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Induction of the *Candida albicans* Filamentous Growth Program by Relief of Transcriptional Repression: A Genome-wide Analysis[D]

David Kadosh and Alexander D. Johnson

Department of Microbiology and Immunology, University of California–San Francisco, San Francisco, CA 94143-2200

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***Candida albicans*, the major human fungal pathogen, undergoes a reversible morphological transition from blastospores (round budding cells) to filaments (elongated cells attached end-to-end). This transition, which is induced upon exposure of *C. albicans* cells to a number of host conditions, including serum and body temperature (37°C), is required for virulence. Using whole-genome DNA microarray analysis, we describe 61 genes that are significantly induced (≥2-fold) during the blastospore to filament transition that takes place in response to exposure to serum and 37°C. We next show that approximately half of these genes are transcriptionally repressed in the blastospore state by three transcriptional repressors, Rfg1, Nrg1, and Tup1. We conclude that the relief of this transcriptional repression plays a key role in bringing the *C. albicans* filamentous growth program into play, and we describe the framework of this transcriptional circuit.**

INTRODUCTION

The yeast *Candida albicans* is the major human fungal pathogen. Although normally present as a commensal organism in the human digestive tract, *Candida* can cause oral and vaginal thrush, as well as a variety of more serious mucosal and systemic infections. Unlike most pathogens, *Candida* is capable of infecting all the major organs and tissues of the human body and has no known reservoir outside of warm-blooded animals (Odds, 1988, 1994a, 1994b; Dupont, 1995; Weig *et al.*, 1998). Candidiasis is now the fourth-leading cause of hospital-acquired infections in the world with an attributable mortality of up to 35% (Edmond *et al.*, 1999). Immunocompromised individuals, such as cancer patients undergoing chemotherapy, AIDS patients, and organ transplant recipients are particularly susceptible to *Candida* infections (for reviews see Shepherd *et al.*, 1985; Dupont, 1995; Weig *et al.*, 1998). Currently, approximately $1 billion per year is spent on the treatment of patients with hospital-acquired infections in the United States alone (Miller *et al.*, 2001).

Several properties of *C. albicans* are known to contribute to its virulence. These include adhesiveness to host cells, secretion of degradative enzymes, and—the subject of this article—the ability to undergo a reversible morphological transition from the blastospore (single-celled) to filamentous (elongated cells attached end-to-end) forms (Lo *et al.*, 1997; Mitchell, 1998; Brown and Gow, 1999; Ernst, 2000a, 2000b; Calderone and Fonzi, 2001; Brown, 2002b; Calderone and Gow, 2002; Saville *et al.*, 2003). The filamentous forms of *C. albicans* encompass two distinct morphologies, pseudohyphae and hyphae. In pseudohyphae, the cells are attached end-to-end but each cell has an elliptical shape with constrictions at the septa. In true hyphae, these constrictions are absent and a row of cells show a relatively uniform width. Pseudohyphae and hyphae also differ in other respects, including the precise ways that mitosis and cell division are carried out (Odds, 1985, 1988; Sudbery *et al.*, 2004). In this article, we use the term filamentous to include both the pseudohyphal and hyphal forms. This term is useful because many of the same conditions that induce the hyphal form also induce the pseudohyphal form; indeed, mixed populations of pseudohyphae and hyphae are a common outcome of filament-inducing conditions (Odds, 1985, 1988; Brown, 2002a).

The blastospore-filament transition is accompanied by the induction of many genes. Although some of these genes are required for the morphological transition per se, others are not (Braun *et al.*, 2000; Nantel *et al.*, 2002). This latter class of genes presumably encode proteins that give the filamentous forms their distinctive properties. Although the blastospore-filament transition is clearly required for virulence (Lo *et al.*, 1997; Braun *et al.*, 2000; Braun *et al.*, 2001; Murad *et al.*, 2001; Saville *et al.*, 2003), the relative importance of the morphological forms themselves, compared with the changes in gene expression that accompany them, is not known.

Candida undergoes the transition from blastospores to filaments in response to a wide variety of laboratory conditions, many of which mimic the environment of mammalian host tissues. These conditions include growth at elevated (body) temperature (37°C), at neutral pH or in the presence of serum or certain human hormones (Kinsman *et al.*, 1988; Bramley *et al.*, 1991; Caticha *et al.*, 1992; Mitchell, 1998; Brown and Gow, 1999; Ernst, 2000a). One of the strongest sets of filament-inducing conditions is the combination of body temperature (37°C) and serum. These conditions trigger a signal transduction pathway that ultimately results in

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Address correspondence to: Alexander D. Johnson (ajohnson@cgl.ucsf.edu).

the activation of a complex transcriptional program important for *C. albicans* filamentous growth and virulence (for reviews, see Ernst, 2000b; Brown, 2002b).

In this article, we address the mechanism by which the filament-specific genes become active, and we focus on three key negative regulators, Rfg1, Nrg1, and Tup1. As previously described, strains missing any one of these regulators are highly attenuated for virulence and grow filamentously under non–filament-inducing conditions. Moreover, each of these deletion strains inappropriately expresses at least some of the filament-specific genes in the absence of any inducer (Braun and Johnson, 1997; Braun *et al.*, 2000; Braun *et al.*, 2001; Kadosh and Johnson, 2001; Khalaf and Zitomer, 2001; Murad *et al.*, 2001). In the well-studied baker's yeast, *Saccharomyces cerevisiae*, Nrg1 and Rox1 (the homolog of Rfg1) are sequence-specific DNA-binding proteins and Tup1 is a global transcriptional repressor recruited to genes via interaction with promoter-specific DNA-binding proteins such as Rox1 and Nrg1 (Keleher *et al.*, 1992; Deckert *et al.*, 1995; Tzamarias and Struhl, 1995; Park *et al.*, 1999; Smith and Johnson, 2000). Although not directly demonstrated, a similar relationship is very likely to hold for *C. albicans* Rfg1, Nrg1, and Tup1.

In this article, we address the following questions: what is the full spectrum of genes transcriptionally induced during the blastospore to filament transition? How much of this program is due to relief of negative control by Nrg1, Rfg1, and Tup1? What is the relationship (superimposing, overlapping, or distinct) between the Rfg1- and Nrg1-repressed genes? Do Rfg1 and Nrg1 act solely through Tup1 or do they have other mechanisms available to them? Are Nrg1, Rfg1, and Tup1 devoted exclusively to the regulation of the filamentous growth program or do they have other roles in the cell as well? To answer these questions, we utilized whole-genome microarrays to analyze the blastospore-filament transition, to identify all genes repressed by Rfg1, Nrg1, and Tup1 and to establish the functional relationships among these three transcriptional repressors.

MATERIALS AND METHODS

Strains

In all experiments, the wild-type strain used was CAF2-1 (Δ*ura3;imm434/URA3*; Fonzi and Irwin, 1993). All *rfg1*, *nrg1*, and *tup1* strains used were homozygous deletion mutants and have been described previously (Braun and Johnson, 1997; Braun *et al.*, 2001; Kadosh and Johnson, 2001). Where possible, multiple isolates of each mutant strain were used for experimental repetitions.

Media and Growth Conditions

In all experiments performed to compare gene expression in *rfg1*, *nrg1*, and *tup1* mutant strains with the wild-type strain, cells were grown under standard noninducing conditions: yeast extract-peptone-dextrose (YEPD) medium at 30°C (Guthrie and Fink, 1991). For all strains, a saturated overnight culture was diluted to OD_{600} = 0.2 in 1 L of YEPD medium and allowed to grow at 30°C until $OD_{600} \sim 1.0$, at which point cells were harvested for RNA preparation. In half of the *nrg1* and *tup1* experiments, a saturated overnight culture was diluted into 1 L of YEPD medium and grown at 30°C overnight to $OD_{600} \sim 1.0$ on the following day; at this point cells were collected for RNA preparation.

In the serum- and temperature-induction experiments, a saturated overnight culture of the wild-type (CAF2-1) strain was diluted into 2 L of YEPD medium and allowed to grow at 30°C overnight until cells reached an OD_{600} of 12.6 (experiment 1) or 14.4 (experiment 2). An aliquot of this culture (the zero time point) was then harvested to prepare RNA and additional 200-ml aliquots were diluted into 2 L of fresh, prewarmed YEPD medium in the presence or absence of 10% fetal calf serum (FCS) and grown at 30 or 37°C. Cells were harvested for RNA preparation at 1-, 2-, 3-, and 5-h time points. The OD_{600} of the zero time point was critical for optimal filament induction (see *Results* section).

DNA Microarray Experiments and Analysis

C. albicans DNA microarray construction, preparation of RNA and cDNA, hybridization of coupled cDNA to microarrays and DNA microarray analysis were performed as described previously (Bennett *et al.*, 2003). Data normalized by NOMAD (http://ucsf-nomad.sourceforge.net/) were filtered to include only spots showing a median signal intensity ≥500 in at least one channel. The *rfg1* experiment was performed four times, and the *nrg1* and *tup1* experiments were done six times (all from independently grown cultures). In all four of the *rfg1* experiments and in half (three) of the *nrg1* and *tup1* experiments, each hybridization contained a mutant sample (labeled with Cy5 dye) and wild-type sample (labeled with Cy3 dye). In half (three) of the *nrg1* and *tup1* experiments and in both serum and temperature induction experiments, each hybridization contained an experimental sample (labeled with Cy5 dye) and a universal reference sample (labeled with Cy3 dye) generated from cells grown under a wide variety of filament-inducing and noninducing conditions; later, the signal ratios (ratio of the median signal intensities of each spot) for each mutant sample versus reference were divided by the signal ratios for the wild-type sample versus reference and signal ratios for each time point of the serum and temperature-induction experiment versus reference were divided by the signal ratios for the zero time point versus reference. Signal ratio values for wild-type versus reference and the zero time point versus reference represented median values obtained from at least three independent DNA microarray experiments (performed using cDNA obtained from the same total RNA preparation). Significance analysis of microarrays (SAM) was performed as described previously (Tusher *et al.*, 2001), using the One-class Response, K-nearest neighbors settings (10 neighbors) and the default Random Number Seed (1234567). For each data set, 600 permutations were carried out and delta values were selected to calculate low false discovery rates (FDRs) for the median *d*-scores. Gene annotation and analysis was based on the *C. albicans* orf19 assembly (Braun *et al.*, 2005) and, for *YHB* and *ALS* family genes, on the *Candida* Genome Database (Arnaud *et al.*, 2005). Please note that in the case of three *SAP* family genes (*SAP4*, *SAP5*, *SAP6*) and genes in the *MRS512* (*FGR6*) family (including orf19.914, orf19.1099, orf19.5191, orf19.5775, orf19.7005), it was impossible to distinguish between family members by microarray experiments because the genes in these families were nearly identical at the DNA level. Gene classification was carried out based on the *S. cerevisiae* gene ontology (GO) terms (Dwight *et al.*, 2002; Harris *et al.*, 2004).

RESULTS

Optimization of Conditions for Induction of the Blastospore to Filament Transition in Response to Serum and Temperature

Northern analysis has previously shown that several filament-specific genes undergo a large (>20-fold) transcriptional induction when *C. albicans* is treated with serum at 37°C (Birse *et al.*, 1993; Staab *et al.*, 1996; Braun and Johnson, 2000). In our initial experiments to monitor the genome-wide response of *C. albicans* to serum and temperature, we observed much lower induction ratios than those cited above. These low induction ratios were also observed by Nantel *et al.* (2002), who described the first microarray analysis of the response of *C. albicans* to serum and temperature. In our experiments, we found that the low induction ratios were largely due to the presence of mixed populations of blastospores and filamentous forms in both the induced and noninduced cultures. This results in a significant underestimation of the extent of transcriptional induction and obscures genes that are only moderately induced. By experimenting with a variety of different protocols, we found that diluting an overnight culture 1:10 directly into fresh, prewarmed YEPD medium with 10% serum at 37°C gave optimal induction of filamentous growth in large cultures. For reasons we do not understand, the optical density of the overnight culture was critical. Cells induced from a culture at $OD_{600} \sim 10.6$ or lower showed incomplete filament formation and a correspondingly reduced expression of filament-specific genes. In contrast, cells induced from a saturated overnight culture generated filaments even when diluted into noninducing medium (fresh YEPD medium at 30°C), a phenomenon that we term "hyphal shock." Only when the optical density of the overnight culture was $OD_{600} \sim 13$ did we observe 100% filament formation and conse-

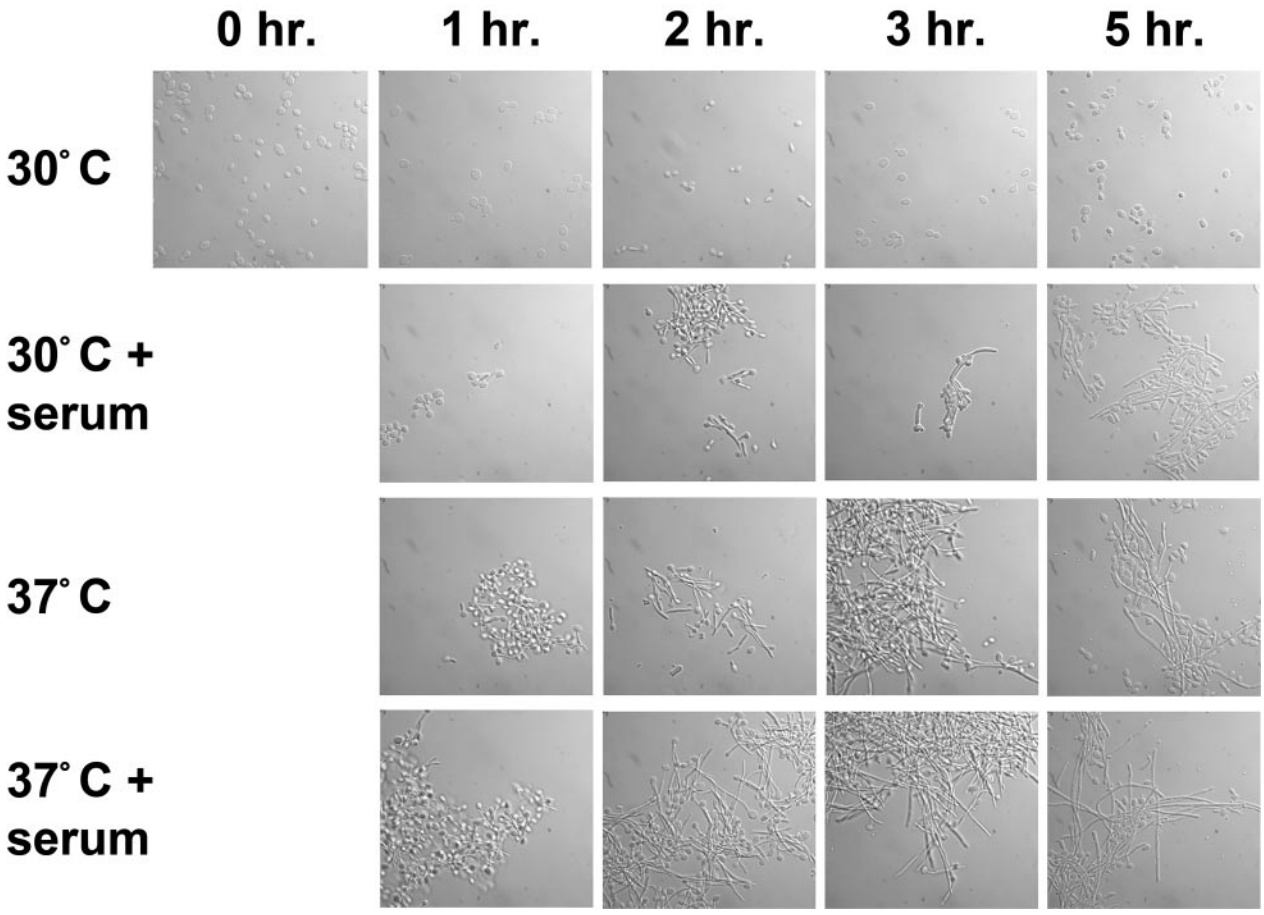

Figure 1. Morphology of cells undergoing the blastospore to filament transition. An overnight culture grown in YEPD medium at 30°C was diluted 1:10 into prewarmed YEPD medium at 30°C or at 37°C in the presence or absence of 10% FCS. Induction time (hrs.) is shown on top. Aliquots of cells were fixed in 4.5% formaldehyde, washed twice with 1 × phosphate-buffered saline, and then visualized by Nomarski optics and photographed under 100× magnification. Please note that the 0-h time point photograph shows cells immediately before induction.

quently high levels of filament-specific gene expression under inducing conditions and little or no filament formation under noninducing conditions. We do not understand why this particular optical density is required for optimal induction, but it is possible that the nutrient availability, quorum sensing, or some other factor plays an important role in priming the cells for efficient serum and temperature induction.

Transcriptional Profile of Genes Induced During the Blastospore to Filament Transition in Response to Serum and Temperature

Using the conditions described above we carried out two independent serum- and temperature-induction experiments with a wild-type *C. albicans* strain (CAF2-1). Over a time course, we observed the response to serum alone, temperature alone (37°C), and serum plus temperature (37°C). For each condition, cells were grown in YEPD medium (plus serum, when appropriate) and harvested at 1-, 2-, 3-, and 5-h time points for RNA extraction and DNA microarray analysis. We also monitored cell morphologies microscopically at each time point. As shown in Figure 1, 100% of the cells were induced to form true hyphae when grown at 37°C in the presence of serum, and most cells remained in this form at the 5-h time point. Cells grown at 30°C in the presence of serum or at 37°C in the absence of serum also showed significant filament formation. However, under these conditions, the morphology change was less pronounced: not all of the cells were in filamentous form, particularly at the 5-h time point, and many of the filamentous cells (especially at 30°C in the presence of serum) were in the pseudohyphal rather than true hyphal form. Nearly all the cells grown in YEPD at 30°C were in the blastospore form for the duration of the experiment (Figure 1).

For the microarray experiments, the results from each time point were normalized to the median value (taken from three microarrays) of the 0 time point (30°C, no serum). We observed 61 genes transcriptionally induced ≥2-fold by serum and temperature (37°C) together when compared with the same time points in the 30°C cultures without serum. Approximately one-third of these genes were induced ≥10-fold (Table 1). The majority of the 61 genes were reproducibly induced in both time courses and at multiple time points within each time course. A few genes were induced only at the earliest time point (t = 1 h); for these genes we relied solely on the reproducibility between the two time courses to assign significance to them. We will refer to these 61 genes as hyphal-specific, because they are differently expressed in 37°C plus serum (where the cells are 100% hyphal) compared with 30°C without serum where the cells are >95% in the blastospore form (Figure 1). Of the 61 hyphal-specific genes, most showed a characteristic tran-

Table 1. Serum- and temperature-induced genes

No. of serum- and temperature-induced genes[b]	No. of these genes up[a]		
	>2-fold	>5-fold	>10-fold
61	61	33	22

[a] Number of genes showing the indicated mean fold induction at the 37°C + 10% serum 1-h time point (n = 2). Please note that a single late-induced gene, orf19.6079, shows a mean fold induction >2-fold at the 37°C + 10% serum 3-h time point.

[b] Criteria for selection: 1) mean induction ≥2-fold (n = 2) at the 37°C + 10% serum, 1-h time point, 2) mean of (37°C +10% serum)/(30°C only) ratio >2.0 for the 1-h time point. Please note that a single late-induced gene, orf19.6079, is an exception to these criteria.

scription profile in response to serum at 37°C, with the highest level of expression observed at the 1-h time point and a progressive decrease at later time points (Figure 2). By a similar analysis, we identified 25 genes whose expression is reduced in the presence of serum at 37°C; see Supplementary Data.

When *C. albicans* was exposed to 37°C in the absence of serum or to serum at 30°C, the majority of the hyphal-specific genes were also induced, but the overall level of induction was lower than that observed for the serum-37°C combination (Figure 2). As described above, at most time points, the 37°C alone and serum alone cultures contained mixtures of blastospores, pseudohyphae, and hyphae. Our microarray results are most consistent with the hyphal-specific genes also being induced in pseudohyphal cells, but the lack of pure populations of pseudohyphae prevent a definitive conclusion. These results indicate that serum or temperature (37°C) alone can bring the filamentous transcription program into play, but that the combination does so more efficiently.

As noted above, Nantel *et al.* (2002) also monitored the response of *C. albicans* to serum and temperature using microarrays. They identified 18 genes induced ≥2-fold, with a single gene induced greater than 10-fold. Ten of the ≥2-fold induced genes are included in our list of 61 hyphal-specific genes. As described above, the growth conditions used in our study enabled us to obtain pure populations of blastospores and hyphae, and this clear separation brought the differentially expressed genes into sharp relief.

Table 2. Total number of genes showing significantly elevated expression in *rfg1*, *nrg1*, and *tup1* mutants

Strain	SAM false discovery rate (%)[a]	No. of genes significantly up
rfg1	3.24	124
nrg1	0.78	124
tup1	0.15	165

[a] Significance analysis of microarrays (SAM) was used to define gene sets based on the indicated false discovery rates (FDRs). See *Materials and Methods* for details.

A Significant Fraction of Genes Induced by Serum and Temperature during the Blastospore to Filament Transition Are under Negative Control by Rfg1, Nrg1, and Tup1

Having identified a defined set of genes that is induced during the blastospore to hyphal transition in response to serum and temperature, we next determined the extent to which these genes are controlled by the Rfg1, Nrg1, and Tup1 repressors. As described in the *Introduction*, these transcriptional repressors are known to control aspects of filamentous growth as a deletion of any one of their genes leads to constitutive filamentous growth on noninducing medium (30°C without serum). To identify the genes controlled by these three repressors, we used *C. albicans* genome-wide arrays to compare the profiles of an isogenic set of strains, each strain deleted for the gene encoding one of the repressors, grown under noninducing conditions (30°C, no serum). As described in *Materials and Methods*, mRNA from each deletion strain was isolated from 4 to 6 independent cultures and compared with mRNA from an isogenic parental strain.

To identify the complete sets of target genes for each repressor, we turned to the significance analysis of microarrays (SAM) program, which ranks target genes by statistical significance (Tusher *et al.*, 2001). Using low false discovery rates, we identified a set of 165 genes showing significantly elevated expression in the Δ*tup1*/Δ*tup1* strain and sets of 124 genes each showing increased expression in Δ*rfg1*/Δ*rfg1* and Δ*nrg1*/Δ*nrg1* strains (Table 2; Supplementary Data). Many of the genes show low (<2-fold), but statistically significant effects, and probably represent secondary effects of deleting these repressors (see below).

To determine which hyphal-specific genes are controlled by Tup1, Nrg1, or Rfg1, we considered all genes induced

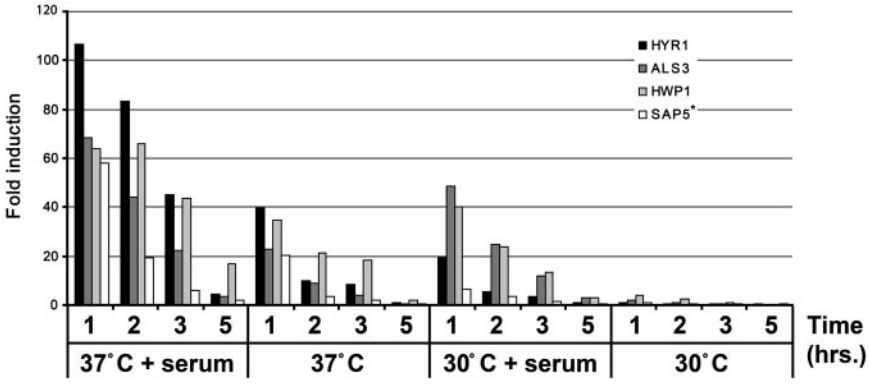

Figure 2. Transcriptional profile of most serum- and temperature-induced genes. Fold induction, relative to the zero time point, is shown for four serum- and temperature-induced genes (*HYR1*, *ALS3*, *HWP1*, *SAP5*) when cells were grown in YEPD medium, at 30 or 37°C in the presence or absence of 10% FCS, at the indicated time points. *Please note that *SAP5* expression levels may reflect, in part, *SAP4* or *SAP6* expression levels due cross-hybridization on the microarray, because these gene family members are nearly identical at the DNA level.

Table 3. Fraction of serum- and temperature-induced genes controlled by Rfg1, Nrg1, and Tup1

	No. of these genes also up ≥2-fold[a]		
No. of genes induced by 37°C + serum[b]	*rfg1*	*nrg1*	*tup1*
61	17 (28)	26 (43)	30 (49)

[a] Median fold effects were determined from four (*rfg1*) or six (*nrg1* and *tup1*) microarray experiments. Genes with less than two data points for any one mutant were excluded from the analysis. Percentage in parentheses.

[b] See Table 1 for selection criteria.

≥2-fold when Tup1, Rfg1, or Nrg1 was deleted and compared this to the list of 61 hyphal-specific genes. By these criteria, about half of the hyphal-specific genes are repressed by one or more of these negative regulators (Table 3). If we consider only hyphal-specific genes that are induced ≥5- or ≥10-fold, the fraction of genes controlled by these repressors is somewhat higher, approximately two-thirds (Figure 3; Supplementary Data). Thus, by any of these criteria the three repressors play a key role in orchestrating the filamentous growth program; they seem particularly important in regulating genes that show the highest hyphal-specific induction.

Functional Relationships among the Rfg1, Nrg1, and Tup1 Repressors

As described in the *Introduction,* Rfg1 and Nrg1 are sequence-specific DNA-binding proteins and Tup1 is a global repressor believed to be recruited by Rfg1, Nrg1, and other DNA-binding proteins. We used the results of the microarray analysis performed on strains deleted for each repressor to further test this idea and to establish the detailed relationships among the three repressors.

Previous microarray studies in *S. cerevisiae* have shown that when the Tup1 transcriptional repressor is deleted, both direct and indirect transcriptional effects can be observed

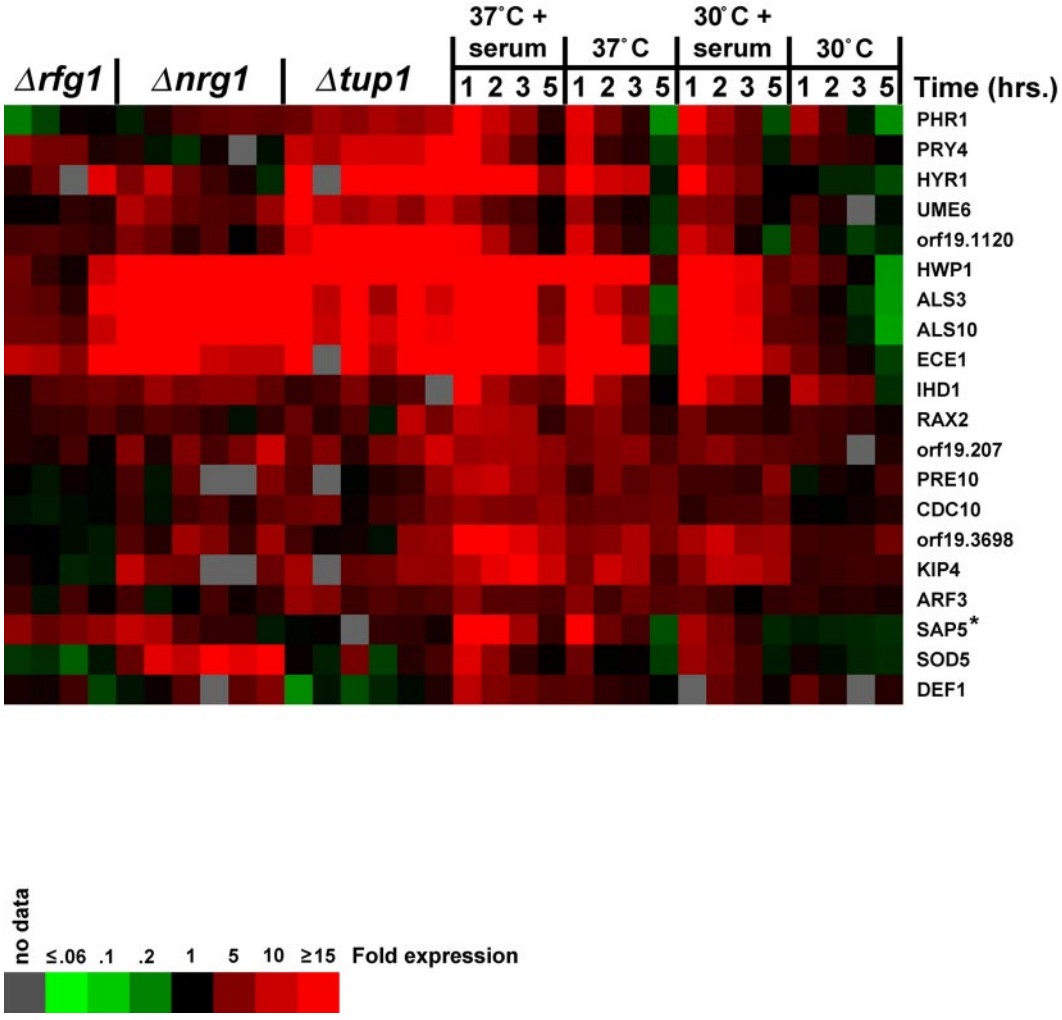

Figure 3. Cluster diagram of the top serum- and temperature-induced genes (≥5-fold mean induction, n = 2, at the 37°C + 10% FCS 1-h time point) with corresponding *rfg1*, *nrg1,* and *tup1* mutant data. Only data from one serum- and temperature-induction experiment and only genes with greater than 89% of data present are shown. Each column represents data from a single microarray experiment. Red, increased expression; green, reduced expression; gray, no data available. *Please note that *SAP5* expression levels may reflect, in part, *SAP4* or *SAP6* expression levels due cross-hybridization on the microarray, because these gene family members are nearly identical at the DNA level.

Table 4. Fraction of top Rfg1- and Nrg1-repressed genes controlled by Tup1

Strain	Total no. of genes up ≥5-fold[a]	No. of these genes with *tup1* mutant data[b]	No. of these genes also up ≥2-fold in *tup1* mutant[c]
rfg1	25	21	12 (57)
nrg1	37	31	29 (94)

[a] Median fold effects were determined from four (*rfg1*) or six (*nrg1*) microarray experiments (comparing the *rfg1* and *nrg1* strains to a wild-type strain). Only genes with two or more data points and the majority of data points ≥2-fold were included in the analysis.

[b] Indicates number of genes with two or more data points for the *tup1* mutant, as determined from six microarray experiments.

[c] Percentage in parentheses.

(DeRisi *et al.*, 1997; Green and Johnson, 2004). Typically the direct effects (genes directly controlled by Tup1) show larger magnitudes of change, although the correlation is not strict. To get a better idea of the relationships among the Rfg1, Nrg1, and Tup1 repressors we wished to minimize indirect effects. To this end, we considered genes showing a median increase in expression ≥5-fold in the deletion strain compared with wild type. By these criteria, Tup1 represses 103 genes; Nrg1 and Rfg1 repress 37 and 25 genes, respectively (Tables 4 and 5). Although these data sets are likely to be incomplete (compared with the SAM-based analysis described above), we believe they are enriched for direct effects and therefore optimal for addressing the question at hand.

Using this strategy, we observed the relationships between the three repressors. Approximately 60% of the genes derepressed ≥5-fold in the Δ*rfg1*/Δ*rfg1* strain also show elevated expression in the Δ*tup1*/Δ*tup1* mutant, suggesting that the majority of Rfg1 repression works via Tup1, but that a significant Tup1-independent pathway also exists (Table 4). Nrg1, in contrast, appears to function almost entirely via Tup1, because 94% of the top Nrg1-controlled genes also show increased expression in the Δ*tup1*/Δ*tup1* mutant (Table 4 and Figure 4). The reciprocal relationship, that is the fraction of Tup1-controlled genes regulated by Rfg1 and Nrg1, was determined in the same way. Approximately half of the genes derepressed ≥5-fold in the Δ*tup1*/Δ*tup1* mutant showed elevated expression in the Δ*nrg1*/Δ*nrg1* strain and approximately one-quarter showed increased expression in the Δ*rfg1*/Δ*rfg1* strain (Table 5). Many of the Rfg1-controlled genes in this set are also controlled by Nrg1, and we estimate that about half of the Tup1 repression observed in *C. albicans*

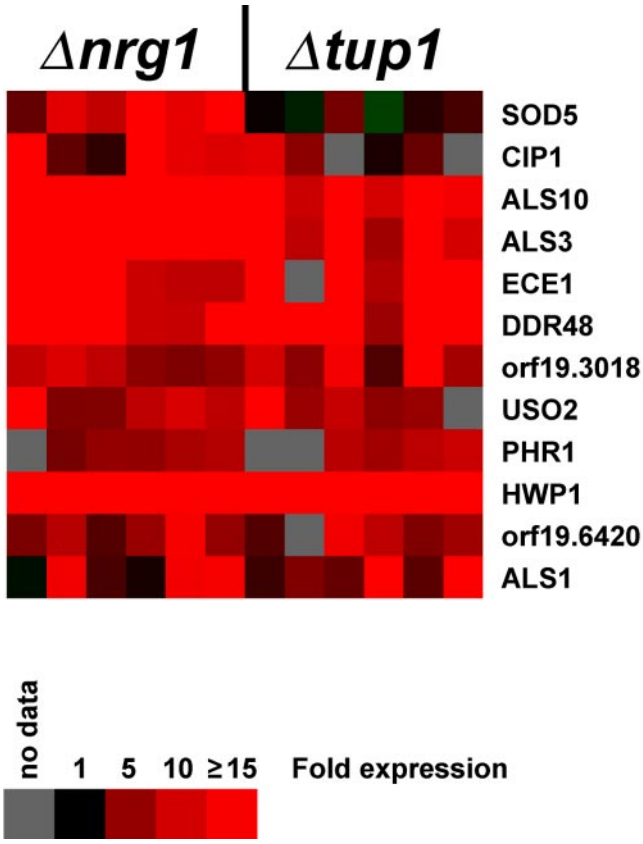

Figure 4. Cluster diagram of genes showing a median elevated expression ≥5-fold (n = 6) in the *nrg1* mutant with corresponding *tup1* mutant data (n = 6). Only genes with greater than 66% of data present are shown. Each column represents data from a single microarray experiment. Red, increased expression; gray, no data available.

occurs through Nrg1 and/or Rfg1; it is therefore likely that several additional sequence-specific DNA-binding proteins also function through Tup1.

Classes of Genes Induced by Serum and Temperature and Repressed by Nrg1, Rfg1, and Tup1

To systematically classify the serum- and temperature-induced and Rfg1-, Nrg1-, and Tup1-controlled target genes, we generated a list of gene classes based on universal gene ontology (GO) terms derived from *S. cerevisiae* (Dwight *et al.*, 2002; Harris *et al.*, 2004). Approximately 75% of target genes

Table 5. Fraction of top Tup1-repressed genes controlled by Rfg1 and Nrg1

Total no. of genes up ≥5-fold in *tup1* mutant[a]	No. of these genes with *nrg1* mutant data[b]	No. of these genes also up ≥2-fold in *nrg1* mutant[c]	No. of these genes with *rfg1* mutant data[b]	No. of these genes also up ≥2-fold in *rfg1* mutant[c]
103	87	42 (48)	70	19 (27)

[a] Median fold effects were determined from six microarray experiments (comparing the *tup1* strain with a wild-type strain). Only genes with two or more data points and the majority of data points ≥2-fold were included in the analysis.

[b] Indicates number of genes with two or more data points for the *nrg1* or *rfg1* mutants, as determined from four (*rfg1*) or six (*nrg1*) microarray experiments.

[c] Percentage in parentheses.

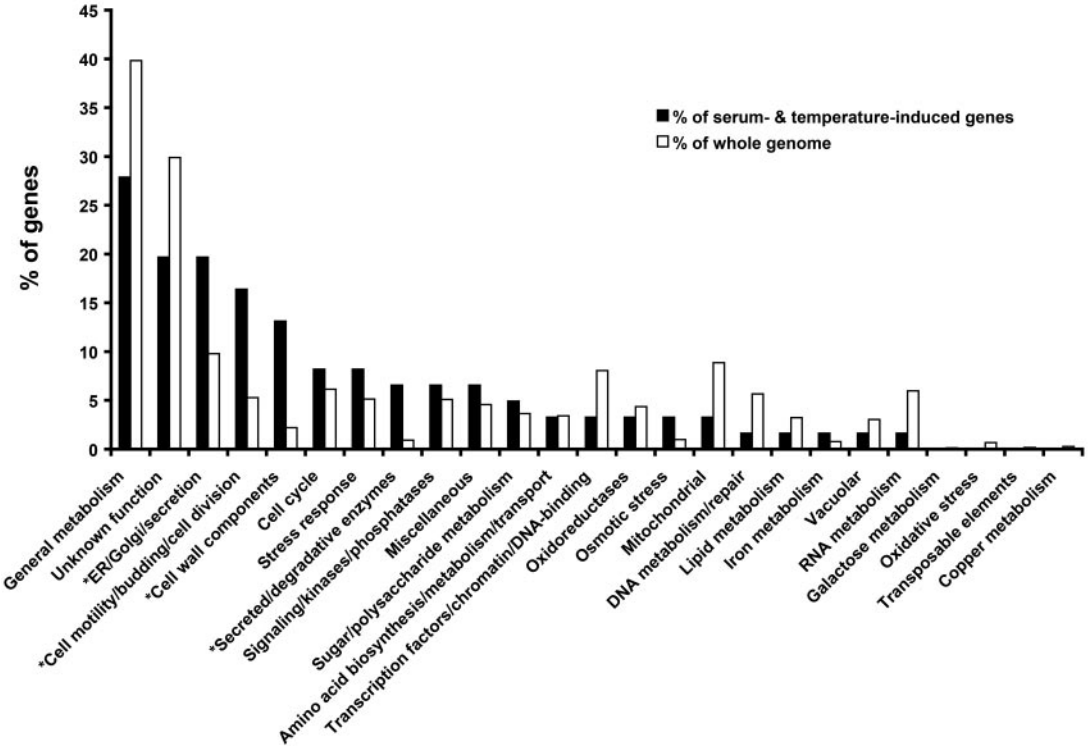

Figure 5. Representation of gene classes induced during the blastospore to filament transition compared with the genome as a whole. Asterisks indicate gene classes which are overrepresented in the serum- and temperature-induced gene set compared with the genome as a whole. Gene classification was based on *S. cerevisiae* gene ontology (GO) terms (Dwight *et al.,* 2002; Harris *et al.,* 2004). Please note that the "unknown function" gene class includes *all* genes of unknown function (with and without *S. cerevisiae* homologues or reciprocal hits).

(depending on the gene set) were classified based on *S. cerevisiae* GO terms (see Supplementary Data for GO terms associated with each gene class). Genes lacking *S. cerevisiae* homologues or reciprocal hits were classified as either "unknown function" (~18%) or "function in other organism" (~7%). Using this system, we identified 25 gene classes, 21 of which describe the genes induced by serum and temperature. As shown in Figure 5, these genes function in a wide variety of biological processes, including ER/Golgi/secretion and the cell cycle.

We next determined whether any gene classes are overrepresented in the serum- and temperature-induced gene set compared with the genome as a whole, and we found four classes: cell wall components, cell motility/budding/cell division genes, secreted/degradative enzymes, and genes involved in ER/Golgi/secretion (Figure 5). The first class, cell wall components, is also overrepresented in all three mutant data sets and the second class, cell motility/budding/cell division genes, is overrepresented in both Nrg1 and Tup1 mutant data sets (see Supplementary Data). The implications of these overrepresentations are presented in the *Discussion.*

DISCUSSION

Induction of the C. albicans Filamentous Growth Program Involves Relief of Transcriptional Repression

Numerous transcriptional regulators have been shown to be important for controlling the transition from the blastospore form to the hyphal form of *C. albicans* (Ernst, 2000b; Brown, 2002b). Here, using whole-genome DNA microarray analysis, we have identified 61 hyphal-specific genes that are induced in response to growth in serum at 37°C, and we show that approximately half of these genes are under negative control by the Rfg1, Nrg1, and/or Tup1 transcriptional repressors (Figure 6). These results, in combination with previous findings (Braun and Johnson, 1997; Braun *et al.,* 2001; Kadosh and Johnson, 2001; Khalaf and Zitomer, 2001; Murad *et al.,* 2001), show that relief of negative regulation plays a key role in the transition from blastospores to hyphae.

We also observed the response of *C. albicans* blastospores to serum at 30°C and to 37°C in the absence of serum. The vast majority, if not all, of the hyphal-specific genes were also induced under these conditions, although the extent of induction of each gene was generally less than that observed for the 37°C plus serum conditions. Although 37°C plus serum gave rise to nearly 100% hyphal cells, 30°C plus serum and 37°C in the absence of serum produced a combination of blastospores, pseudohyphae and hyphae. The simplest interpretation of our result is that many, if not all, of the hyphal-specific genes are also induced in pseudohyphae. Thus, serum or 37°C can bring about the filamentous growth program, but the combination of serum and 37°C does so much more efficiently.

Specific Roles of Rfg1, Nrg1, and Tup1 in Regulating Filamentous Growth

By the criteria described in the results section, Nrg1 and Tup1 work together to repress 20 hyphal-specific genes (Figure 6). As described in the introduction, Nrg1 mRNA is down-regulated by serum and temperature (Braun *et al.,*

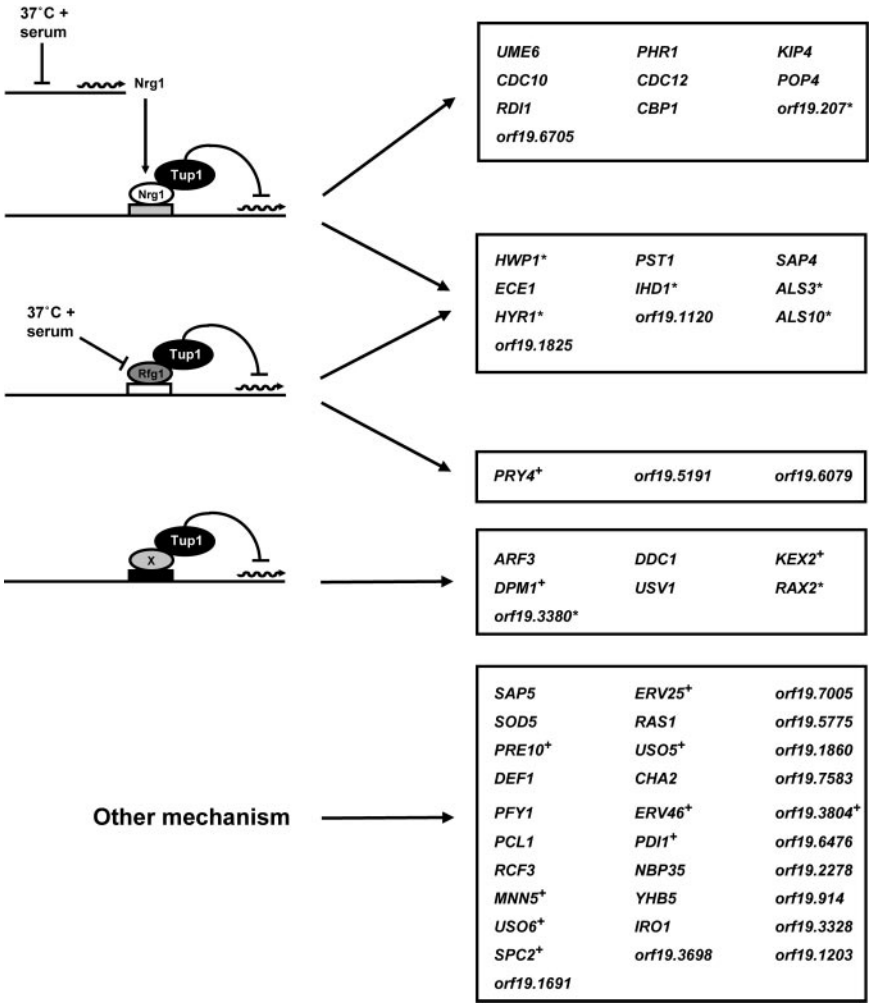

Figure 6. A model for control of genes induced during the blastospore to filament transition by the Rfg1, Nrg1, and Tup1 pathways. Rfg1, Nrg1, and at least one additional DNA-binding protein (X) bind to the promoters of filament- and virulence-specific target genes and direct transcriptional repression via recruitment of the Tup1 corepressor. The Nrg1 transcript itself has previously been shown to be down-regulated in the presence of 10% serum and temperature (Braun *et al.,* 2001; Murad *et al.,* 2001). Rfg1 repression activity is proposed to be regulated by filament-inducing conditions at the posttranslational level (Kadosh and Johnson, 2001). Target genes are indicated in boxed areas. *Cell wall components; $^{+}$ER/Golgi/secretion genes.

2001; Murad *et al.,* 2001); it is likely that the hyphal-specific genes controlled by Nrg1-Tup1 are induced as a direct result of this down-regulation. Rfg1 and Tup1 together repress 13 hyphal-specific genes, although it is not currently known how Rfg1 repression is lifted during the blastospore-hyphal transition. A significant number of hyphal-specific genes (10) are controlled by both Rfg1 and Nrg1, indicating some degree of overlap between these two pathways, whereas other hyphal-specific genes are controlled by only one of these two regulators. Finally, our microarray results predict the existence of at least one additional sequence-specific DNA-binding protein (unknown at this point) that recruits Tup1 and represses 7 hyphal-specific genes (Figure 6).

Although Rfg1, Nrg1, and Tup1 clearly have a key role in hyphal-specific gene regulation, our results also indicate that about half of the genes induced by serum and temperature are controlled independently of them. It is likely that direct transcriptional activation is responsible for much of this gene regulation, as a number of transcriptional activators have been implicated in the filamentous growth program (for reviews, see Ernst, 2000b; Brown, 2002b). We also note that in the case of some genes controlled by Rfg1-Tup1 or Nrg1-Tup1 (such as *HYR1* and *PHR1*), the level of expression is often higher during filamentous growth than it is in a strain grown in noninducing conditions but deleted for the appropriate repressor. Thus, at least some of the hyphal-specific genes that are negatively controlled by Nrg1, Rfg1, and Tup1 are likely also to be under direct positive control during filamentous growth.

Genes Involved in a Wide Variety of Biological Processes Are Induced during Filamentous Growth by Relief of Transcriptional Repression

Many of the genes induced in the blastospore-hyphal transition made conceptual sense. For example, a high proportion of the hyphal-specific genes encode cell wall components (Figures 5 and 6), some of which are known to function as adhesins important for adherence to host cells (Staab *et al.,* 1999; Zhao *et al.,* 2004); it has been known for many years that hyphae and blastospores differ in their adherence properties (Odds, 1985, 1988). A number of these cell wall components, such as *HWP1, ALS3,* and *HYR1* have been studied directly (Bailey *et al.,* 1996; Hoyer *et al.,* 1998; Staab *et al.,* 1999); the role of others, such as *PST1* and *IHD1*, have not been addressed experimentally. The majority of the cell wall class of proteins are under negative control by Rfg1, Nrg1, and Tup1 (Figure 6).

We also found that several of the hyphal-specific genes repressed by Rfg1, Nrg1, and Tup1 encoded proteins important for cell division. These genes included *KIP4*, a kinesin heavy-chain homolog (Hoyt *et al.,* 1992); *CDC10*, a septin involved in cytokinesis (Warenda and Konopka, 2002); and *RAX2*, a homolog of a membrane protein involved in bipolar

budding (Kang *et al.,* 2004). It is well known that the mechanisms of cell division differ between blastospores, pseudohyphae, and hyphae (Sudbery *et al.,* 2004), so this class of hyphal-specific genes makes intuitive sense. A number of hyphal-specific genes also encode secreted/degradative enzymes, such as members of the secreted aspartyl protease (*SAP*) family, which have long been known to play a role in *C. albicans* virulence (Hube, 1996; Hube and Naglik, 2002).

Several hyphal-specific genes controlled by Rfg1, Nrg1, and/or Tup1 appear to function in processes not previously implicated in filamentous growth and virulence. For example, *CBP1* encodes a human corticosteroid-binding protein with a putative oxidoreductase activity (Malloy *et al.,* 1993); this could be important in allowing hyphae to sense the local environment. *DCC1* functions as a putative DNA damage checkpoint protein (Chang *et al.,* 2002) that could be important for enabling *C. albicans* to cope with oxidative stress in the host environment. Two genes induced by serum and temperature (*UME6* and *USV1*) encode putative transcriptional regulators (Tzung *et al.,* 2001; Segal *et al.,* 2003), and it is possible they are responsible for activating a subset of the hyphal-specific genes through a regulatory cascade.

We also note the presence of one additional hyphal-specific gene class, ER/Golgi/secretion, in which the majority of genes do not appear to be under negative control by Rfg1, Nrg1 or Tup1 (Figure 6). The role of this gene class in filamentous growth and virulence is less obvious, but may signal a general need for increased protein expression and secretion during the morphological change.

Finally, a significant number of hyphal-specific genes, some of which are under negative control by the repressors described in this study, encode proteins of unknown function (Figure 6). Most of these genes are unique to *C. albicans* (at least so far), suggesting they mediate specific aspects of commensalism or pathogenesis.

Relationship between Virulence and the Blastospore-Hyphal Transition

Of the 61 genes induced by serum and temperature, 7 are known to be important for virulence in a mouse model of systemic candidiasis to our knowledge (Ghannoum *et al.,* 1995; Sanglard *et al.,* 1997; Staab *et al.,* 1999; Braun *et al.,* 2000; Warenda *et al.,* 2003; Martchenko *et al.,* 2004); virulence studies of the remaining genes, at least to our knowledge, have not been reported. It is also known that mutations that affect the regulation of hyphal-specific genes also lead to attenuated virulence (Lo *et al.,* 1997; Kadosh and Johnson, 2001; Saville *et al.,* 2003). Thus the blastospore-hyphal transition, which includes the induction of the 61 genes identified here, is clearly an important aspect of *C. albicans* virulence. The work in this article shows the key importance of negative transcriptional control in regulating this process and identifies a large set of genes that are implicated in specific aspects of virulence.

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