/S circuit. While the basic outline shares

similarities with other fungi, including *S. cerevisiae*, the results uncover several key differences. The data also identified hyphal signaling factors that are potential candidates for linking G1 phase with hyphal development, and suggest that the G1/S circuit may be coordinated with other another developmental programs, including the opaque cell formation.

3.2 Functional analyses of Swi6p, Swi4p and Mbp1p

In order to further define the G1/S regulatory circuit and identify potential mediators of Cln3p function in *C. albicans*, we next utilized a directed, genetic approach that involved characterization of *SWI6* (ORF19.4725), *SWI4* (ORF19.4545) and *MBP1* (ORF19.5855). Orthologues in *S. cerevisiae* are components of the SBF/MBF transcription factor complex, which is crucial for mediating CDK/cyclin activity to initiate Start (71). Swi6p is the regulatory subunit for both SBF and MBF, while Swi4p and Mbp1p are the DNA-binding subunits of SBF and MBF, respectively (84).

3.2.1 Cells lacking *MBP1* do not show any significant change in growth or phenotype.

C. albicans ORF19.5855 is annotated as *MBP1* (*Candida* Genome Data Base, <http://candidagenomedatabase.org>), and shares 29% identity at the protein level with Mbp1p from *S. cerevisiae*. In order to determine the function of *C. albicans* *MBP1*, the two alleles were replaced with *URA3* and *HIS1* markers in strain BWP17 (Fig. 6). Transformants were screened by PCR and Southern (Fig. 7), and strain BH261 was used for further analysis. An isogenic control strain was created by transforming strain BWP17

with plasmids pBS-Ca*URA3* and pBS-Ca*HIS1*, resulting in strain BH440. To confirm the deletion strain results, a conditional strain was created by replacing one copy of *MBP1* with *HIS1*, and placing the second copy under control of the *MET3* promoter, resulting in strain KmCa4a.

When strains BH261 and BH440 were incubated on solid (SD) minimal medium for 48 hr at 30°C, the colonies of strain BH261 were round and smooth, similar to control strain BH440 (data not shown). To determine if there was any change in individual cell phenotype, overnight cultures of the strains were diluted and incubated in fresh liquid medium at 30°C for 7 h. The majority of cells of strain BH261 were of normal size and morphology, comparable to those in strain BH420 (Fig. 8, Table 6). However, 6.9% (n=144) of cells lacking *MBP1* were slightly elongated in shape, compared to 1.2% (n=243) in strain BH420. In comparison, absence of *MBP1* in *S. cerevisiae* did not result in any dramatic change in cell shape but produced a 20% increase in cell volume and 5% increase in the proportion of budded cells (84). To confirm that absence of *MBP1* did not dramatically influence growth, cells of strain KmCa4a were grown overnight in inducing medium (-MC), then diluted into fresh inducing or repressing (+MC) medium for 7 h at 30°C. Under repressing conditions, most cells were in a normal morphology, with 7.6% (n=315) showing minor elongation, compared to 5.5% (n=362) under inducing conditions. Thus, Mbp1p is not essential for normal cell growth but has a mild influence on morphology in *C. albicans*.

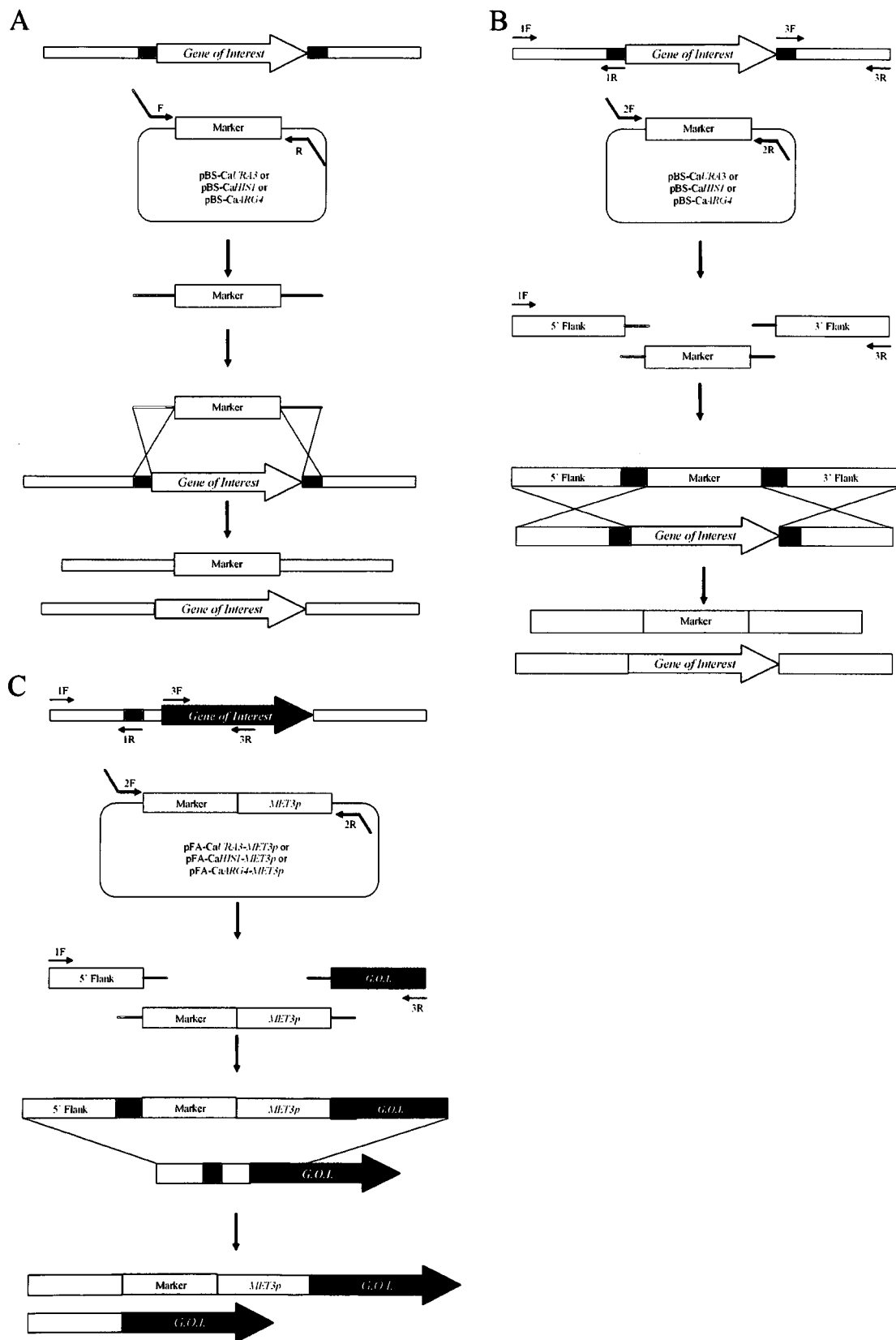


Figure 6. Strategies for gene deletion and promoter replacement. (A) One-step strategy for producing a deletion construct by PCR amplifying a marker using oligonucleotides with 80nt homology to the flanking regions of the gene of interest. (B) 2-step PCR fusion protocol to produce a construct for gene deletion. PCR was used to amplify 0.5-1 Kb fragments flanking the gene of interest, using primers 1F,R and 3F,R, as well as a marker using oligos 2F,R. The latter contained the reverse complement sequence to primers 1R and 3F, respectively. The final fusion product was created using all 3 PCR products as templates in a reaction using oligos 1F and 3R. (C) Promoter replacement strategy to replace a promoter using a similar 2-step PCR fusion protocol.

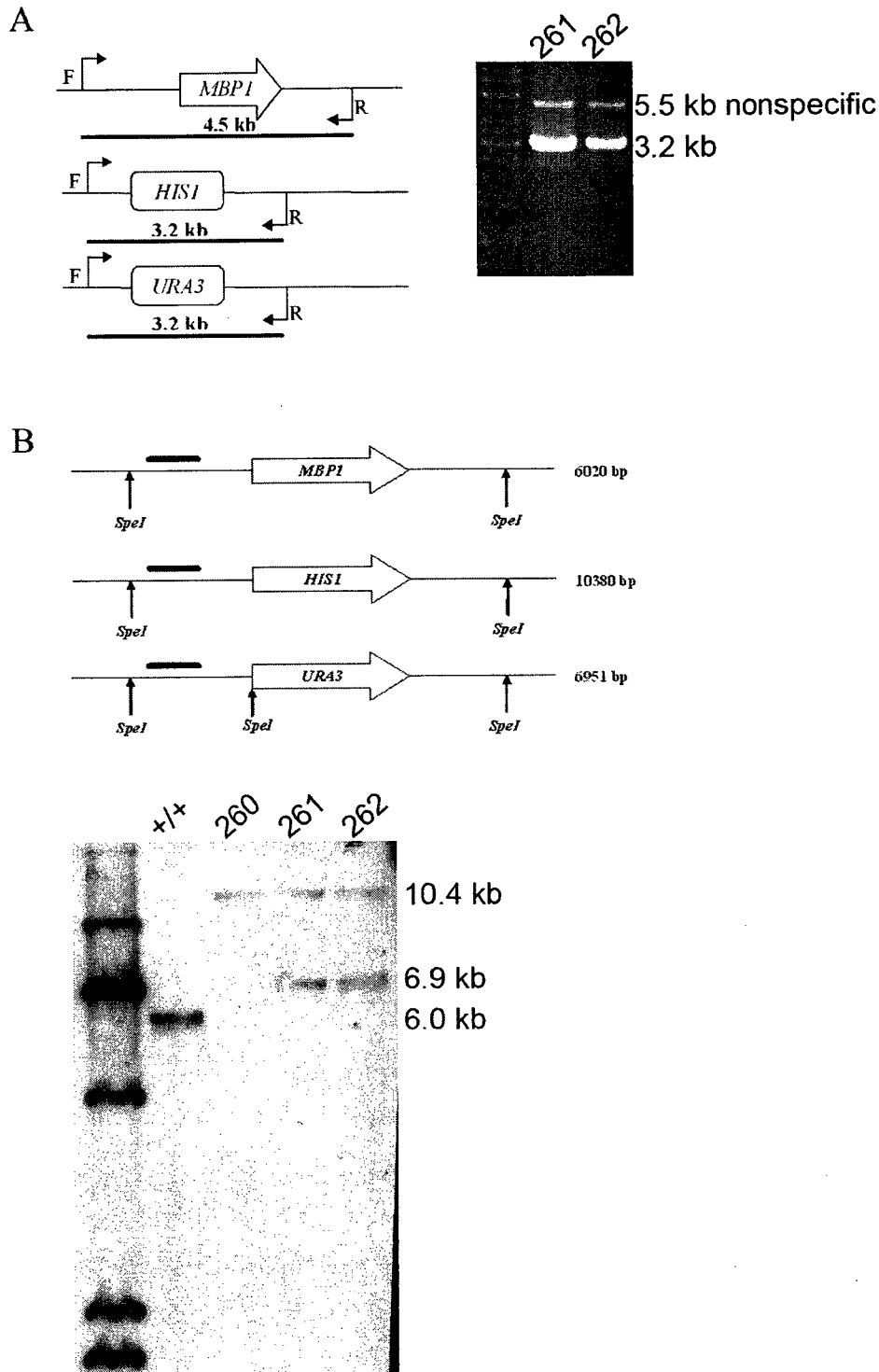


Figure 7. PCR and Southern screens confirming *MBP1* deletion strains. (A) Map and gel of PCR screen, showing a 3.2 kb band for *mbp1Δ::URA3* or *mbp1Δ::HIS1*, and a 4.5 kb band for *MBP1*. (B) Map for Southern analysis indicating position of probe, and blot confirming construction of strains. Digestion of gDNA with *SpeI* produced a wildtype band at 6.0 kb, an *mbp1Δ::HIS1* deletion band at 10.4 Kb, and an *mbp1Δ::URA3* deletion band at 6.9 kb.

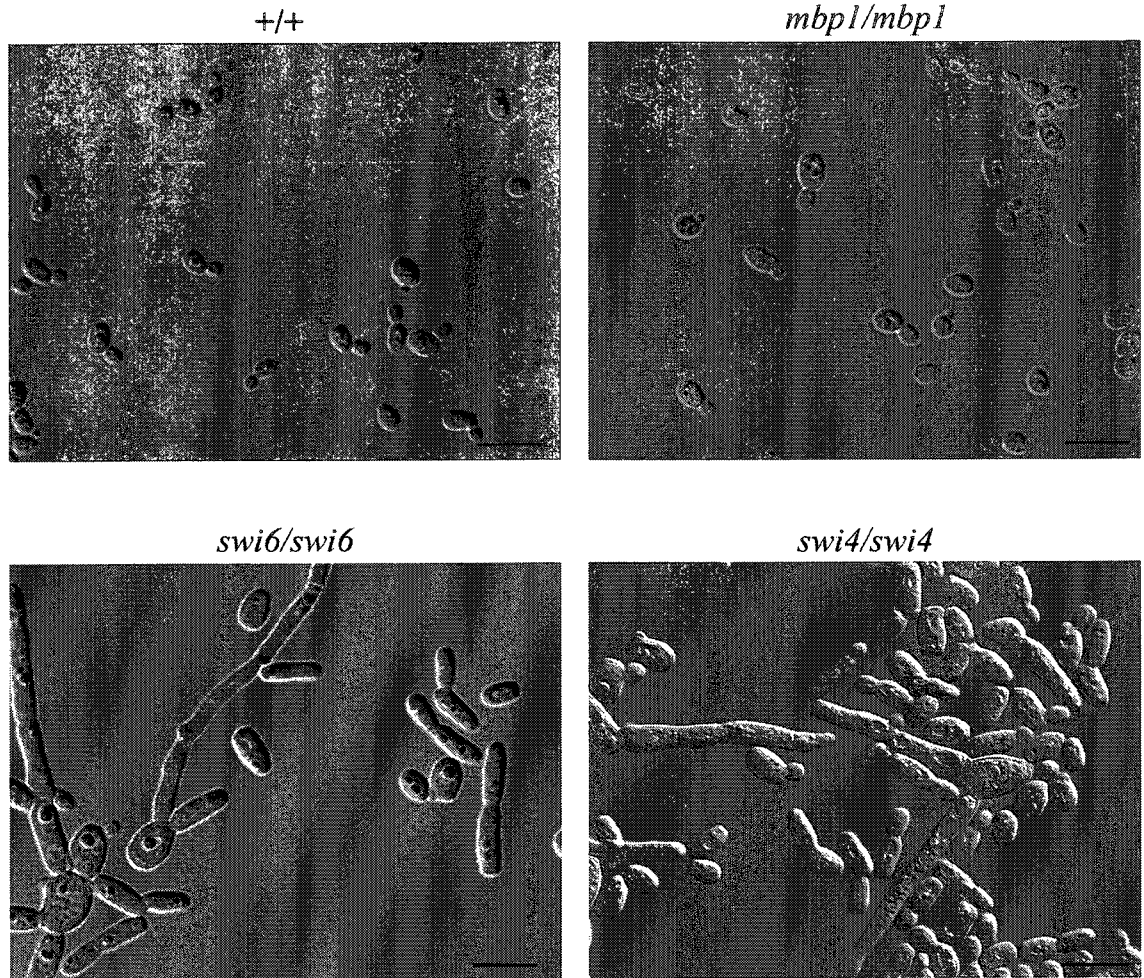


Figure 8. Deletion of *SWI4* or *SWI6* results in dramatic changes in morphology under yeast growth conditions, in contrast to the *MBP1* deletion. Cells from strains BH261 (*mbp1* Δ ::*URA3/mbp1* Δ ::*HIS1*), BH120 (*swi6* Δ ::*URA3/swi6* Δ ::*HIS1*), BH185 (*swi4* Δ ::*URA3/swi4* Δ ::*HIS1*) and BH420 (*MBP1/MBP1*, *SWI6/SWI6*, *SWI4/SWI4*) were incubated in SD medium overnight, then diluted into fresh SD medium and incubated for 7 h at 30°C. Bar: 10 μ m.

Table 6: Proportion of different morphologies in cells lacking SBF/MBF components.¹

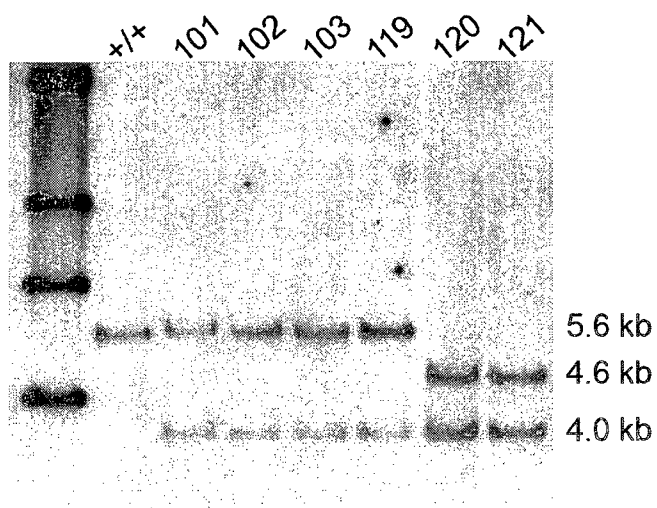
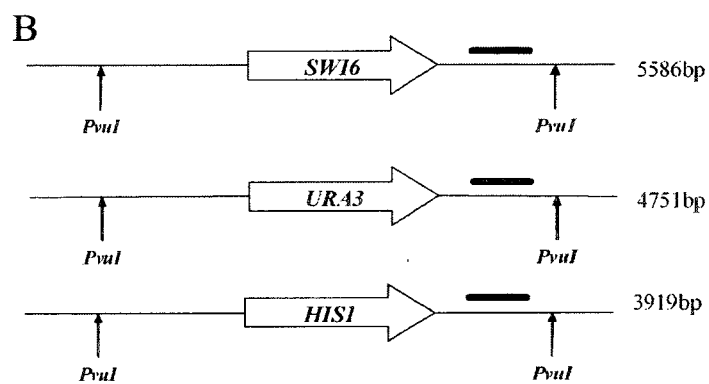
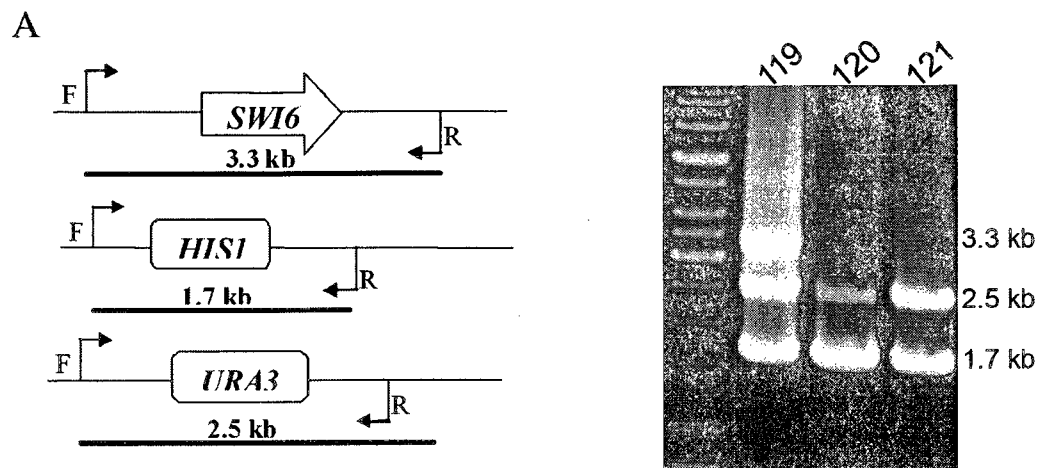
		2	3	4	5	6	7	8	Pseudohyphae ⁹	Hyphae ¹⁰
+/+										
	0h	94.0	0.7	2.5	1.1	1.8	-	-	-	-
	7h +MC	92.6	1.2	2.7	1.2	1.9	-	-	0.4	-
<i>mbp1/mbp1</i>										
	0h	99.1	0.9	-	-	-	-	-	-	-
	7h +MC	91.0	6.9	-	-	-	-	-	2.1	-
<i>swi6/swi6</i>										
	0h	23.4	52.2	3.4	1.5	2.4	8.3	1.0	6.8	1.0
	7h +MC	28.0	51.4	3.3	1.4	2.3	4.2	3.3	5.1	1.0
<i>swi4/swi4</i>										
	0h	33.2	45.1	3.1	1.3	2.2	5.3	0.9	8.8	-
	7h +MC	26.7	40.8	3.7	1.6	2.6	4.7	3.1	15.2	1.6
<i>swi4/MET3::SWI4 swi6/swi6</i>										
	0h	38.8	48.2	2.5	1.1	1.8	4.7	-	2.9	-
	7h +MC	31.3	24.6	5.2	2.2	3.7	3.0	5.2	22.4	2.2
<i>swi4/MET3::SWI4 mbp1/mbp1</i>										
	0h	78.0	8.5	3.1	1.3	2.2	1.8	-	4.9	-
	7h +MC	57.8	20.0	3.8	1.6	2.7	1.1	2.7	9.7	0.5

¹All proportions are in percentages. ²Small budding yeast. ³Enlarged, extended yeast. ⁴Opaque or Rectangular-looking yeast; enlarged. ⁵Enlarged, rectangular cell budding off corner of the mother cell. ⁶Rod shaped cell. ⁷Elongated bud. ⁸Chains or pseudohyphae composed of rod-shaped cells. ⁹Pseudohyphae & chains of cells. ¹⁰True hyphae, including any with double germination.

3.2.2 Absence of *SWI4* or *SWI6* results in cell enlargement and a pleiotropic phenotype, including development of hyphae, under yeast growth conditions.

Orthologues of *SWI4* (ORF19.4545) and *SWI6* (ORF19.4725) were identified in CGD. Swi4p and Swi6p are 23% and 26% identical to their counterparts in *S. cerevisiae*, respectively. To investigate the function of these factors, deletion strains were created by replacing alleles with *URA3* and *HIS1* markers. Transformants were screened by PCR and Southern Blot analysis (Fig. 9A, B and Fig. 10A, C, D). Strains BH185 (*swi4Δ::HIS1/swi4Δ::URA3*) and BH120 (*swi6Δ::HIS1/swi6Δ::URA3*) were used for further investigations. In order to confirm the deletion phenotypes, conditional strains carrying a single copy of *SWI4* or *SWI6* under control of the *MET3* promoter were also constructed. Strains BH150 (*swi4Δ::hisG/MET3::SWI4*) and CB552 (*swi6Δ::URA3/MET3::SWI6*) were confirmed by PCR and Southern Blot analysis (Fig. 9C, D and Fig. 10B, C, D)

When strains BH185, BH120, and control strain BH440 were incubated on solid medium for 48 h at 30°C, colonies of strains BH185 and BH120 showed some filamentation at the periphery compared to the round and smooth colonies of strain BH440 (data not shown). To explore the phenotype further, the strains were grown overnight in liquid medium at 30°C, diluted into fresh medium and incubated for 7 h. Cells lacking *SWI4* (BH185) or *SWI6* (BH120) demonstrated a general increase in cell size but also a pleiotropic phenotype, consisting of small budding yeast, pseudohyphae and some true hyphal cells (Fig. 8, Table 6).



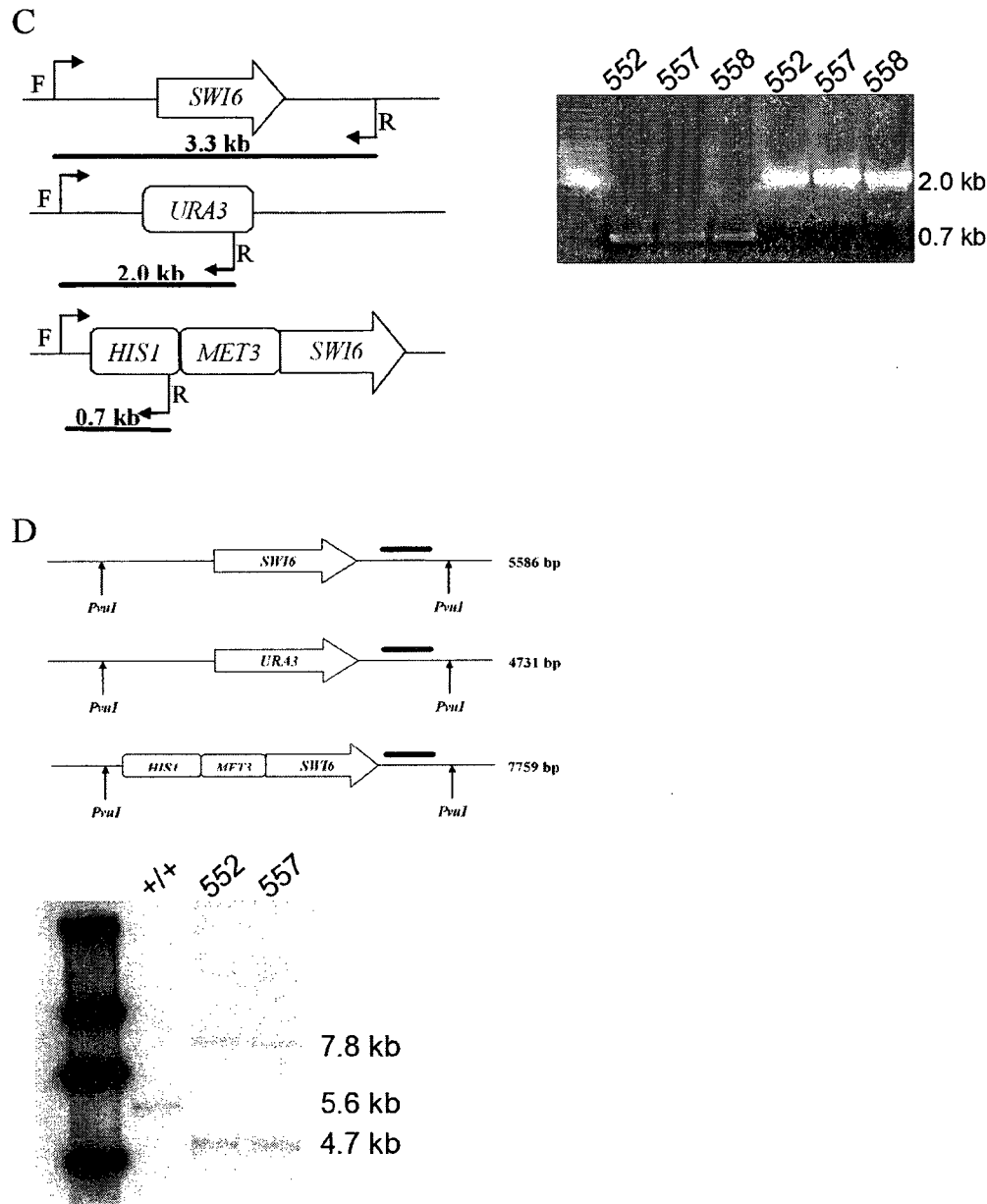
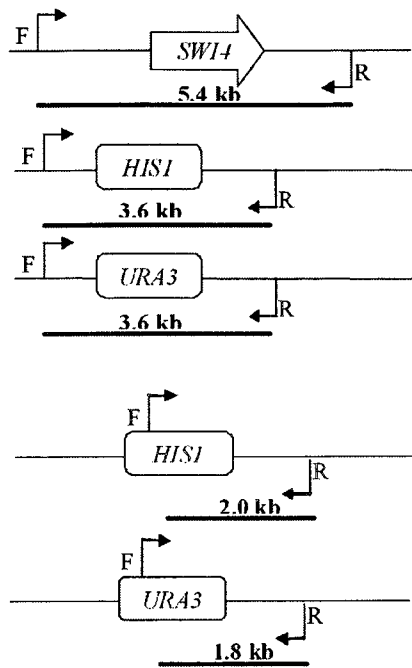
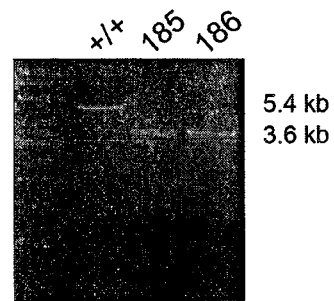


Figure 9. PCR and Southern screens confirming creation of *SWI6* deletion and conditional strains. (A) Map and gel of PCR screen, showing a 2.5kb band for *swi6Δ::URA3*, a 1.7 kb band for *swi6Δ::HIS1*, and a 3.3 kb band for *SWI6*. BH119 was negative, while BH120 and BH121 were positives deletion strains. (B) Map for Southern analysis indicating position of probe, and blot confirming construction of strains. Digestion of gDNA with *PvuI* produced a wildtype band at 5.6 kb, a *swi6Δ::HIS1* band at 4.0 kb , and a *swi6Δ::URA3* band at 4.6 kb. (C) Map and gel of PCR screen using nested oligos, showing a 2.0 Kb band for *swi6Δ::URA3*, and a 0.7 Kb band for *MET3::SWI6*. BH552, 557, and 558 are positive conditional strains. (D) Map for Southern analysis indicating position of probe, and blot confirming construction of strains. Digestion of gDNA with *PvuI* produced a wildtype band at 5.6 Kb. A *swi6Δ::URA3* band at 4.7 Kb, and a *MET3::SWI6* band at 7.8 Kb confirming strains CB552 and CB557.

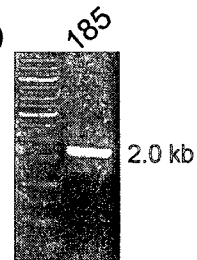
A



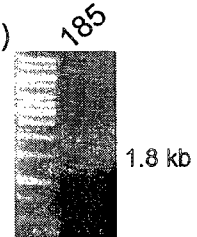
(i)



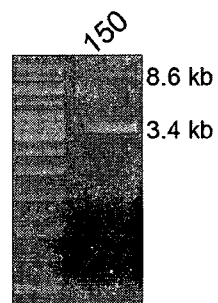
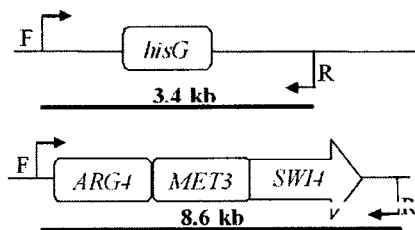
(ii)



(iii)



B



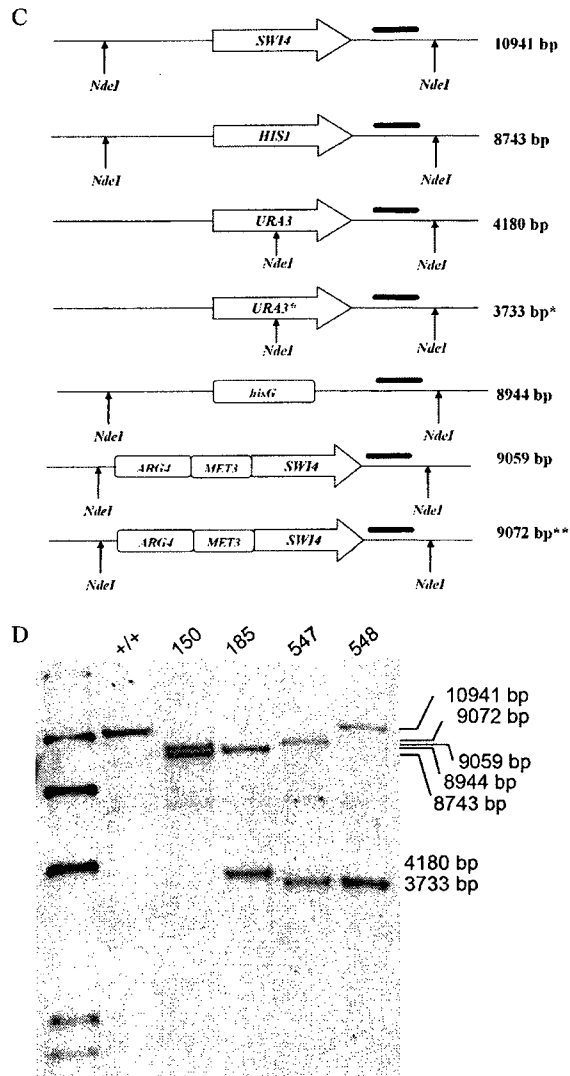


Figure 10. PCR and Southern screens confirming deletion and conditional *SWI4* strains. (A) Map and gel of PCR screen. (i) Gel 1 demonstrates a 5.4kb band for wildtype, and a 3.6kb band for *swi4Δ::URA3* or *swi4Δ::HIS1*. Gels 2 (ii) and 3 (iii) show a 2.0 kb or a 1.8kb bands for *swi4Δ::HIS1* or *swi4Δ::URA3*, respectively, using nesting oligos. (B) Map and gel of PCR screen for conditional strains, showing a 3.4kb band for *swi4Δ::hisG*, and a 8.6kb band for *MET3::SWI4*. (C) Map for Southern analysis indicating position of probe, and blot confirming construction of strains. (D) Digestion of gDNA with *NdeI* produced a wildtype band at 10.9 kb, a *swi4Δ::HIS1* band at 8.7 kb, and a *swi4Δ::URA3* band at 4.2 kb. Strain BH185 was positive. A *swi4Δ::hisG* band at 8.9 kb, and a 9.1 kb band for *MET3::SWI4* were shown in strain BH150. CB547, a separate conditional strain, demonstrated a 3.7 kb band for *swi4Δ::URA3* and a 9.1 kb band for *MET3::SWI4*. CB548 was negative. The difference between strains BH150 and CB547 is due to the fact that the *swi4Δ::URA3* deletion construct was produced with different oligos, CB122F,R resulting in a *swi4Δ::URA3* band at 3.7 kb. In addition, oligos CB123F,R were used to produce the *MET3::SWI4* replacement construct, resulting in a slightly larger band size at 9072 bp as opposed to the 9059 bp band representing *MET3::SWI4* produced using BH10F,R, BH11F,R, and BH12F,R.

Intriguingly, there were also a significant proportion of enlarged oval or rod-shaped cells that resembled opaque cells (Fig. 8, Table 6). In contrast, cells of control strain BH440 were in a normal yeast morphology (Fig. 8, Table 6). To confirm that the phenotypes were due to absence of *SWI4* or *SWI6*, the conditional strains BH150 (*swi4Δ::hisG/MET3::SWI4*) and CB552 (*swi6Δ::HIS1/MET3::SWI6*) were incubated in inducing medium overnight, diluted into fresh inducing or repressing medium, and incubated for 7 h. Under inducing conditions, strain BH150 produced normal yeast cells, while in repressing medium, the same range of phenotypes as observed in deletion strain BH185 were present (Fig. 11). Thus, absence of Swi4p has a dramatic effect on cell morphology and influences cell size. Strain CB552 grew as normal yeast cells under inducing conditions, but in repressing medium, only minor morphological defects were observed compared to the deletion strain, including wide or enlarged bud necks and an increase in cell size (Fig. 11). The disagreement between the deletion and conditional strain under repressing conditions is not due to transformant-specific behavior, since several transformants of each strain were examined and produced consistent phenotypes. Alternatively, the difference could reflect some leakiness of the *MET3* promoter at the *SWI6* locus.

Overall, these results suggest that Swi4p and Swi6p are important for proper yeast growth and morphogenesis, and may influence these processes in a similar manner. The increase in cell size in a proportion of cells suggests a delay in G1 phase and thus possible role for these factors in G1/S regulation. In comparison, *SWI4* null mutants in *S. cerevisiae* show cell enlargement, defects in bud emergence and slow growth, but do not

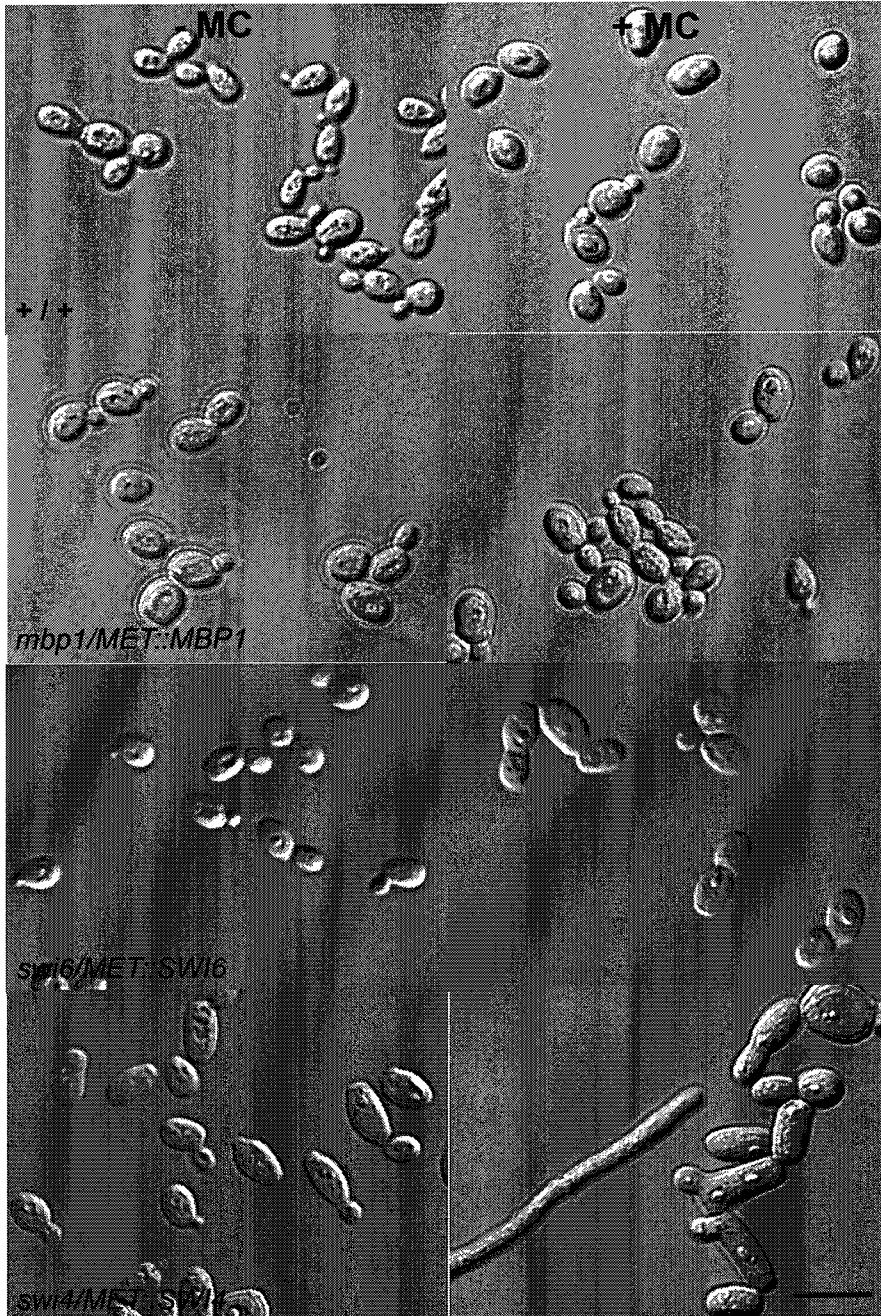


Figure 11. Repression of *MBP1* or *SWI4* produced phenotypes similar to deletion of the genes, in contrast to repression of *SWI6*.

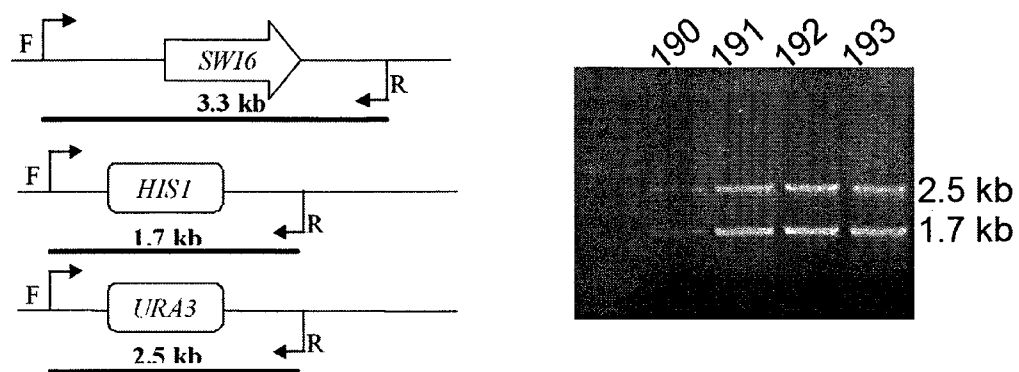
Strains KmCa4 (*mbp1* Δ ::*URA3/MET3*::*MBP1*), CB557 (*swi6* Δ ::*URA3/MET3*::*SWI6*), and CB547 (*swi4* Δ ::*URA3/MET3*::*SWI4*) were incubated in inducing medium overnight, then diluted into fresh inducing (-MC) or repressing medium (+MC) and incubated for 5-7h at 30°C. Bar: 10 μ m.

produce filaments (85, 86), while *SWI6* null mutants were enlarged and elongated, but did not form filaments or switch cell fate, as seen in *C. albicans* cells lacking *SWI6* (33, 87, 88). Since cells can divide without Swi6p in *C. albicans*, comparable to those lacking Swi6p in *S. cerevisiae* (87, 89) but in contrast to absence of Cdc10p in *S. pombe* (34, 90), other factors must be capable of activating Swi4p and possibly Mbp1p.

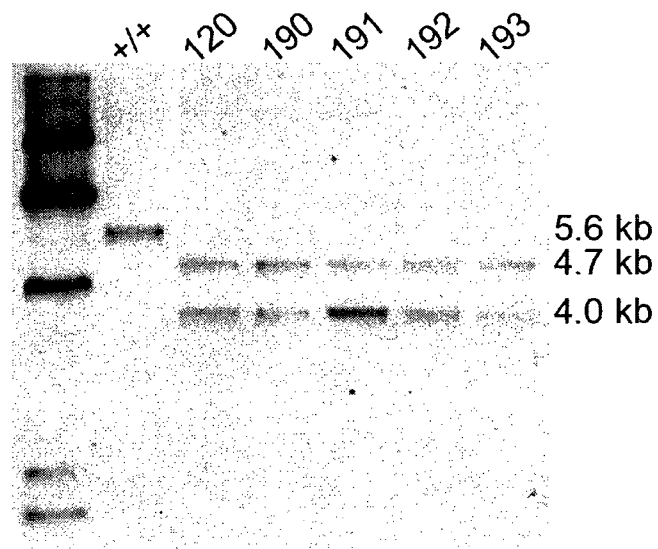
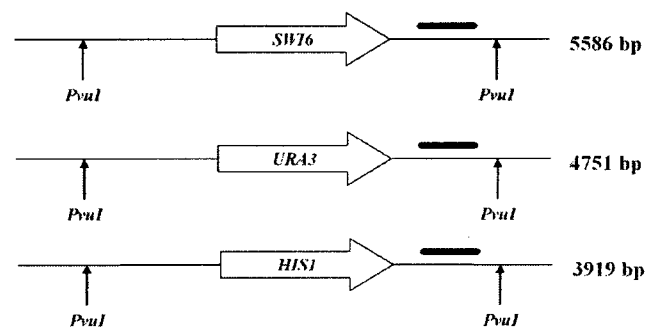
3.2.3 Cells lacking both *SWI6* and *SWI4* are viable and show enhanced filamentous growth but do not resemble cells depleted of Cln3p

In order to further explore the potential role of SBF/MBF factors in G1/S regulation in *C. albicans*, strains lacking both *SWI6* and *SWI4* were constructed. In *S. cerevisiae*, a *swi4/swi6* double mutant is lethal, inferring that SBF is crucial for cell cycle entry (33, 84). To create a strain lacking Swi6p and Swi4p, one allele of *SWI4* was substituted with the *hisG-URA3-hisG* cassette. Following loop out of *URA3*, alleles of *SWI6* were replaced with *URA3* and *HIS1* markers, while the remaining copy of *SWI4* was placed under control the *MET3* promoter containing an *ARG* marker (Fig. 6), creating strain BH190. An isogenic control strain, BH420, was created by transforming strain BWP17 with pRM100 and pBS-Ca*ARG4* plasmids. Strains were confirmed with PCR and by Southern Blot analysis (Fig. 12).

A



B



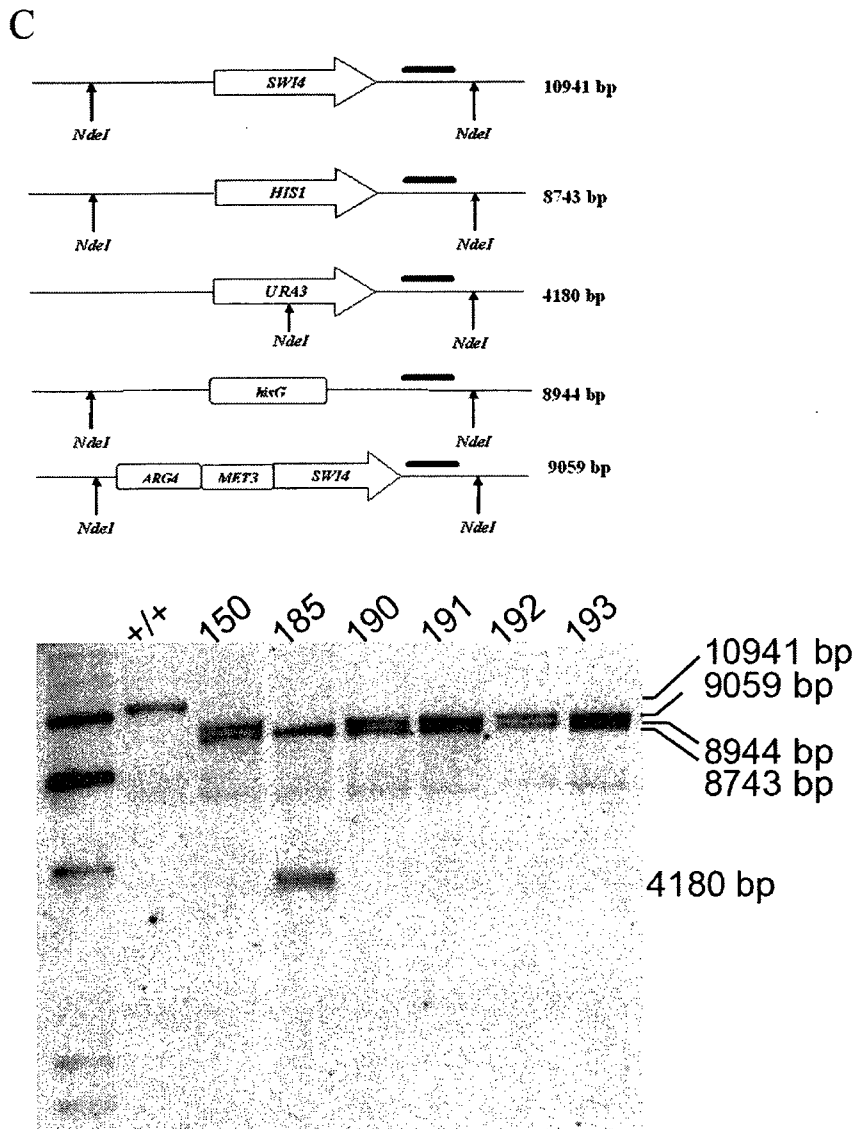


Figure 12. PCR and Southern screens confirming *swi4/swi6* double mutant strains. (A) Map and gel of PCR screen of strains deleted for *SWI6* showing a 2.5 kb band for *swi6Δ::URA3*, a 1.7 kb band for *swi6Δ::HIS1*, and a 3.3 kb band for *SWI6*. (B) Map for Southern analysis indicating position of probe, and blot confirming construction of strains. Digestion of gDNA with *PvuI* produced a wildtype band at 5.6 Kb, a *swi6Δ::HIS1* band at 4.0 Kb, and a *swi6Δ::URA3* band at 4.7 kb. BH190-193 are positive, and BH120 (*swi6::HIS1/swi6::URA3*) is included for comparison (C) Map for Southern analysis indicating position of probe, and blot confirming deletion of one *SWI4* allele, and replacement of the other with the *MET3* promoter. Digestion of gDNA with *NdeI* produced a wildtype band at 10.9 kb, a *swi4Δ::hisG* band at 8.9 kb, and a *MET3::SWI4* band at 9.1 kb. Strains BH150 (*swi4Δ::hisG/MET3::SWI4*) and BH185 (*swi4Δ::HIS1/swi4Δ::URA3*) were included for comparison.

In order to determine the phenotype of cells lacking *SWI4* and *SWI6*, strains BH190 and BH420 were incubated on solid inducing (-MC) or repressing (+MC) medium at 30°C for 72 h. Both strains formed colonies on inducing and repressing medium (data not shown), suggesting that cells from strain BH190 were viable. To explore the phenotype further, strains were grown overnight in inducing medium at 30°C, then diluted into fresh inducing or repressing medium for 7 h at 30°C. The overnight culture of strain BH190 in inducing medium consisted of the same range of phenotypes described for the *SWI4* or *SWI6* deletion strains, but with a higher proportion of enlarged and elongated yeast cells (Table 6, Fig. 13). After 7 h in repressing medium, the proportion of enlarged cells decreased while there was an increase in the number of rod-shaped and rectangular cells. In addition, an almost ten-fold increase in the number of filamentous cells, including pseudohyphae, chains of elongated cells, and hyphae, was observed (Table 6, Fig. 13). Furthermore, a significant number of rod or oval-shaped cells showed a multi-budding phenotype, suggesting defects in cell separation, with the buds maintaining polarized growth. Finally, many chained cells were composed of a variety of cell types (Fig. 13). DAPI staining demonstrated that most cells contained a normal number of nuclei, supporting the notion that cells were not blocked in G1 phase, (Fig. 14), although some examples of multinucleation were observed. The enlarged size, however, suggests a delay in G1 phase. In comparison, cells of control strain BH420 were in a normal yeast form. Thus, Swi4p and Swi6p are important, but not essential, for G1/S progression and in mediating a portion of Cln3p activity. That they have a synergistic effect on morphogenesis suggests that these factors may act in additional, separate pathways.

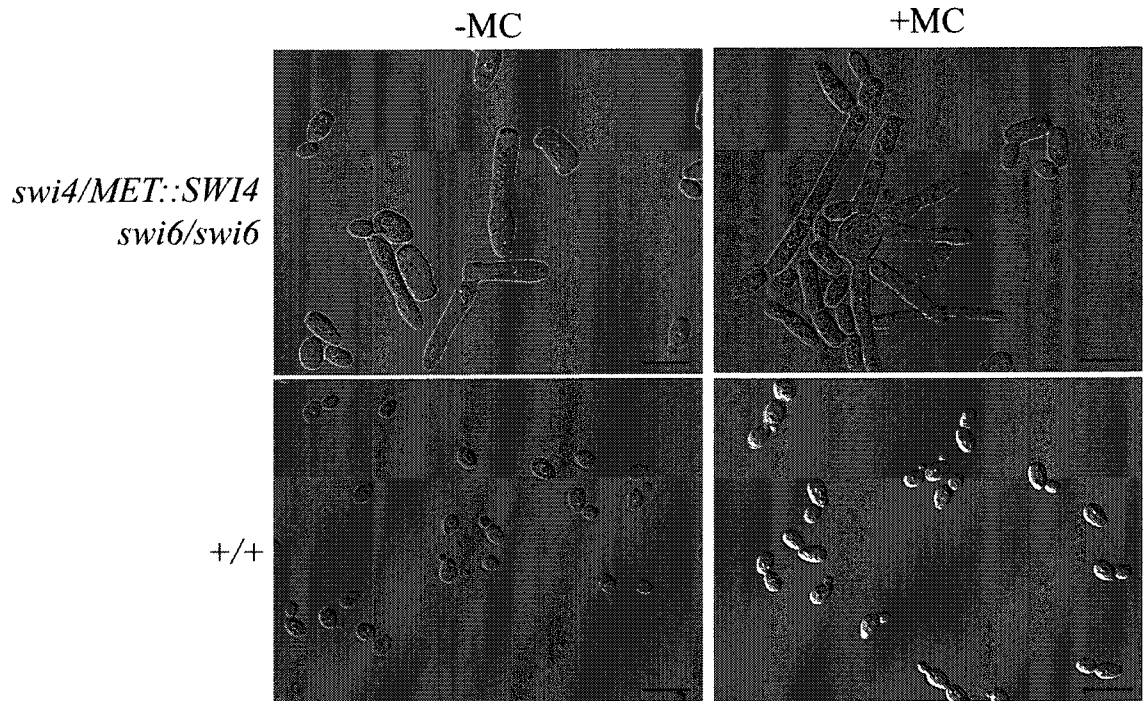


Figure 13. Cells lacking both *SWI4* and *SWI6* under yeast growth conditions do not arrest in G1 phase and show enhanced filamentation and rod/oval-shaped cells compared to the single mutants. Cells from strain BH190 (*swi6* Δ ::*URA3/swi6* Δ ::*HIS1*, *swi4* Δ ::*hisG/MET3*::*SWI4*) and wild type strain BH420 (*SWI6/SWI6*, *SWI4/SWI4*) were incubated in inducing medium overnight, diluted into SD inducing (-MC) or repressing medium (+MC) and incubated for 7 h at 30°C.

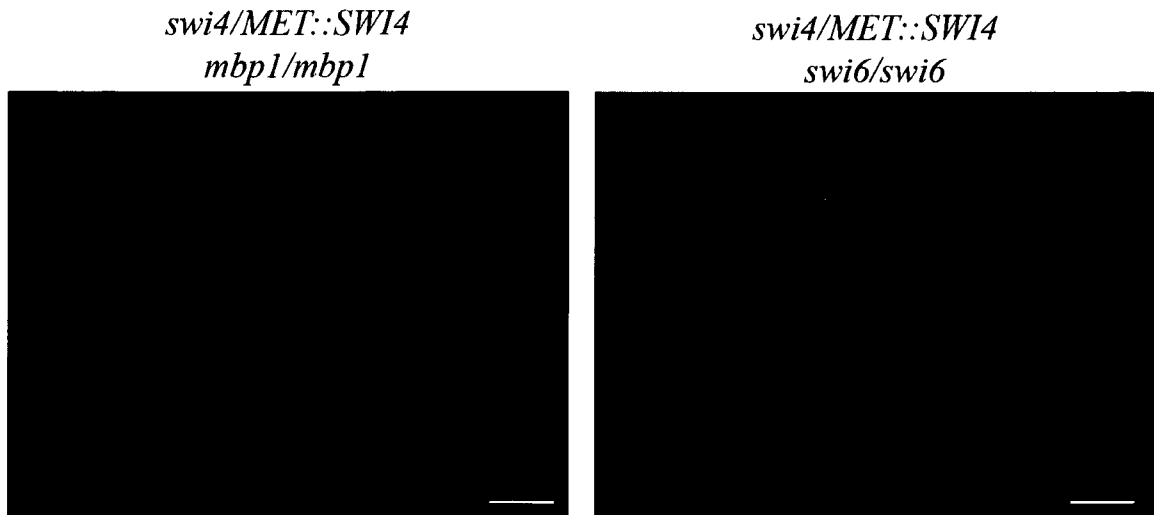


Figure 14. Cells lacking *SWI4* and *SWI6* or *SWI4* and *MBP1* under yeast growth conditions undergo nuclear division. Cells from strains BH190 (*swi6::URA3/swi6::HIS1*, *swi4::hisG/MET3::SWI4*) and BH276 (*mbp1::URA3/mbp1::HIS1*, *swi4::hisG/MET3::SWI4*) were incubated in SD repressing medium (+MC) for 7 h, then fixed and stained with DAPI and Calcofluor. Bar: 10 μ m.

3.2.4 Cells lacking *MBP1* and *SWI4* resemble the *swi4* deletion strain and do not arrest in G1 phase.

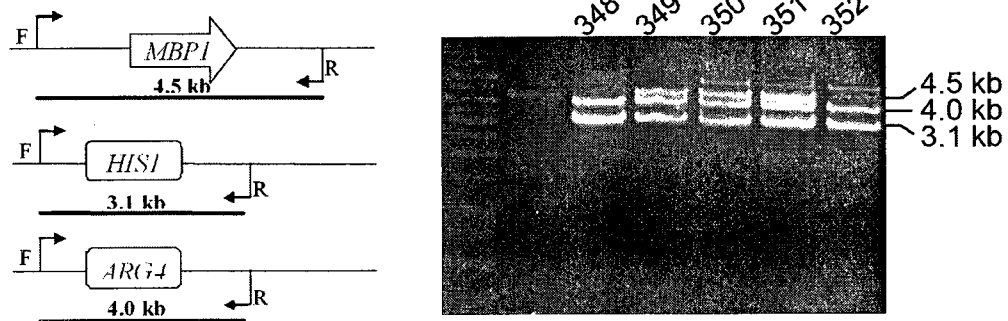
We next wanted to determine whether both DNA-binding components, Swi4p and Mbp1p, were essential for the G1/S transition, as seen in *S. cerevisiae*. To investigate this possibility, alleles of *MBP1* were replaced with *URA3* and *HIS1* markers in a *swi4Δ::hisG/SWI4*, *MBP1/MBP1* strain, while the remaining allele of *SWI4* was placed under control of the *MET3* promoter (Fig. 6). The resulting strain BH277 was confirmed by PCR and Southern Blot analyses (Fig. 15D, E).

Cells of strain BH277 and control strain BH420 were plated on solid repressing or inducing medium for 72 h at 30°C. Surprisingly, strain BH277 grew under repressing conditions, much like that seen under inducing conditions and in comparison to control strain BH420, although some of the colonies were wrinkled in appearance (data not shown). To investigate the cell phenotype, the strains were subsequently grown overnight in inducing medium, then diluted into inducing or repressing medium and incubated for 7 h at 30°C (Fig. 16A). Prior to shutting off *SWI4*, strain BH277 was predominantly composed of normal yeast cells, with only 8.5% showing an extended or enlarged phenotype, in agreement with the *MBP1* deletion strain (Table 6). After shutting off *SWI4*, however, cells were viable and did not arrest in G1 phase, in contrast to that seen in *S. cerevisiae* (87). Alternatively, a pleiotropic phenotype as seen with the *SWI4* deletion strain BH185 was observed, including enlarged, and rod-shaped yeast, as well as some pseudohyphae (Table 6, Fig. 16A). However, the proportions of different cell phenotypes were not as high as that seen in strain BH185 (Table 6), which could be due to some leakiness of the *MET3* promoter. DAPI staining showed that most cells

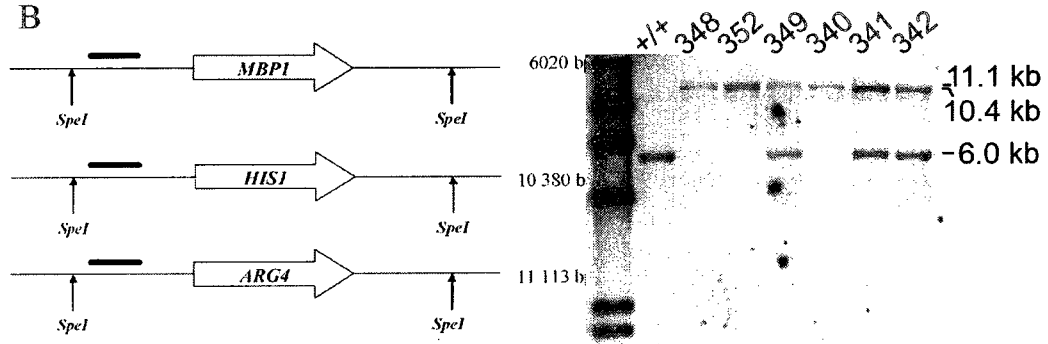
contained a normal number of nuclei, indicating successful progression through the cell cycle (Fig. 14). However, the increase in cell size suggests a delay in G1 phase (Fig. 16A). Thus, Swi4p and Mbp1p are not essential for the G1/S progression, in contrast to that seen in *S. cerevisiae*. Since absence of both factors did not result in a major synergistic effect on morphogenesis or growth, the data further support the notion that Mbp1p's contribution to growth control at the G1/S transition is minor, at best.

In order to confirm the shut-off phenotype, a strain was constructed where both copies of *SWI4* and *MBP1* were deleted. *MBP1* alleles were replaced with the *HIS1* and *ARG4* markers. One allele of *SWI4* was replaced with a *hisG-URA3-hisG* cassette, and after looping out of the *URA3* marker, the remaining copy was replaced with the *URA3* marker. The resulting strains were confirmed by PCR and Southern analysis (Fig. 15A, B, C) and strain BH348 was used for subsequent analysis. When incubated on solid medium for 72 h at 30°C, strain BH348 was viable, and formed some wrinkled, puckered colonies compared to the smooth and round colonies of control strain BH420 (data not shown). After growing in liquid medium for 7 h, strain BH348 exhibited the same variety of cell morphologies as noted in strain BH277 under repressing conditions, though there was a marked increase in the proportion of enlarged yeast, suggesting a slower rate of proliferation (Fig. 16B). The surprising lack of a G1 phase arrest and generation of only a small proportion of hyphal cells compared to Cln3p-depleted cells further suggest that Swi4p and Mbp1p are not essential for cell proliferation, and highlight that Swi4p and Swi6p mediate only a portion of the essential Cln3p activity at the G1/S transition, unlike that seen in *S. cerevisiae* and in *S. pombe* (91, 92).

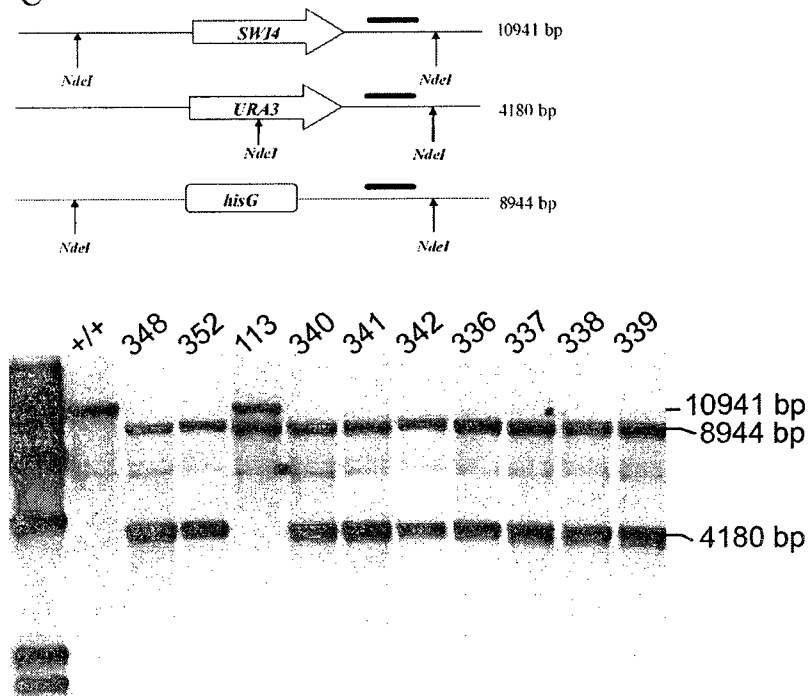
A



B



C



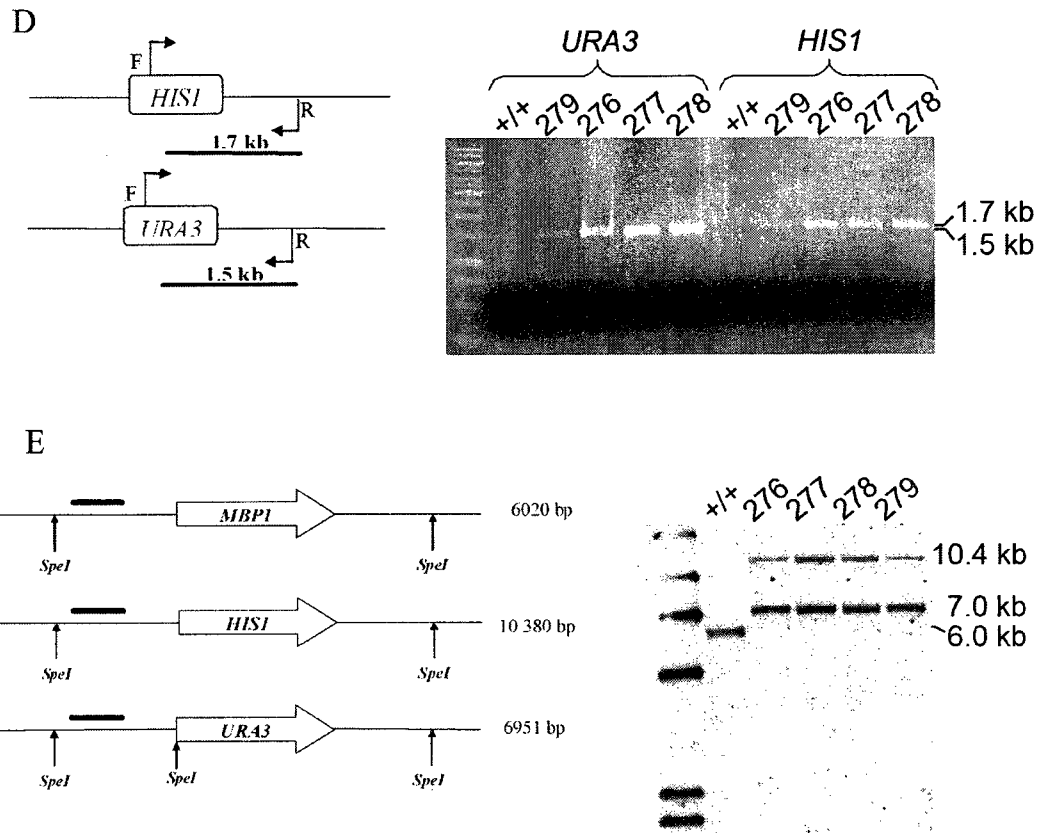


Figure 15. PCR and Southern screens confirming *swi4/mbp1* double mutant strains

(A) Map and gel of PCR screen of deletion of *MBP1*, showing a 3.1 kb band for *mbp1Δ::HIS1*, a 4.0 Kb band for *mbp1Δ::ARG4*, and a 4.5 kb band for *MBP1*. **(B)** Map for Southern analysis indicating position of probe, and blot confirming construction of strains. Digestion of gDNA with *SpeI* produced a wildtype band at 6.0 kb, an *mbp1Δ::HIS1* band at 10.4 kb, and an *mbp1Δ::ARG4* band at 11.1 kb. BH348 and BH352 were positive, while BH349 was negative. Strains BH341 and BH342 were confirmed as positive *MBP1* heterozygotes (*swi4Δ::hisG/swi4Δ::URA3 mbp1Δ::HIS1/MBP1*). **(C)** Map for Southern analysis of *SWI4* and blot confirming strains, indicating position of probe used. gDNA digested with *SpeI*, produced a wildtype band at 10.9 kb, a *swi4Δ::hisG* band at 8.9 kb and a *swi4Δ::URA3* band at 4.2 kb band, confirming strains BH348 and BH352. For comparison strains BH113 (*swi4Δ::hisG/SWI4*) and BH336-339 (*swi4Δ::hisG/swi4Δ::URA3*) were included. **(D)** Map and gel of PCR screens of *MBP1*. For *mbp1Δ::HIS1/mbp1Δ::URA3*, *swi4Δ::hisG/MET3::SWI4* strains, CaURA3F or CaHIS1F nested oligonucleotides were used with BH32R. A 1.5 kb band represents *mbp1Δ::URA3*, while a 1.7 kb band represents *mbp1Δ::HIS1*. **(E)** Map for Southern analysis of *MBP1* indicating position of probe, and blot confirming construction of strains. Digestion of gDNA with *SpeI* produced a wildtype band at 6.0 kb, an *mbp1Δ::HIS1* band at 10.4 kb, and an *mbp1Δ::URA3* band at 7.0 kb, confirming the *mbp1* deletion in strains BH276-279.

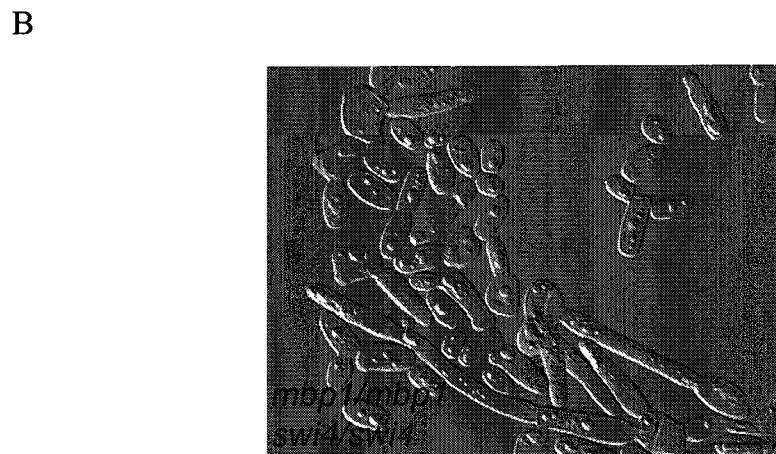
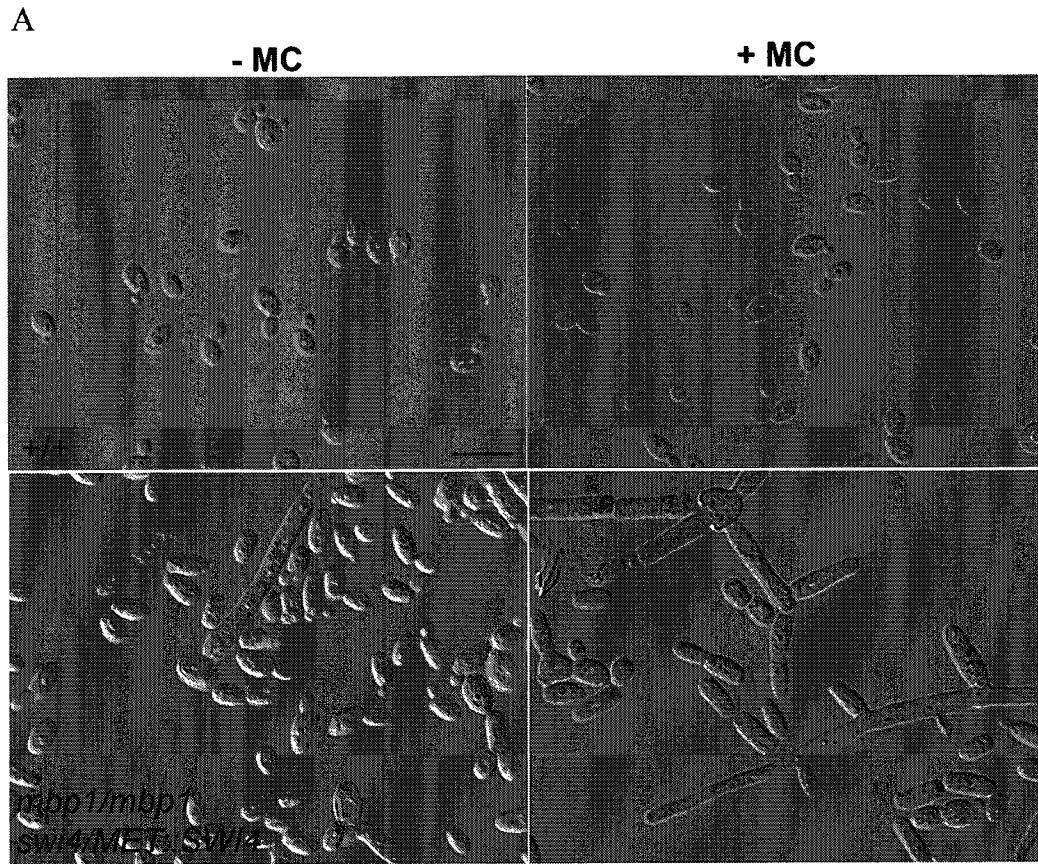


Figure 16. Cells lacking *SWI4* and *MBP1* do not arrest in G1 phase, and resemble the *swi4* deletion strain. (A) Cells from conditional strain BH276 (*mbp1* Δ ::*URA3/mbp1* Δ ::*HIS1*, *swi4* Δ ::*hisG/MET3*::*SWI4*) and wild type strain BH420 (*MBP1/MBP1*, *SWI4/SWI4*) were incubated in inducing medium overnight, then diluted into fresh inducing (-MC) or repressing medium (+MC) for 7h at 30°C. (B) The double deletion strain BH348 (*mbp1* Δ ::*HIS1/mbp1* Δ ::*ARG4*, *swi4*::*hisG/swi4* Δ ::*URA3*), was incubated in minimal medium for 7h at 30°C. Bar: 10 μ m.

3.2.5 Transcription profiles of cells lacking *SWI6* and *SWI4* support a role for these factors in regulating G1/S progression and in mediating a part of Cln3p activity

In order to identify potential targets of Swi6p/Swi4p and further characterize cells lacking these factors, transcription profiles of cells depleted of Swi6p and Swi4p were obtained. Strains BH190 and BH420 were incubated in inducing medium overnight, diluted into repressing medium, incubated for 7 h at 30°C, and processed for microarray analysis. From 4 independent samples, 1108 significantly modulated genes were obtained, based on a 1.5 fold cut-off and p-value <0.05 (Fig. 17A, B).

Expression patterns were enriched for repression of genes involved in cell cycle functions, DNA replication/repair, chromatin remodeling, ribosome biosynthesis and translation. For example, genes constituting putative SBF/MBF targets, such as *CUP9* and *HCM1* (71), other cell cycle-associated factors with putative roles in G1 and S phase including *WHI3*, *PES1*, *CDC6*, *FKH2*, *CTR9*, and numerous RSC complex genes, TAF's, and RNA helicases, were repressed (Tables 7, S7). The G1 cyclin orthologues *PCL2* and *CCN1* (44) were not modulated, but Northern analysis confirmed their repression in strains BH190 (C. Bachewich, unpublished data). Some genes associated with DNA synthesis and repair were mildly induced, including *RNR1*, *DUN1*, *RAD16*, 53 and 57, (Tables 8, S8) but this could be due to the fact that cells were pleiotropic in nature. The overall expression patterns were consistent with a delay in G1 phase taking place, and provide additional evidence that Swi6p and Swi4p contribute to the G1/S regulatory circuit in *C. albicans*. TOR-dependent nutrient response regulators of cell size and proliferation, including *SFP1* and *SCH9*, were also repressed (Tables 7, S7). Additional expression patterns related to G1/S regulation included the unexpected induction of the

putative SBF target *YOX1*, and the cyclin *CLN3* (Tables 8, S8). *YOX1* is activated by SBF in *S. cerevisiae*, and contributes to the negative regulation of *CLN3* expression (71, 93). Comparing the significantly modulated genes with those depleted of Cln3p for 6 h (Fig. 17D) demonstrated a similar number of up-regulated genes, but 2.5 times more down-regulated genes under either condition than repressed genes in common. This suggests that Swi4p and Swi6p may have regulatory inputs other than Cln3p, and additional functions which may not be restricted to G1/S, as seen from the pleiotropic mutant phenotypes. Thus, the data further suggest re-wiring in the G1/S circuit of *C. albicans* compared to *S. cerevisiae* (Fig. 18). A large group of modulated genes included the unknowns, which has important implications for future drug target discovery if any are found to be required for controlling growth.

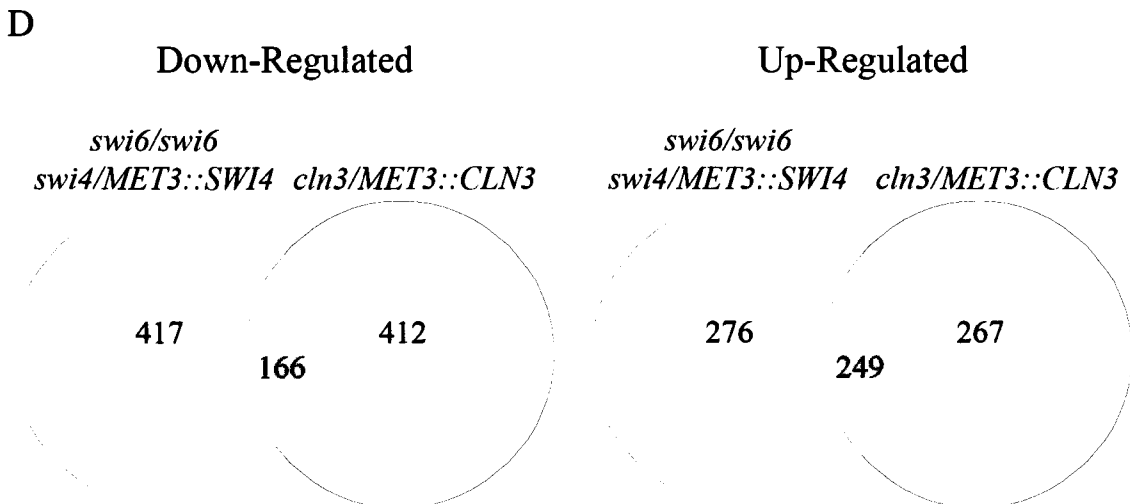
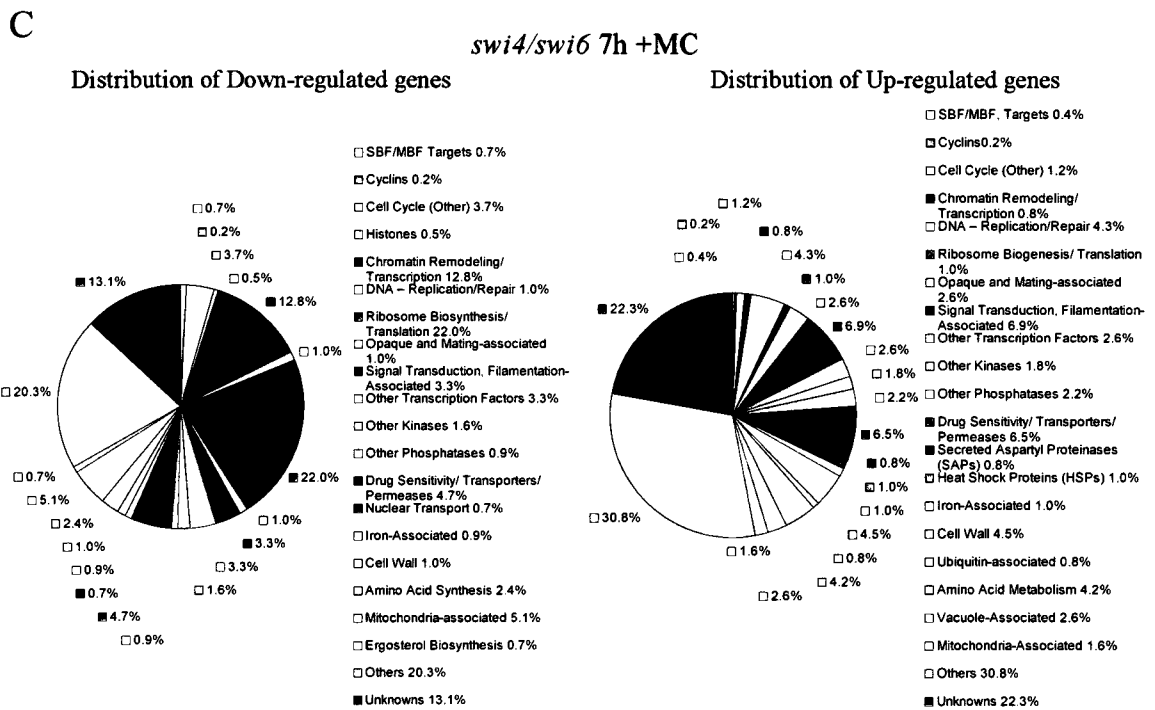
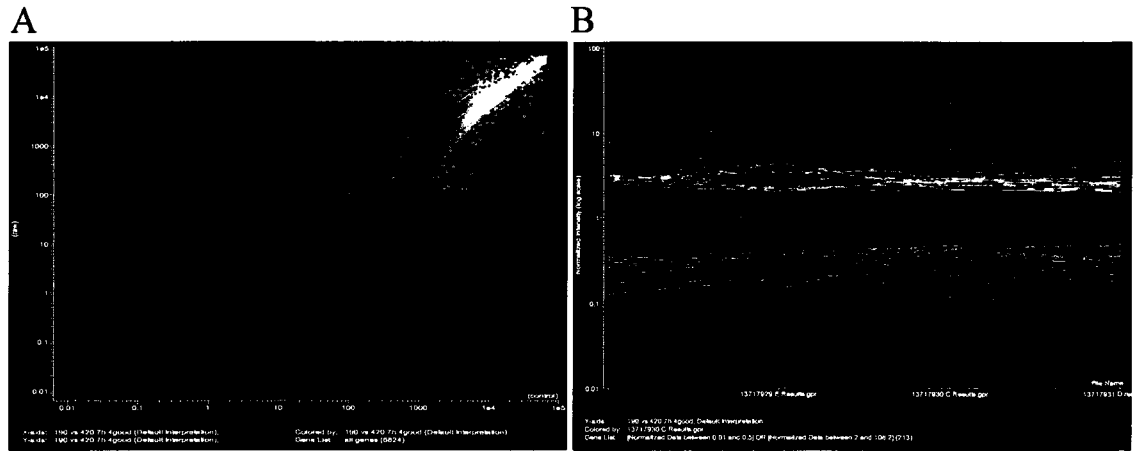


Figure 17. Distribution of significantly modulated genes in Swi4p and Swi6p-depleted cells. (A) Strain BH190 (*swi4Δ::hisG/MET3::SWI4*, *swi6Δ::HIS1/swi6Δ::URA3*, experimental), and strain BH420 (*SWI4/SWI4*, *SWI6/SWI6*, control), were incubated in repressing medium (+MC) for 7 h, and processed for microarray analysis. Scatterplot shows total data collected from 4 microarray chips representing 4 separate samples. (B) Normalized intensities (Lowess) of significantly modulated genes (1.5 fold cut-off, t-test, $p < 0.05$; Genespring software), across the 4 chips showing consistency in the data obtained. (C) Pie charts showing distribution of significantly modulated genes grouped into functional categories. Gene names and function were identified through Genespring analysis, and manually verified using the *Candida* Genome Database (CGD) at <http://candidagenome.org/>. Different colours represent different categories, as indicated. Genes were categorized according to a single function, although some genes may have several functions. Full categorized gene list in Appendix Tables S7, S8. (D) VENN diagrams showing the proportion of genes significantly modulated in cells depleted of Cln3p for 6 h, versus those lacking Swi4p and Swi6p at 7 h.

Table 7: Select genes down-regulated in cells lacking Swi4p and Swi6p.¹

Functional Category	Common Gene Name
SBF/MBF-associated	<i>SWI4, SWI6, CUP9, HCM1</i>
Cyclins	<i>BUR2</i>
Cell Cycle (Other)	<i>PES1, WHI3, FKH2, CTR9, CDC6, MCM1, SAP190, GIN4, CDC14, TUB4</i>
Histones	<i>HHO1, HTB2, HTA3</i>
DNA Repair/Replication	<i>POL5, TOP1, HMII, SIM1, PAN3</i>
Chromatin Remodeling	<i>RSC4, RSC58, RSC9, RSC2, RSC8, SDC1, SWD1, ISW2, ITC1, ASH2, NPL6, TAF60, TAF5, TAF12</i>
Ribosome Biogenesis/Translation	<i>NOC2, NOC3, NOC4, NOG1, NOG2, NOP2, NOP4, NOP5, NOP8, NOP13, NOP14, NOP15, UTP15, UTP4, UTP5, RRS1, RPF1, RPF2, RPL39, RRP6, RRP7, RRP8, RRP12, CFT1, ERF1, SUI3, CDC33, TIF3, NIP1, SUI3</i>
Opaque-associated	<i>CTA9, MDN1, HBR1, ARG11, NCE103</i>
Hyphal: Filamentation-associated	<i>GIS2, ENP1, EAP1, RBE1</i>
Hyphal: Regulatory	<i>CZF1, SWI1, PDE2, GCN4, TPK1, MNL1, HAP5, FGR39, FGR50</i>
TOR pathway-associated	<i>SFP1, SCH9, TBF1</i>
Drug sensitivity, transporters	<i>PDR16, FLU1, TPO4,5, ALP1, GAP1, OPT9</i>
Iron-regulated, uptake	<i>YAH1, PGA6, HEM3, HBR1, HBR3</i>

¹Experimental (*swi4/MET3::SWI4*; *swi6/swi6*) and prototrophic control cells (*SWI4/SWI4 SWI6/SWI6*) were incubated in repressing medium (2.5mM Methionine, 0.5mM Cysteine) for 7 h. results are based on 4 microarray chips representing 4 separate samples. Significant genes were selected based upon a 1.5 fold cut-off, and t-test function with $p < 0.05$. For full gene list with fold changes, see Appendix Table S7

Table 8: Select genes up-regulated in cells lacking Swi4p and Swi6p.¹

Functional Category	Common Gene Name
SBF/MBF-associated	<i>YOX1, STB1</i>
Cyclins	<i>CLN3</i>
Cell Cycle (Other)	<i>SOL1, SLK19, BUB3</i>
DNA Repair/Replication	<i>RNR1, DUN1, PMS1, RAD16, RAD57, RAD51, RAD53, MPH1, MGT1, CDC8, SMC6, MLH1, HSM3</i>
Opaque, Mating-associated	<i>ORF19.2202, CMK1, FAV1, IFF6, ALD6, IDP2, ORF19.2506, SOD4, MDH1, SAP8, ORF19.3461</i>
Hyphal: Filamentation-associated	<i>RBT1, RBR1, RBR2, HWP1, IHD1, IHD2, PCK1, PHR1, CHK1, ERV25, PBI2, ALS2, ALS4, ORF19.4749, ORF19.2903</i>
Hyphal: Regulatory	<i>TFS1, RFX2, ASR1, RD11, KIC1, CHK1, FGR51, STE13, ORF19.4459</i>
Drug sensitivity, transporters	<i>CDR1, TAC1, OPT4, OPT6</i>
Iron-regulated, uptake	<i>CFL5, SAP99, SAP10, HSP12, ALS2, FET31, SIT4, SMF3, HOL4</i>

¹Experimental (*swi4/MET3::SWI4*; *swi6/swi6*) and prototrophic control cells (*SWI4/SWI4 SWI6/SWI6*) were incubated in repressing medium (2.5mM Methionine, 0.5mM Cysteine) for 7 h. Data is based on 4 microarray chips representing 4 separate samples. Significant genes were selected based upon a 1.5 fold cut-off, and t-test function with $p < 0.05$. For full gene list with fold changes, see Appendix Table S8

3.3 Cells lacking Swi4p and Swi6p show opaque cell features

Intriguingly, the transcription profiles demonstrated that a large number of opaque-associated genes were also modulated (Tables 7, 8, S7, S8; Fig. 17C), albeit both up and down. Although classic opaque phase cell markers were not induced, such as *CDR3*, *HBR1*, which negatively regulates the opaque state through unknown means, was mildly repressed. In addition, the white-specific gene *WH11* was paradoxically highly induced relative to the control yeast cells, as in *Cln3p*-depleted cells, but this expression pattern could reflect opaque cells switching back to the white phase (79). Since many of the *swi6/swi4* cells resembled oblong, opaque cells, the data suggests that Swi4p/Swi6p function may contribute to the regulation of the opaque state, or at least a subset of its features, implying that the G1/S transition is linked to more than one developmental pathway in a manner that can be uncoupled from the normal environmental controls (Fig. 18).

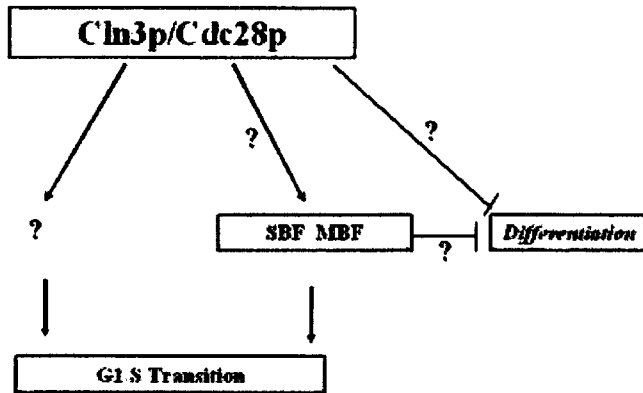


Figure 18. Potential model for the role of Cln3p in the regulation of the G1/S circuit and differentiation in *Candida albicans*.

Since the *swi4/swi6* double mutants were viable, and the phenotype did not closely resemble that of Cln3p-depleted cells, SBF/MBF may not be the only major targets of Cln3p function. In addition, transcription profiles of cells lacking Cln3p demonstrated only partial overlap with those of Swi4p and Swi6p-depleted cells. The phenotypes of cells lacking Swi4p and Swi6p suggest that these factors may be important for regulating development of different cell types, possibly including opaque cells, further demonstrated by the modulation of opaque or mating-associated genes. Thus, regulation of G1 phase in *C. albicans* involves some unique features, and may be linked to different differentiation pathways through Swi6p, Swi4p and Cln3p function.

3.4 Cells lacking *SWI4*, *SWI6* or *MBP1* can form hyphae in response to serum

Since Swi6p and Swi4p significantly influence morphology and growth of yeast cells, we next asked if they played similar roles in environmentally-induced hyphae. Cells from strains BH120 (*swi6Δ::HIS1/swi6Δ::URA3*), BH185 (*swi4Δ::HIS1/swi4Δ::URA3*), BH261 (*mbp1Δ::HIS1/mbp1Δ::URA3*), BH190 (*swi6Δ::URA3/swi6Δ::HIS1, swi4Δ::hisG/MET3::SWI4*), BH276 (*mbp1Δ::URA3/mbp1Δ::HIS1, swi4Δ::hisG/MET3::SWI4*), and the prototrophic control strain BH420 were incubated in SD inducing medium (-MC) overnight, diluted into repressing medium supplemented with 10% serum, incubated for 3.5 h at 37°C, fixed and then stained with DAPI (Fig. 19). Cells lacking *SWI4*, *SWI6*, or *MBP1* formed normal hyphae (Fig. 19) with divided nuclei (data not shown) in the presence of serum, although the actual dynamics of nuclear division and septation were not quantified. Strains lacking *SWI4* and *SWI6* or *SWI4* and *MBP1* also formed hyphae, but many cells appeared to be slightly enlarged, particularly when originating from misshapen parent yeast cells (Fig. 19). Thus, Swi6p and Swi4p are not essential for the cell cycle in hyphal cells, but appear to influence G1/S progression, as seen with an increase in cell size, and morphogenesis.

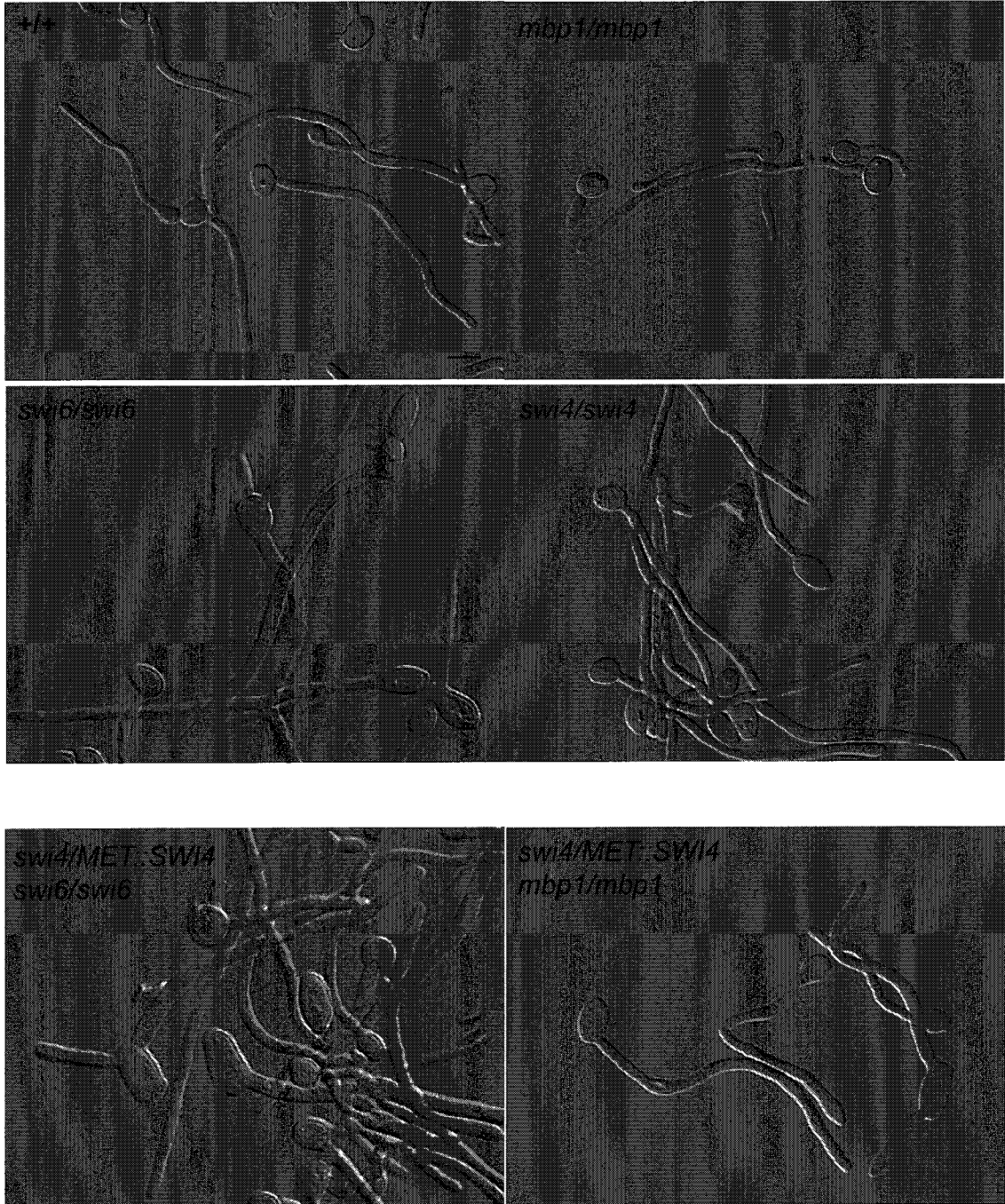


Figure 19. Cells lacking *SWI4*, *SWI6* or *MBP1* can form hyphae in the presence of serum. Cells from strains BH120 (*swi6Δ::HIS1/swi6Δ::URA3*), BH185 (*swi4Δ::HIS1/swi4Δ::URA3*), BH261 (*mbp1Δ::HIS1/mbp1Δ::URA3*), BH190 (*swi6::URA3/swi6::HIS1*, *swi4::hisG/MET3::SWI4*) and BH276 (*mbp1::URA3/mbp1::HIS1*, *swi4::hisG/MET3::SWI4*) were incubated in SD inducing medium (-MC) overnight, then diluted into repressing medium supplement with 10% serum, and incubated for 3.5 h at 37°C. Bar: 10 μm.

4. Discussion

4.1 Transcription profiles of Cln3p-depleted cells support the occurrence of a G1 phase arrest and provide a framework for the G1/S circuit and associated factors.

Little is known about the G1/S regulatory circuit in *C. albicans* due to the poor characterization of the cell cycle in this organism. Transcription plays a critical regulatory role at the transition in most organisms, cell-cycle dependent transcription profiles of *C. albicans* white phase have not been obtained due to problems with synchronization (44, 94). Transcription profiles of synchronized opaque phase yeast cells of *C. albicans* were recently reported (94), and when compared with the model yeast *S. cerevisiae* ((71), a basic outline of the G1/S circuit could be inferred. However, functional studies on individual components and their interactions with other players are lacking. In addition, it is not clear that the regulatory circuit would be identical to that in white phase yeast cells, since aspects of cell cycle regulation can differ between cell types of *C. albicans*, including yeast and hyphae, for example ((47, 95). To address these issues, and screen for potential G1/S-associated genes and putative targets of Cln3p activity in white phase yeast, we obtained transcription profiles of cells lacking Cln3p. Although a large number of genes were modulated, and not all were expected to be functionally important due to secondary responses to Cln3p depletion, the data uncovered several significant expression patterns that highlighted important features of the cells and the putative G1/S circuit.

First, the expression profiles supported the notion that cells lacking Cln3p were arrested in G1 phase, based on repression of cyclins, histones, SBF/MBF putative targets,

and DNA replication factors. Although this was previously inferred from the cell phenotype, the precise arrest stage had not been validated by other methods. Thus, the data confirms that Cln3p is essential for the G1/S transition in *C. albicans* yeast cells. In contrast, Cln3p is not essential in *S. cerevisiae*, due to the additional function of Bck2p, which *C. albicans* lacks, in activating Start (96-98).

Second, although not inclusive, the data provide the first comprehensive picture of factors associated with G1/S phase in white yeast cells of *C. albicans*. For example, in *S. cerevisiae* and higher organisms, SBF/MBF or E2F, respectively, control the initial burst in transcription at G1/S through regulating numerous downstream transcription factors (29, 71). In *C. albicans* cells depleted of Cln3p, homologues of SBF/MBF targets, including *TOS4*, *HCM1*, *TOS8*, and *YOX1*, were repressed, consistent with a role in the G1/S transition. Targets of SBF/MBF in *S. cerevisiae* regulate additional transcription factors (71), homologues of which were also repressed in Cln3p-depleted cells, including *GCN4*, *FKH2*, *STB1* and *RME1*, which function in a diversity of processes, including DNA replication/repair and chromatin remodeling (99, 100). Another essential function of SBF/MBF and E2F is to activate G1 cyclins, including *CLN1* and *CLN2* or Cyclin E, respectively (29, 31, 87, 101). The importance of this step is underscored by the fact that overexpression of *CLN2* can rescue a G1 phase arrest (102, 103). In *C. albicans*, equivalent G1 cyclins have not been confirmed, but our expression data supports the suggestion that the *CLN1* homologue *CCN1* and the cyclin *PCL2* are candidates, since both were repressed during Cln3p depletion (47). On the other hand, the *CLN2* homologue *HGC1*, which is required for hyphal growth and does not have any known cell cycle function in yeast (42), was not repressed. Additional evidence

supporting a role for Ccn1p and Pcl2p in the G1/S circuit could be obtained by determining whether overexpression of either partially suppresses the G1 phase arrest upon Cln3p depletion, allowing re-entry into the yeast cell cycle. Ultimately, chIP-chip or chIP-seq coupled with additional functional studies on individual players will determine the relationships between the various factors and levels of regulation within the G1/S circuit.

The transcription profiling data suggests that the basic structure of the G1/S circuit shares a high degree of similarity with that of *S. cerevisiae*, but also contains several unique features. For example, many genes of unknown function, often *C. albicans*-specific, constituted one of the largest group of modulated factors during Cln3p depletion. Since several of these were repressed or induced as early as 1 h Cln3p depletion, they could potentially play a role in G1/S control. In agreement with this, many of these genes were transcriptionally regulated in a G1/S-dependent manner in opaque phase cells (94). It will thus be informative to explore these genes further, since the identification of novel factors required for controlling growth of the pathogen has important implications for new drug target discovery. A second unique feature of the G1/S circuit highlighted by the transcription profiling data was the observation that the CDK *CDC28* and the SBF/MBF components *SWI6* and *MBP1* were repressed in Cln3p-depleted cells, suggesting that these factors may be transcriptionally regulated at G1/S, in contrast to that seen with orthologues in *S. cerevisiae*. In agreement with this suggestion, Côte *et al.*, (2009) found that *CDC28*, *SWI6* and *SWI4*, but not *MBP1*, were cell cycle-regulated at the transcriptional level, albeit in opaque phase cells. Thus, these results

suggest further re-wiring of the G1/S network in the pathogen, compared to a related, but non-pathogenic, model fungus.

In comparison, the G1/S circuit in hyphal cells is less understood, but is known to have some critical differences from yeast. For example, Cln3p and the pescadillo homologue Pes1p are essential for yeast but not hyphal growth (47, 48, 95). It would thus be informative to elucidate and compare G1/S circuitry in the different cell types to gain more insight on how cell-type specific proliferation is controlled.

4.2 Linking Cln3p to hyphal development

The mechanisms linking Cln3p depletion to hyphal and pseudohyphal growth, independent of normal environmental cues, remain unclear. Hyphae are normally induced by a variety of environmental cues, such as higher temperature and serum, for example, which are mediated in a complex manner by a number of different signaling pathways (2, 10). The transcription profiling data revealed modulation of several hyphal regulatory factors, suggesting that Cln3p activity may impinge on the hyphal regulatory networks. For example, the transcription factor *UME6* is sufficient for stimulating hyphal growth (77) and was up-regulated in Cln3p-depleted cells, while *NRG1*, a negative regulator of hyphal development (104, 105), was repressed. To date, neither of these factors have been previously linked to cell cycle regulation. *EFG1*, which can induce or repress hyphal development depending on the environmental conditions (82, 83), was also repressed. Repression of other inducers of hyphal growth, including *YAK1* and *FLO8*, was unexpected, but the complexity of the hyphal signaling pathways precludes a comprehensive understanding of the functions of known players and the

extent of cross-talk (10, 67). It is possible, for example, that signaling factors could be modulated in response to others or as a consequence of hyphal growth itself. That *EFG1*, *YAK1* and *FLO8* were modulated regardless of the presence of Ras1p, however, suggests that their expression pattern is not a response to hyphal growth, and thus may be functionally significant. Further investigations of any of these genes would first require validation of expression levels using qPCR or Northern analysis at early vs. late stages of Cln3p-depletion, followed by screening for suppression of Cln3p-dependent hyphal development by overexpression or deletion.

Less clear is the nature of the initial signal stemming from depletion of Cln3p, leading to the generation of hyphae. One possibility is that Cln3p activity, mediated in part through MBF, negatively regulates hyphal development. This model is consistent with the conserved relationship between G1 phase and development in other organisms, and supported by the fact that cell cycle regulators, such pRB and E2F, have additional roles in regulating developmental genes in higher organisms (29). However, a link between G1 phase and hyphal development is currently controversial, where one study showed that hyphae can be induced at any cell cycle stage in *C. albicans*, at least in the presence of serum (43), while another showed that hyphae could only initiate during G1 phase (45). However, blocking *C. albicans* yeast cells at other cell cycle stages does not induce true hyphal growth (44, 45, 47, 50), and intriguingly, blocking G1 phase in *S. cerevisiae* and many other organisms is not sufficient to trigger development (39, 40, 51-53) in contrast to *C. albicans*. It is thus possible that a bias for hyphal formation exists during G1 phase as a vestige of evolution, but the pathogen evolved additional mechanisms to form hyphae, an important virulence trait, at any cell cycle stage while in

the presence of strong environmental inducers. Alternatively, hyphal development could be an indirect response to G1 phase arrest or delay due to reaching a threshold concentration of some regulatory factor, or achieving a certain cell size (44). To distinguish between models, future investigations could include testing the effects of elevated or stabilized Cln3p on serum-induced hyphal growth, determining whether Cln3p/Cdc28p kinase activity is modulated during environmental-induced hyphal growth, and exploring whether a G1 phase arrest created through other means while maintaining Cln3p activity results in hyphal formation. To date, however, the only known method to create a clear G1 phase arrest without withholding nutrients in *C. albicans* involves depletion of Cln3p (47, 48).

4.3 Swi6p and Swi4p are important, but not essential, for mediating Cln3p activity, highlighting significant re-wiring in the G1/S network

In testing a model of the G1/S circuit, we provided direct evidence that orthologues of SBF/MBF mediate a portion Cln3p activity during the G1/S transition. The SBF/MBF transcription factor complex is composed of ankyrin domain-containing proteins specific to fungi, which serve as the gateway to commitment to mitosis. In *S. cerevisiae*, SBF (Swi6p/Swi4p) mediates G1/S early events such as cyclin expression, budding, and cell wall deposition, whereas MBF (Swi6p/Mbp1p) mediates DNA replication (71, 106, 107). In *S. pombe*, MBF mediates most G1/S events, and is composed of Cdc10p, the functional equivalent of Swi6p, and at least two DNA binding elements Res1p and Res2p (89, 92, 108, 109). We provide functional evidence that Swi4p and Swi6p mediate a portion Cln3p activity during the G1/S transition. First, cells

lacking these factors were significantly enlarged in size, suggesting a delay in G1 phase. Second, transcription profiles of cells lacking both factors showed repression of genes associated with the G1/S transition, including putative SBF targets, DNA replication/chromatin remodeling factors, and translation/ribosome biogenesis genes, for example. G1 cyclins were also repressed, as shown by Northern analysis (C. Bachewich, unpublished data). Third, some of the modulated genes corresponded to factors that cycle at the transcriptional level during the G1/S and S/G2 transitions in synchronized opaque phase cells of *C. albicans* (94). Not all of the relevant cycling genes identified by Côte *et al.*, (2009) were modulated in Swi6p/Swi4p-depleted cells, which is likely due to variability in the mutant phenotype and issues with microarray sensitivity, but collectively our data support the notion that a G1 phase delay was taking place and that Swi4p and Swi6p participate in the G1/S circuit.

Our results highlight several key differences with respect to the proposed function and potential regulation of ankyrin-repeat proteins in G1/S control compared to other fungi. For example, Mbp1p appears to play a minor role, since deletion resulted in minor effects on growth and phenotype compared to deletion of *SWI4*, and the *SWI4*, *MBP1* double deletion strain showed a mild synergistic effect. More importantly, however, was the fact that deletion of *MBP1* was not synthetically lethal in combination with deletion of *SWI4*. In contrast, *S. cerevisiae* cells lacking both *SWI4* and *MBP1* are not viable (33). Our genetic data thus suggests that Swi6p and Swi4p are the dominant ankyrin-repeat proteins participating in G1/S control, and that Swi4p and Mbp1p do not significantly overlap in function. Côte *et al.*, (2009) demonstrated that *C. albicans* G1/S cycling genes contain MCB, not SCB, binding sites, suggesting that MBF should be the regulatory

complex in *C. albicans*, as seen in *S. pombe*. Our genetic data suggests that MBF would thus be composed of Swi6p in combination with Swi4p, not Mbp1p (94). In the absence of direct binding studies, we cannot rule out the possibility that other noncanonical regulatory elements mediate putative Swi6p/Swi4p binding and activity. Additional common regulatory elements were not identified in G1/S cycling genes (94), but Bean *et al.*, (2005) demonstrated that Swi4p and Mbp1p could regulate many of the same genes, and this functional overlap did not correlate well with presence or absence of clear MCB or SCB binding sequences.

Another critical difference in *C. albicans* is that ankyrin-repeat proteins are not essential for the G1/S transition. While lack of Swi4p and Swi6p, or Swi4p and Mbp1p in *S. cerevisiae*, results in non-viable cells (33, 110) and absence of Cdc10p or both Res1p and Res2p while in *S. pombe* blocks cell division (34, 35), *C. albicans* yeast cells lacking Swi6p and Swi4p or Swi4p and Mbp1p were viable and continued to grow, albeit more slowly. Thus, ankyrin-domain containing proteins do not mediate all of the essential activity of Cln3p at the G1/S transition, suggesting that additional modes of regulation exist. In comparison, some residual G1/S transcription was reported in a normally lethal *swi4/mbp1* double mutant in *S. cerevisiae* that was alternatively held viable through overexpression of Cln2p (84), but the fact that *C. albicans* cells are viable without Swi4p and Mbp1p suggests that a more prominent mechanism is involved in mediating the remainder of Cln3p activity. If at the transcriptional level, one possibility is the involvement of Efg1p or Efh1p (111), which belong to the APSES domain-containing family of proteins involved in regulating developmental events in fungi (112). Intriguingly, Efg1p and to a lesser extent Efh1p, can bind *MluI* sites via the APSES

domain (113), but there is no evidence supporting a role for either factor in regulating cell proliferation (16). Alternatively, residual Cln3p activity may be mediated at a post-translational level. The identification of Cln3p/Cdc28p targets will shed light on this important question.

The expression patterns of the putative MBF target *YOXI* and the cyclin *CLN3* highlight another difference in potential MBF function in *C. albicans*. In *S. cerevisiae*, *YOXI* expression is activated by SBF, and in turn represses the Mcm1p-dependent activation of *CLN3* expression (71). Consistent with this, *YOXI* was repressed upon Cln3p-depletion in *C. albicans* (C. Bachewich, unpublished observations), peaked at G1/S in opaque phase cells, and has an MCB binding element. (94). That *YOXI* and *CLN3* were induced in *C. albicans* cells lacking Swi6p and Swi4p suggests that *YOXI* expression may be regulated by Cln3p activity, but not solely through MBF, if at all, and that Yox1p may alternatively have a positive influence on *CLN3* expression. Thus, the data provide functional evidence for the involvement of MBF in G1/S control, but further demonstrate that significant re-wiring has taken place in the G1/S circuit (94), particularly with respect to putative MBF function and regulation.

4.4 Swi4p and Swi6p influence cell morphogenesis and development

Absence of Swi4p and Swi6p had a significant effect on morphology. The pleiotropic effects indicate that the deletions were not 100% penetrant, in line with several other deletions in *C. albicans* and in other organisms (33, 87, 114, 115). It is possible that varying levels of G1 cyclins or other regulatory factors resulted in differences in the timing of transition through G1/S phase, and perhaps generation of

different cell shapes and types. Swi6p/Swi4p-depleted cells appeared strikingly similar to mutants of *GRR1*, an F-box protein that directs SCF ubiquitin ligase activity (116, 117), except the latter did not form true hyphae. Since absence of *GRR1* in *C. albicans* lead to stabilization of Ccn1p and Cln3p (116), and cells lacking Swi6p and Swi4p showed induction of *CLN3*, it is possible that increased levels of Cln3p underlie the common phenotype. However, overexpression of a semi-stabilized form of *CLN3* lacking the PEST domains did not result in a similar phenotype (C. Bachewich, unpublished results), suggesting the involvement of other mechanisms. The budding defects in cells lacking Swi4p and/or Swi6p could generate many of the abnormal cell shapes seen within the pleiotropic population, but the presence of distinct cell types, including true hyphae, indicates that the factors may also be linked to developmental pathways. Strong expression of hyphal-associated markers and virulence genes, despite the pleiotropic cell population, coupled with the presence of unconstricted septa in filamentous cells, support the notion that true hyphae were generated. Although *swi4/swi4*, *swi6/swi6* cells did not completely phenocopy Cln3p-depleted cells, these factors could mediate in part the influence of Cln3p on hyphal development. It is not yet clear whether expression patterns of hyphal regulatory factors, including *CZF1*, are functionally significant, but they provide potential leads in determining how hyphal development may be linked to Swi6p and Swi4p, if this occurs at a direct transcriptional level. Thus, the molecular basis of the interplay between Cln3p, MBF, and hyphal development remains to be determined, but the identification of Cln3p/Cdc28p and MBF targets will help clarify this important question.

The data suggest that Swi4p and Swi6p may also be linked to regulation of the opaque cell fate. First, many cells lacking these factors were elongate and bean-shaped, similar to opaque cells. Second, transcription profiles demonstrated modulation of several genes associated with or regulated during the opaque state, albeit both up and down and excluding classic opaque markers such as *CDR3* and *OP4*. Further, the white phase-specific gene *WH11* was highly up-regulated in *swi4/swi4*, *swi6/swi6* cells relative to the control. Such a pattern was predicted for opaque cells switching back to the white phase, although it was not observed (79). Additional opaque-like features have since been identified in these strains (C. Bachewich, unpublished results), lending support to our hypothesis. This data raises the possibility that MBF may influence the opaque cell regulatory program, or at least aspects of it. The pleiotropy in cell phenotype of the mutant strains and instability of the opaque state, particularly at 30°C (17, 25) could explain the variability in opaque-like characteristics in the cell population. Attempts to grow cells at 24°C for several days did not enhance the opaque-like phenotype (data not shown), but given the complex regulation of opaque differentiation, additional variables could be influencing the response.

For example, white to opaque switching requires changes at the mating type-like locus (*MTL*) of white phase cells from a heterozygous to a homozygous or hemizygous state, which involves either chromosome loss or dramatic rearrangements in chromosome structure, including gene conversion or mitotic recombination (18-20). Cells heterozygous at *MTL* express the $\alpha 1/\alpha 2$ heterodimer, which acts as a general repressor of the opaque state. The infrequent occurrence of generating homozygosity at the *MTL* leads to stochastic increases of Wor1p, a master opaque regulator, above a threshold, allowing

white cells to switch to the opaque state (21, 22). Thus, it is possible that Swi6p and Swi4p play a role in negatively regulating at least a subset of opaque features, in addition to functioning in G1/S regulation. In support of this, a target of SBF activity in *S. cerevisiae*, *TOS4*, regulates developmental genes (71, 84). However, absence of SBF alone does not activate development, as seen in *C. albicans*.

Thus, our results significantly advance our understanding of the composition of the G1/S regulatory circuit in *C. albicans*, which includes unique features, and provide evidence for the existence of a regulatory link between G1 phase and development that can bypass the normal environmental inputs. Specifically, we show that Swi4p and Swi6p may mediate only a part of the essential activity of Cln3p activity, and may play a dual role in controlling the G1/S transition and repressing development. Since a relationship between G1 phase and hyphal development is controversial (44), and current models of white/opaque switching do not include the involvement of cell cycle factors (119), the data introduce potential new modes of regulation for the differentiation of these cells. Our results also highlight the concept that *C. albicans* has seemingly acquired the ability to influence developmental pathways through cell cycle-related cues that can bypass the normal environmental signals, unlike that seen in many other organisms, including *S. cerevisiae*. Additional routes leading to differentiation may reflect an adaptation to enhance developmental plasticity within the host, directly contributing to virulence potential. Future work addressing the direct functions and regulation of Swi6p and Swi4p, and elucidating the Cln3p-dependent, MBF-independent pathway in G1/S control will significantly advance our understanding of how basic cell proliferation is

regulated and linked with developmental events, which are critical for virulence in an important human pathogen.

5. References

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Appendix I

Supplementary Appendix tables S1-S8.

Full lists of significantly modulated genes (1.5 fold cut-off, t-test $p < 0.05$) in (a) Cln3p-depleted cells after 1, 3, or 6h (b) Cells lacking Swi4p and Swi6p

Please see attached CD.