UME6, a Novel Filament-specific Regulator of *Candida albicans* Hyphal Extension and Virulence

Mohua Banerjee,* Delma S. Thompson,* Anna Lazzell,† Patricia L. Carlisle,* Christopher Pierce,† Carlos Monteagudo,‡ José L. López-Ribot,† and David Kadosh*

*Department of Microbiology and Immunology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229-3900; †Department of Biology, University of Texas at San Antonio, San Antonio, TX 78249; and ‡Department of Pathology, Universidad de Valencia, 46010 Valencia, Spain

The specific ability of the major human fungal pathogen *Candida albicans*, as well as many other pathogenic fungi, to extend initial short filaments (germ tubes) into elongated hyphal filaments is important for a variety of virulence-related processes. However, the molecular mechanisms that control hyphal extension have remained poorly understood for many years. We report the identification of a novel *C. albicans* transcriptional regulator, *UME6*, which is induced in response to multiple host environmental cues and is specifically important for hyphal extension. Although capable of forming germ tubes, the *ume6Δ/ume6Δ* mutant exhibits a clear defect in hyphal extension both in vitro and during infection in vivo and is attenuated for virulence in a mouse model of systemic candidiasis. We also show that *UME6* is an important downstream component of both the *RFG1-TUP1* and *NRG1-TUP1* filamentous growth regulatory pathways, and we provide evidence to suggest that Nrg1 and Ume6 function together by a negative feedback loop to control the level and duration of filament-specific gene expression in response to inducing conditions. Our results suggest that hyphal extension is controlled by a specific transcriptional regulatory mechanism and is correlated with the maintenance of high-level expression of genes in the *C. albicans* filamentous growth program.

INTRODUCTION

Candida albicans, the most common human fungal pathogen, is responsible for a wide variety of mucosal and systemic infections. Mucosal infections include oral and vaginal thrush, whereas systemic infections can occur in nearly every organ and tissue of the human body (Odds, 1988; Dupont, 1995; Weig *et al.,* 1998). *Candida* spp. are the fourth-leading cause of hospital-acquired bloodstream infections in the United States, with an attributable mortality rate approaching 35% (Edmond *et al.,* 1999). In this country approximately $1 billion per year is spent on antifungal treatments for patients with hospital-acquired *Candida* infections (Miller *et al.,* 2001). Immunocompromised individuals, including AIDS patients, organ transplant recipients, cancer patients on chemotherapy, and recipients of artificial joints and prosthetic devices are especially vulnerable to infection (for reviews see Shepherd *et al.,* 1985; Dupont, 1995; Weig *et al.,* 1998).

  C. albicans possesses several virulence properties including the ability to undergo a reversible morphological conversion from single round budding yeast cells (blastospores) to elongated cells attached end-to-end (filaments) (for reviews see Mitchell, 1998; Brown, 2002; Calderone and Gow, 2002). *C. albicans* filaments are known to occur in two distinct forms: pseudohyphae and hyphae. Pseudohyphal cells

are elliptical in shape and have constrictions at cell junctions, whereas hyphal cells have parallel sides and true septa (lacking constrictions); for a more complete description of the differences between these two forms see Sudbery *et al.* (2004). Tissues infected with *C. albicans* typically contain a mixture of blastospores, pseudohyphae, and hyphae (Odds, 1988).

  The *C. albicans* blastospore-to-filament transition is required for virulence and is known to occur in response to a wide variety of inducing conditions present in host tissues including serum, body temperature, *N*-actetylglucosamine, neutral pH, amino acids, and certain human hormones (Odds, 1988; Lo *et al.,* 1997; Brown, 2002; Saville *et al.,* 2003). When *C. albicans* cells encounter these conditions, they initially form small projections, termed "germ tubes." Subsequent cell division at the apical tip of the germ tube allows *C. albicans* to form extended filaments (Odds, 1988; Sudbery *et al.,* 2004).

  The ability to form and extend hyphal filaments has been associated with a number of virulence-related processes in *C. albicans* as well as several other pathogenic fungi. Studies on clinical isolates have shown a clear correlation between extensive invasion of oral epithelial mucosal surfaces and increased number of hyphal filaments (Bartie *et al.,* 2004). Other studies have reported that although *C. albicans* hyphae can be found within epithelial cells, blastospores are generally found either between these cells or on the epithelial cell surface, strongly suggesting that extended hyphae are the invasive form (Scherwitz, 1982; Ray and Payne, 1988; Filler and Sheppard, 2006). Indeed, epithelial cell invasion is believed to be caused by the mechanical force exerted by hyphal extension (Kumamoto and Vinces, 2005b). *C. albicans*

Address correspondence to: David Kadosh (kadosh@uthscsa.edu).

hyphal filament extension is critical for the process of thigmotropism (guidance of hyphal growth along ridges of the substratum, which is believed to be an adaptation for tissue invasion) and also plays an important role in the ability of *C. albicans* to breach endothelial cells and lyse macrophages and neutrophils when endocytosed (Zink *et al.*, 1996; Lo *et al.*, 1997; Jong *et al.*, 2001; Korting *et al.*, 2003; Kumamoto and Vinces, 2005a,b). In the widespread fungal pathogen *Aspergillus fumigatus*, as well as Zygomycete fungi such as *Rhizopus oryzae*, hyphal filament extension is specifically important for the process of angioinvasion (Filler and Sheppard, 2006). Although the importance of hyphal extension to virulence in *C. albicans* and other pathogenic fungi has been well-established, little is known about the molecular mechanisms that control this process or the specific molecular properties of *C. albicans* hyphal filaments that are associated with enhanced virulence.

To address these questions, we previously identified, by DNA microarray analysis, a transcriptional program of 61 genes that is induced in the presence of one of the strongest filament-inducing conditions, serum at 37°C (Kadosh and Johnson, 2005). Although certain genes in this program do not appear to be involved in filamentation per se, they do carry out several known virulence functions (e.g., adherence to host cells and degradation of host cell membranes), suggesting that filament formation and hyphal extension are coregulated with other virulence properties. In this study we examine the basis for hyphal filament extension by focusing on the mechanisms that are important for controlling induction and maintaining expression of the *C. albicans* filamentous growth program. We have previously demonstrated that approximately half of all genes in this program are under negative control in the blastospore form by three key transcriptional repressors, Rfg1, Nrg1, and Tup1 (Kadosh and Johnson, 2005). Strains deleted for any of these regulators are filamentous in the absence of filament-inducing conditions and are highly attenuated for virulence in a mouse model of systemic candidiasis (Braun and Johnson, 1997; Braun *et al.*, 2000, 2001; Kadosh and Johnson, 2001; Khalaf and Zitomer, 2001; Murad *et al.*, 2001). In *Saccharomyces cerevisiae*, the *RFG1* and *NRG1* homologues encode DNA-binding proteins, which are known to direct transcriptional repression of target genes by recruitment of the Tup1 corepressor (Keleher *et al.*, 1992; Deckert *et al.*, 1995; Tzamarias and Struhl, 1995; Park *et al.*, 1999; Smith and Johnson, 2000). Numerous lines of evidence have suggested that in *C. albicans* these regulators function in a similar manner to direct repression of filament- and virulence-specific target genes (Braun *et al.*, 1997, 2000, 2001; Kadosh and Johnson, 2001; Khalaf and Zitomer, 2001; Murad *et al.*, 2001). Previous studies have demonstrated that the *C. albicans* *NRG1* transcript is down-regulated in response to serum at 37°C (Braun *et al.*, 2001; Murad *et al.*, 2001). Down-regulation of *NRG1* appears to be an important step in the blastospore to filament transition because strains expressing *NRG1* at high constitutive levels are locked in the blastospore form and are highly attenuated for virulence in a mouse model of systemic candidiasis (Braun *et al.*, 2001; Saville *et al.*, 2003).

Here, we report the identification of a novel filament-specific transcriptional regulator of *C. albicans* hyphal extension and virulence, *UME6*, which is important for controlling the level and duration of *NRG1* down-regulation as well as maintaining expression of filament-specific genes in response to inducing conditions. We examine the functional relationships between *UME6* and both the *RFG1-TUP1* and *NRG1-TUP1* filamentous growth regulatory pathways. The identification of *UME6* also provides us with a unique op-portunity to examine the specific transcriptional regulatory mechanisms that control the important, but poorly understood, *C. albicans* virulence trait of hyphal filament extension.

MATERIALS AND METHODS

Strains and DNA Constructions

Wild-type (CAF2-1), *rfg1Δ/rfg1Δ*, *nrg1Δ/nrg1Δ*, and *tup1Δ/tup1Δ* strains have been described previously (Fonzi and Irwin, 1993; Braun and Johnson, 1997; Braun *et al.*, 2001; Kadosh and Johnson, 2001), and the genotypes of all strains used in this study are listed in Table 1. The *ume6Δ/ume6Δ* strain was generated using a fusion PCR strategy previously described by Noble and Johnson (Noble and Johnson, 2005). Briefly, 5′ and 3′ flanking sequences immediately outside of the *UME6* coding region were generated using primers 1 and 2 for the upstream flank and primers 3 and 4 for the downstream flank (see Table S1 for a listing of primers used in this study). These flanks were then used in a second round of fusion PCR with *LEU2* and *HIS1* markers (obtained using primers 5/6 and plasmids pSN40 and pSN52) to generate *ume6Δ::LEU2* and *ume6Δ::HIS1* knockout PCR products. To construct the strain MBY1 (*ume6Δ/+*), SN152 (*leu2⁻, his1⁻, arg4⁻*; Noble and Johnson, 2005) was first transformed with *ume6Δ::LEU2*, and wild-type alleles of *C. albicans* *ARG4* (generated using primers 33/34) and *HIS1* (generated using the primers 35/36) were also subsequently added back to this strain. Our wild-type control strain, DK318, was constructed by adding back wild-type alleles of *C. albicans* *ARG4* and *HIS1* to strain SN95 (*his1⁻, arg4⁻*; Noble and Johnson, 2005) using the PCR products described above. The *ume6Δ/ume6Δ* strain (DK312) was generated by transforming SN152 with *ume6Δ::LEU2*, followed by *ume6Δ::HIS1* and finally by a wild-type allele of *C. albicans* *ARG4*. To construct the *ume6Δ/ume6Δ::UME6* add-back strain, a 4.3-kb BamHI-XhoI fragment containing *Candida dubliniensis ARG4* was cloned from pSN69 to pSN75. Roche Expand High Fidelity Plus polymerase (Roche Diagnostics, Basel, Switzerland) was next used to generate a 4.3-kb BamHI-BamHI PCR fragment containing *UME6* (using primers 7/8), which was subsequently cloned into pSN75-*ARG4* cut with BamHI. The resulting construct was linearized by digestion with SmaI and integrated at the *UME6* promoter of a *ume6Δ/ume6Δ* (*arg4⁻*) strain (DK244). The *nrg1Δ/nrg1Δ ume6Δ/ume6Δ* (MBY61) and *rfg1Δ/rfg1Δ ume6Δ/ume6Δ* (MBY79) strains were constructed as follows: first, *rfg1Δ::ARG4* and *nrg1Δ::ARG4* PCR knockout products were generated by fusion PCR (using primers 9/10 and 11/12 for *NRG1* flanks, primers 17/18 and 19/20 for *RFG1* flanks and primers 5/6 for plasmid pSN69 containing the *ARG4* marker) and were used to transform *ume6Δ/ume6Δ* (*arg4⁻*) strain (DK244). To delete the second copy of each gene, PCR was used to generate 5′ and 3′ flanks for *RFG1* (with primers 21/22 and 23/24) and *NRG1* (with primers 13/14 and 15/16). The 5′ *RFG1* and *NRG1* flanks were digested with KpnI and XhoI, and the 3′ flanks were digested with NotI and SacII; the resulting fragments were cloned into vector pSFS2 (Reuss *et al.*, 2004) digested with the appropriate restriction enzymes. Finally, the resulting constructs were digested with SacII and KpnI to release *rfg1Δ::SAT1* and *nrg1Δ::SAT1* fragments (5.2 kb each), which were used to transform *rfg1Δ/+ ume6Δ/ume6Δ* and *nrg1Δ/+ ume6Δ/ume6Δ* strains, respectively; the *SAT* marker was subsequently looped out of the final double mutant strains as described previously (Reuss *et al.*, 2004). The *tup1Δ/tup1Δ ume6Δ/ume6Δ* strain (MBY93) was generated as follows: PCR was used to generate *UME6* 5′ and 3′ flanks (with primers 25/26 and 27/28). The 5′ *UME6* flank was digested with BamHI and PstI, and the 3′ *UME6* flank was digested with BglII and KpnI; the resulting fragments were cloned into pBB510 (Braun and Johnson, 2000) digested with the appropriate restriction enzymes. A 4.9-kb PstI-KpnI fragment containing the 5′ and 3′ *UME6* flanking regions and the *URA3* marker was then released from this construct and used to disrupt the first copy of *UME6* in strain BCa2-5 (*tup1Δ/tup1Δ, ura3⁻*; Braun and Johnson, 1997). To delete the second copy of *UME6* in this strain, 5′ and 3′ *UME6* flanks (obtained by PCR with primers 29/30 and 31/32) were digested with KpnI-XhoI and NotI-SacII, respectively, and cloned into pSFS2 cut with the appropriate restriction enzymes. The resulting construct was digested with KpnI and SacII to release a 5.2-kb *ume6Δ::SAT1* fragment, which was used to generate the final *tup1Δ/tup1Δ ume6Δ/ume6Δ* strain (MBY93). Correct integration of all disruption constructs was verified by whole cell PCR across the 5′ and 3′ disruption junctions and absence of the open reading frame (ORF) in homozygous deletion strains was confirmed by PCR using internal ORF primers.

Media and Growth Conditions

Standard non–filament-inducing growth conditions for *C. albicans* were yeast extract-peptone-dextrose (YEPD) medium at 30°C (Guthrie and Fink, 1991). Serum medium consisted of YEPD plus 10% fetal calf serum (FCS). Spider and Lee's media were prepared as described previously (Lee *et al.*, 1975; Liu *et al.*, 1994). Liquid Spider-, Lee's pH 6.8-, serum- and temperature-induction experiments were carried out, with certain modifications, using previously described protocols (Kadosh and Johnson, 2005). For DNA microarray experiments, saturated overnight cultures of both wild-type (DK318) and *ume6Δ/ume6Δ* (DK312) strains were diluted into 2 L of YEPD medium and grown at 30°C overnight until cells reached an OD$_{600}$ of ~8.5 (first experiment) or

Table 1. Strains used in this study

Strain	Genotype	Source
CAF2-1	*ura3Δ::imm[434]/URA3 iro1Δ::imm[434]/IRO1*	Fonzi and Irwin (1993)
SN95	*arg4Δ/arg4Δ his1Δ/his1Δ ura3Δ::imm[434]/URA3 iro1Δ::imm[434]/IRO1*	Noble and Johnson (2005)
SN152	*arg4Δ/arg4Δ leu2Δ/leu2 Δ his1Δ/his1Δ ura3Δ::imm[434]/URA3 iro1Δ::imm[434]/IRO1*	Noble and Johnson (2005)
DK318 (WT)	*arg4Δ::ARG4/arg4Δ his1Δ::HIS1/his1 Δ ura3Δ::imm[434]/URA3 iro1Δ::imm[434]/IRO1*	This study
MBY1 (*ume6Δ/+*)	*ume6Δ::C.m.LEU2/UME6 arg4Δ::ARG4/arg4Δ leu2Δ/leu2Δ his1Δ::HIS1/his1Δ ura3Δ ::imm[434]/URA3 iro1Δ ::imm[434]/IRO1*	This study
DK244	*ume6Δ::C.m.LEU2/ume6Δ::C.d.HIS1 arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ ura3Δ::imm[434]/URA3 iro1Δ::imm[434]/IRO1*	This study
DK312 (*ume6Δ/Δ*)	*ume6Δ::C.m.LEU2/ume6Δ::C.d.HIS1 arg4Δ::ARG4/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ ura3Δ::imm[434]/URA3 iro1Δ::imm[434]/IRO1*	This study
MBY16 (*ume6Δ/Δ::UME6*)	*ume6Δ::UME6:C.d.ARG4:C.m.LEU2/ume6Δ::C.d.HIS1 arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ ura3Δ::imm[434]/URA3 iro1Δ::imm[434]/IRO1*	This study
MBY79 (*ume6Δ/Δ rfg1Δ/Δ*)	*ume6Δ::C.m.LEU2/ume6Δ::C.d.HIS1 rfg1Δ::ARG4/rfg1Δ::frt arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ ura3Δ::imm[434]/URA3 iro1Δ::imm[434]/IRO1*	This study
MBY61 (ume6Δ/Δ nrg1Δ/Δ)	*ume6Δ::C.m.LEU2/ume6Δ::C.d.HIS1 nrg1Δ::ARG4/nrg1Δ::frt arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ ura3Δ::imm[434]/URA3 iro1Δ::imm[434]/IRO1*	This study
MBY93 (*ume6Δ/Δ tup1Δ/Δ*)	*ume6Δ::URA3/ume6Δ::SAT1 tup1Δ/tup1Δ ura3Δ::imm[434]/ura3Δ::imm[434] iro1Δ::imm[434]/iro1Δ::imm[434]*	This study
BCa2-10 (*tup1Δ/Δ*)	*tup1Δ/tup1Δ::URA3 ura3Δ::imm[434]/ura3Δ::imm[434] iro1Δ::imm[434]/iro1Δ::imm[434]*	Braun and Johnson, (1997)
BCa23-3 (*nrg1Δ/Δ*)	*nrg1Δ/nrg1Δ::URA3 ura3Δ::imm[434]/ura3Δ::imm[434] iro1Δ::imm[434]/iro1Δ::imm[434]*	Braun *et al.* (2001)
DK129 (*rfg1Δ/Δ*)	*rfg1Δ/rfg1Δ::URA3 ura3Δ::imm[434]/ura3Δ::imm[434] iro1Δ::imm[434]/iro1Δ::imm[434]*	Kadosh and Johnson (2001)

C.d., *C. dubliniensis*; C.m., *C. maltosa*.

~10.5 (second experiment). At this point (the zero time point) for each strain was harvested for RNA preparation, and additional 200-ml aliquots were diluted into 2 L of fresh pre-warmed YEPD medium at 30°C or YEPD medium plus 10% serum at 37°C. Cells were harvested at 1-, 2-, 3- and 5-h time points for RNA preparation (please note that this protocol is similar to our previously described optimized protocol for serum and temperature induction (Kadosh and Johnson, 2005)). A similar procedure was used to prepare cells for the Nrg1-Ume6 kinetics experiment (Figure 6) except that cells were grown overnight in YEPD medium to an OD_{600} of ~ 4.0 and diluted 1:10 into 150 ml of pre-warmed YEPD at 30°C or YEPD plus 10% serum at 37°C, and 5-ml aliquots were harvested for RNA preparation (we have noticed that the optimal OD_{600} for serum and temperature induction can vary depending on culture volume). For the experiment testing the effect of various filament-inducing conditions on *UME6* transcript levels (Figure 5), cells were grown overnight in YEPD to an OD_{600} of ~4.0, and diluted 1:25 into 150 ml of each inducing medium (pre-warmed), and 40-ml aliquots were harvested for RNA preparation at 30 min after induction. In the Nrg1-Tup1-Ume6 epistasis experiment (Figure 8B), strains were grown overnight under non–filament-inducing conditions (YEPD medium at 30°C) to an OD_{600} of ~1.0, at which point 5-ml aliquots of cells were collected to prepare RNA. For the Rfg1-Ume6 epistasis experiment (see Figure 8C), a saturated overnight culture of each strain was diluted in YEPD medium to an OD_{600} of 0.2 and grown at 30°C to an OD_{600} of ~1.0. For both epistasis experiments 5-ml aliquots of cells were collected for RNA preparation (although data for one wild-type strain (CAF2-1) is shown for the epistasis experiments, we also obtained very similar results using the DK318 wild-type strain as well).

RNA Preparation and Analysis

For both DNA microarray and Northern analysis, total RNA was prepared using hot acid phenol (as described previously by Ausubel *et al.,* 1992) and quantified by spectrophotometer. For Northern analysis 3 μg of RNA from each sample was loaded onto a formaldehyde denaturing gel and transferred by capillary action to a GeneScreen Plus nylon membrane (Perkin Elmer, Waltham, MA). Probes for Northern analysis were generated by PCR, purified using a Qiaquick column and labeled with α-^{32}P-dATP using a random priming kit (GE Healthcare Biosciences, Piscataway, NJ) (see Table S1 for a complete listing of primers used to generate the probes). Blots were hybridized overnight at 42°C, washed twice for 10 min at 59°C using the Church protocol (Church and Gilbert, 1984), and scanned using a Molecular Dynam-

ics Typhoon 9400 phosphorimager (Sunnyvale, CA). Autoradiographs were visualized and quantified using Image Quant 2.0 software (Molecular Dynamics).

DNA Microarray Analysis

Construction of whole-genome *C. albicans* DNA microarrays, poly(A) RNA selection, cDNA preparation, and hybridization of coupled cDNA to microarrays as well as DNA microarray analysis were carried out as previously described (Bennett *et al.,* 2003; Kadosh and Johnson, 2005). All data were normalized by NOMAD (http://ucsf-nomad.sourceforge.net) and filtered so as to include only spots with a median signal intensity ≥500 in at least one channel. The serum- and temperature-induction experiment was carried out twice using both wild-type and *ume6/ume6Δ* strains (note that microarray experiment 2 (Supplementary Data) was performed using oligonucleotide-based arrays). Each hybridization contained a Cy5-labeled experimental sample and a Cy3-labeled universal reference sample, which has been previously described (Kadosh and Johnson, 2005). For both wild-type and *ume6/ume6Δ* samples, signal ratios (ratio of the median signal intensity of each spot) for each time point versus reference were divided by the signal ratio of the zero time point (for each strain, respectively) versus reference. Signal ratio values for the zero time point versus reference (for both strains) represented median values from three independent DNA microarray experiments (using cDNAs from the same total RNA preparation). Gene annotation was based on the *C. albicans* orf19 assembly (Braun *et al.,* 2005) and on the *Candida* Genome Database (Arnaud *et al.,* 2005).

Virulence Experiments and Histology

Overnight cultures (grown in YEPD medium at 25°C) of wild-type (DK318), *ume6Δ/ume6Δ* and *ume6Δ/ume6Δ::UME6* strains were harvested by centrifugation and washed three times in sterile pyrogen-free saline. Based on cell counts made using a hemocytometer, appropriate dilutions were carried out, and 200 μl of cell suspension from each strain was injected by lateral tail vein into individual 6–8-wk-old BALB/c mice (eight mice per group). Mice were monitored for survival for 21 d after infection (moribund mice were killed and were recorded as dying on the next day). The Kaplan-Meier log rank test was used to determine statistical differences between groups and analyses were carried out using Prism and GraphPad Software (San Diego, CA).

For all animals, one kidney was removed at the time of death for histological analysis. Briefly, these kidneys were fixed in 10% buffered Formalin, embedded in paraffin, sectioned, and stained with Grocot-Gomori methenamine-silver (GMS).

RESULTS

UME6 Is Important for C. albicans Filamentous Growth in Response to a Variety of Filament-inducing Conditions

UME6 was first identified as a component of the *C. albicans* filamentous growth program that is induced in response to the host filament-inducing conditions of serum and body temperature, 37°C (Kadosh and Johnson, 2005). The *S. cerevisiae UME6* homolog encodes a zinc-finger DNA-binding protein that is known to function as a key transcriptional regulator of early meiosis-specific genes and is also important for controlling arginine catabolism, peroxisomal function, and DNA repair (Strich *et al.,* 1994; Bowdish *et al.,* 1995; Einerhand *et al.,* 1995; Steber and Esposito, 1995; Sweet *et al.,* 1997). *C. albicans UME6* (orf19.1822) encodes a 843-amino acid protein with a zinc-finger DNA-binding domain that is 41% identical to that of *S. cerevisiae* Ume6. Although *C. albicans* is not known to undergo meiosis, we hypothesized that *CaUME6* may play a role in filamentous growth because this gene is induced by serum and 37°C and is repressed by the Nrg1-Tup1 pathway in blastospores under non–filament-inducing conditions (Kadosh and Johnson, 2005). To further explore this possibility, we generated both heterozygous and homozygous *ume6* deletion mutants and tested for filamentation on a variety of solid filament-inducing media (wrinkled or rough colonies typically indicate filamentation). As shown in Figure 1 the *ume6Δ/ume6Δ* mutant is significantly defective for filamentation in response to Spider medium (nitrogen and carbon starvation), Lee's medium, pH 6.8 (neutral pH), and YEPD medium plus 10% serum at 37°C com-
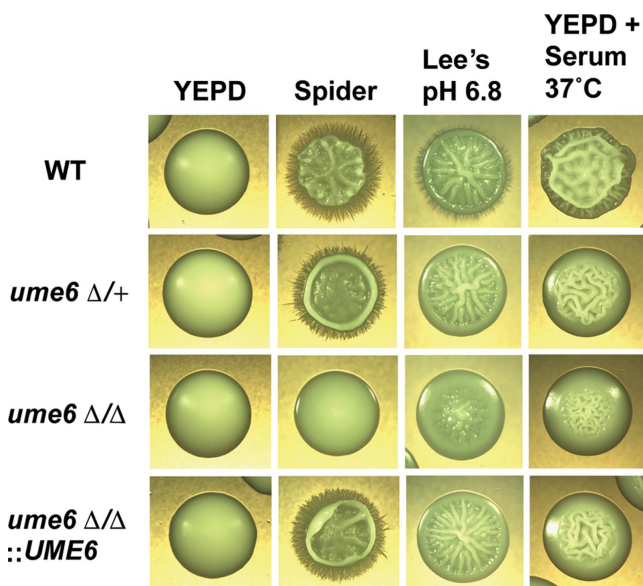
pared to a wild-type strain under these same growth conditions (microscopic examination revealed that under all filament-inducing conditions the *ume6Δ/ume6Δ* mutant formed filaments that were significantly shorter in length than those of the wild-type strain). In contrast, the *ume6Δ/+* strain showed a milder filamentation defect, suggesting that the *ume6* mutant phenotype is dosage-dependent. We also generated a strain in which a single wild-type copy of *UME6* is added back to the *ume6Δ/ume6Δ* mutant. The *ume6Δ/ume6Δ::UME6* "add-back" strain showed clear complementation of the *ume6Δ/ume6Δ* filamentation defect and appeared phenotypically similar to the *ume6Δ/+* strain under all filament-inducing conditions tested (Figure 1), confirming that the original defect was due to a loss of *UME6* function.

Because filamentation is known to be important for *C. albicans* biofilm formation (for reviews see Ramage *et al.,* 2005; Blankenship and Mitchell, 2006), we also tested the strains described above for their ability to form biofilms on an inert substrate (polystyrene wells). The *ume6Δ/ume6Δ* mutant showed a partial defect in biofilm formation that was complemented in the *ume6Δ/ume6Δ::UME6* "add-back" strain (Figure S1).

UME6 Is Specifically Important for Hyphal Filament Extension

Because *UME6* is induced by serum and temperature, we next sought to determine the extent to which this regulator is required for hyphal filament formation in liquid medium under these conditions. Using a previously optimized protocol in which nearly all cells are induced to form hyphal filaments (Kadosh and Johnson, 2005), we carried out a serum- and temperature-induction time course experiment to compare wild-type and *ume6Δ/ume6Δ* strains. Aliquots of cells from both strains were harvested at 0-, 1-, 2-, 3-, and 5-h time points, fixed, and examined by microscopy (Figure 2). In contrast to the wild-type strain, the *ume6Δ/ume6Δ* mutant exhibited a clear defect in the extension of hyphal filaments. Many cells formed germ tubes within the first 2 h of induction. At the 3-h time point many of these cells remained as germ tubes or very short filaments and by the 5-h time point, a large fraction of *ume6Δ/ume6Δ* cells appeared to take on an elongated blastospore-type morphology. The long extended hyphal filaments normally present in the wild-type strain at the 3- and 5-h time points were absent in the *ume6Δ/ume6Δ* mutant (Figure 2). Thus, although not required for initial germ tube formation, Ume6 appears to be specifically important for hyphal filament extension.

UME6 Controls the Level and Duration of Gene Expression in the C. albicans Filamentous Growth Program

To examine the expression levels of filament-specific transcripts, both wild-type and *ume6Δ/ume6Δ* mutant cells were harvested at each time point during the serum- and temperature-induction experiment described above, and RNA was prepared for DNA microarray analysis. As indicated by the cluster diagram shown in Figure 3A, although nearly all of the top filament-specific target genes were induced in both wild-type and *ume6Δ/ume6Δ* strains, the level and duration of induction was significantly reduced in the *ume6Δ/ume6Δ* mutant. A transcriptional profile (Figure 3B) indicates that although some of the more highly induced genes in the wild-type strain, such as *ALS3*, are also strongly induced in the *ume6Δ/ume6Δ* mutant at the 1-h time point, others (such as *SAP4* and *HYR1*) show reduced expression even at this early stage; most transcripts showed significantly reduced induction in the *ume6Δ/ume6Δ* strain at the 2-, 3- and 5-h
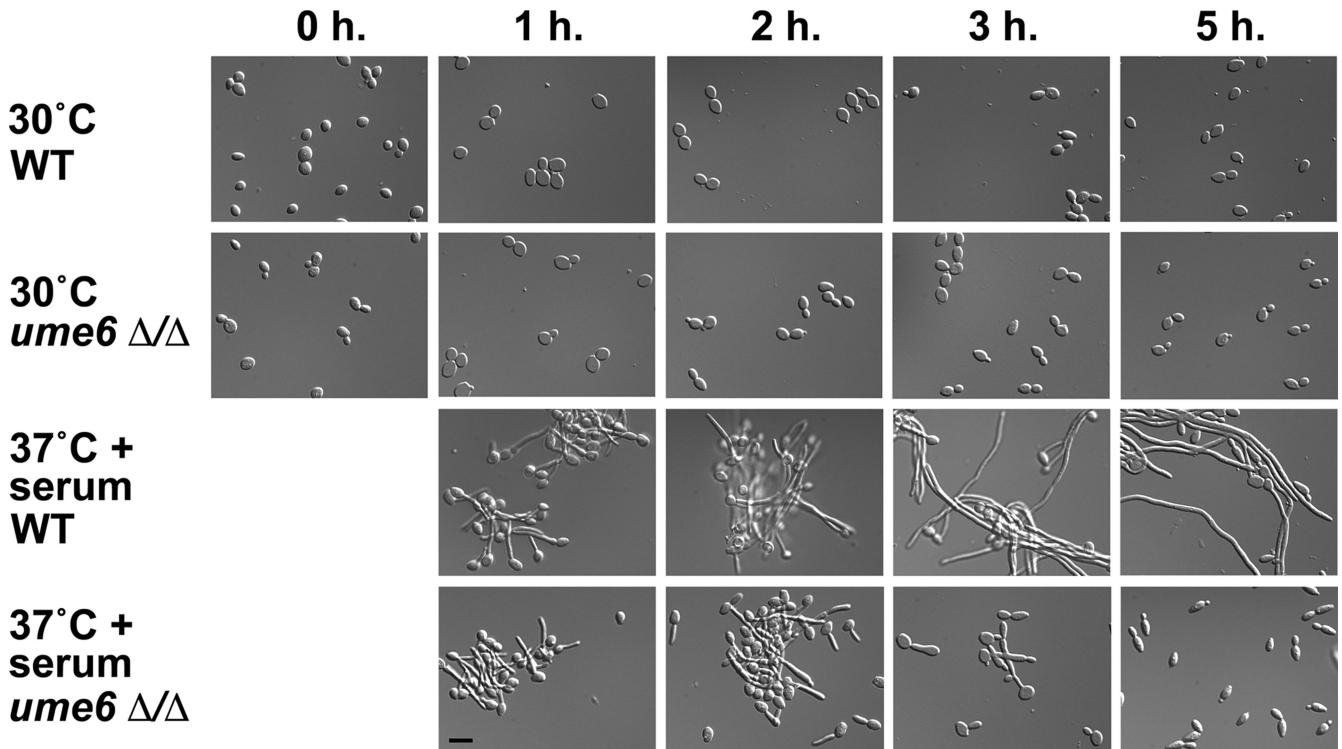

	YEPD	Spider	Lee's pH 6.8	YEPD + Serum 37°C
WT				
ume6 Δ/+				
ume6 Δ/Δ				
ume6 Δ/Δ ::*UME6*				

Figure 1. *ume6Δ/Δ* mutants are defective for filamentous growth in response to a variety of filament-inducing conditions. Colony morphologies of wild-type (WT), *ume6Δ/+*, *ume6Δ/Δ*, and *ume6Δ/Δ::UME6* (add-back) strains grown on solid non–filament-inducing medium (YEPD) and on various solid filament-inducing media. All colonies were grown at 30°C for 5 d, with the exception of cells grown on YEPD + 10% serum plates, which were grown at 37°C for 3 d, and photographed at ∼×20 magnification.

Figure 2. Morphology of wild-type and *ume6Δ/Δ* cells undergoing the blastospore to filament transition. Wild-type (WT) and *ume6Δ/Δ* strains grown under non–filament-inducing conditions (YEPD medium at 30°C) were diluted into pre-warmed YEPD medium at 30°C or 37°C in the presence or absence of 10% fetal calf serum (FCS). Induction time (h) is shown on top. Aliquots of cells were fixed in 4.5% formaldehyde, washed twice in 1× PBS, and then visualized by Nomarski/DIC optics. Please note that the 0-h time point shows cells immediately before induction. Bar, 10 μm.
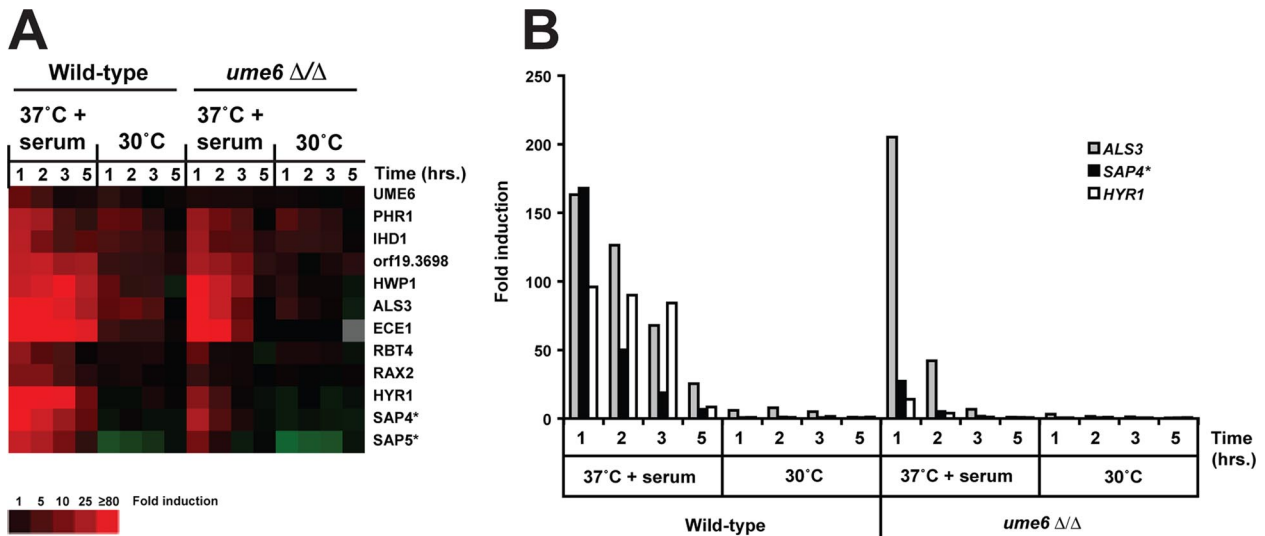
Figure 3. Transcriptional profile of serum- and temperature-induced genes in wild-type and *ume6Δ/Δ* strains. (A) Cluster diagram indicating expression levels of several previously identified (Kadosh and Johnson, 2005) top serum- and temperature-induced transcripts (≥5-fold mean induction in the wild-type strain, n = 2, at the 37°C + 10% FCS 1-h time point). Only data from one serum- and temperature-induction experiment and only genes with greater than 93% of data present are shown. Red, increased expression; green, reduced expression; gray, no data available. Expression levels reflect fold induction relative to the zero time point for each strain. None of the genes showed a significant difference in absolute level of expression between wild-type and *ume6Δ/Δ* strains at the zero time point. (B) Histogram indicates fold induction, relative to the zero time point of wild-type or *ume6Δ/Δ* strains, for three serum- and temperature-induced genes: *ALS3*, an adhesin important for both epithelial and endothelial cell adhesion (Zhao *et al.,* 2004), *SAP4*, a secreted aspartyl protease important for host tissue invasion and virulence (Sanglard *et al.,* 1997; Schaller *et al.,* 1999), and *HYR1*, a putative cell wall glycoprotein (Bailey *et al.,* 1996) described in A. *Note that expression levels of *SAP5* may, in part, reflect those of *SAP4* or *SAP6* (and *SAP4* levels may reflect *SAP5* and *SAP6* levels) due to cross-hybridization on the microarray, since these genes have nearly identical DNA sequences.

time points. These results correlate well with the morphological differences observed between wild-type and *ume6Δ/ume6Δ* strains (Figures 1 and 2) and suggest that Ume6 is required to maintain full expression of the *C. albicans* filamentous growth program in response to serum at 37°C.

The *ume6 Δ/ume6Δ* Mutant Is Attenuated for Virulence and Defective for Hyphal Extension in a Mouse Model of Systemic Candidiasis

In an experiment to determine the effect of the *ume6Δ/ume6Δ* mutation on virulence, wild-type, *ume6Δ/ume6Δ*, and *ume6/ume6Δ::UME6* (add-back) strains were each injected by tail vein into eight 6–8-wk-old female BALB/c mice. Although mice injected with the wild-type strain all died by 5–9 d after infection, mice injected with the *ume6Δ/ume6Δ* strain generally died after a significantly longer time period (p ≤ 0.0005), indicating that the *ume6Δ/ume6Δ* mutant is attenuated for virulence. The *ume6Δ/ume6Δ::UME6* strain did not show a statistically significant difference in virulence compared with the wild-type strain, indicating that the observed virulence defect of the *ume6Δ/ume6Δ* strain was specifically due to a loss of *UME6* function (Figure 4A).
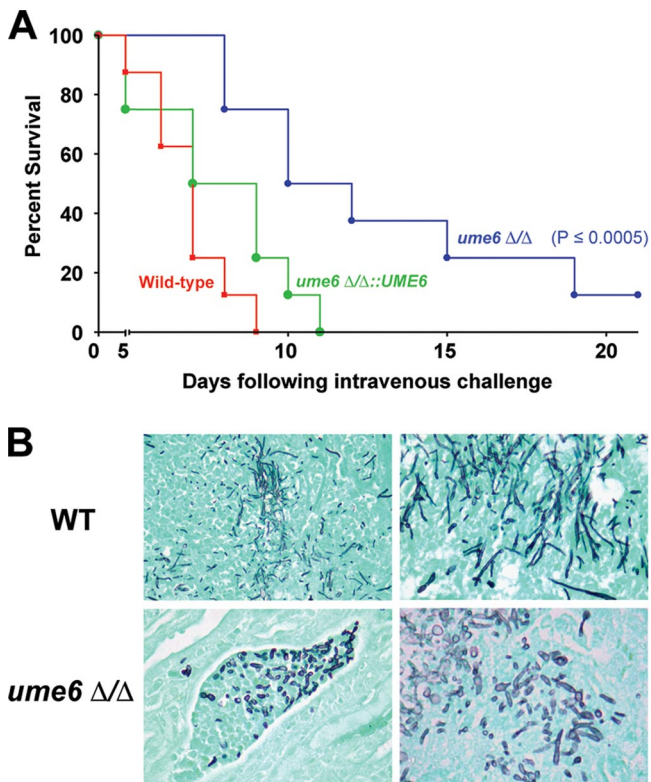
We next carried out histopathology studies to determine whether the virulence defect of the *ume6Δ/ume6Δ* mutant was due to altered *C. albicans* morphology during infection. Mouse kidneys were fixed, sectioned, and stained to visualize fungal cells. In contrast to kidneys of mice infected with a wild-type strain, which showed a mixture of blastospores and extended hyphal and pseudohyphal filaments, kidneys of mice infected with the *ume6Δ/ume6Δ* mutant showed a mixture of blastospores and short, stubby filaments (Figure 4B). These findings are consistent with our previous results (Figure 2) and indicate that the *ume6Δ/ume6Δ* mutant is defective for hyphal filament extension both in vitro as well as during a systemic infection in vivo.

UME6 Is Induced in Response to a Variety of Filament-inducing Conditions

The results described above suggest that induction of *UME6* appears to be critical for proper hyphal filament extension both during infection in vivo and in response to the combination of serum and 37°C in vitro. We next sought to determine the relative contribution of serum and temperature as well as a variety of other filament-inducing conditions present in host tissues to *UME6* induction. We examined induction of *UME6* by Northern analysis in response to YEPD plus 10% serum alone, YEPD at 37°C, YEPD plus 10% serum at 37°C, Spider medium (nitrogen and carbon starvation), and Lee's medium (at both pH 4.5 and 6.8) (Figure 5). We found that serum and 37°C both made separate contributions and showed an additive effect on *UME6* induction. The *UME6* transcript was also induced in the presence of both Spider and Lee's pH 6.8, but not Lee's pH 4.5 media. Interestingly, *NRG1*, which encodes a transcriptional regulator that has been shown to repress *UME6* under non–filament-inducing conditions (Kadosh and Johnson, 2005), is down-regulated in response to serum and temperature as well as neutral pH (Braun *et al.,* 2001; Murad *et al.,* 2001; Lotz *et al.,* 2004). Taken together, these results suggest that *UME6* and *NRG1* may function together in a coordinated manner to control induction of filament-specific target genes in response to a variety of specific inducing conditions and signaling pathways.
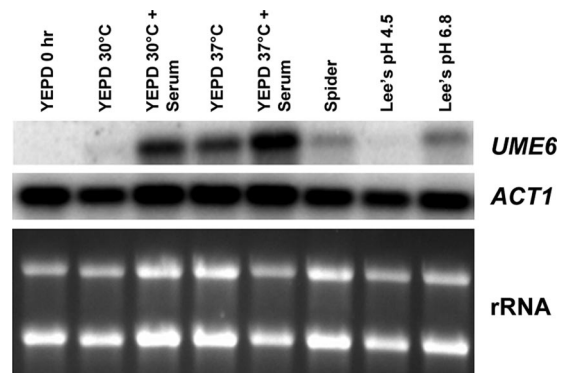

Figure 4. The *ume6Δ/Δ* mutant is attenuated for virulence and defective for hyphal filament extension in a mouse model of systemic candidiasis. (A) Eight female BALB/c mice (6–8 wk old) were each injected with 2 × 10⁵ CFUs of a wild-type, *ume6Δ/Δ*, or *ume6Δ/Δ::UME6* (add-back) strain. Survival was monitored over the course of 21 d. A Kaplan-Meier test was performed to confirm that the difference in virulence between the wild-type and *ume6Δ/Δ* strains is statistically significant (p ≤ 0.0005) (the *ume6Δ/Δ::UME6* strain did not show a statistically significant difference in virulence when compared with the wild-type strain). (B) Kidney tissues from infected mice were fixed, sectioned, and stained with Grocot-Gomori methenamine-silver to visualize fungal cells. Top panels, sections taken from mice infected with the wild-type (WT) strain; bottom panels, sections taken from mice infected with the *ume6Δ/Δ* strain.

Figure 5. Effect of a variety of filament-inducing conditions on induction of *UME6*. A wild-type strain was grown under non–filament-inducing conditions (YEPD medium at 30°C) and diluted into pre-warmed flasks containing either YEPD or the indicated filament-inducing media at 30°C (or 37°C where shown). Cells were harvested at the zero time point (immediately before induction) and at 30 min after induction for total RNA preparation. Northern analysis was carried out using 3 μg of RNA from each sample to assess transcript levels of the indicated genes. The *ACT1* transcript, as well as ribosomal RNA (rRNA) are shown as loading controls.
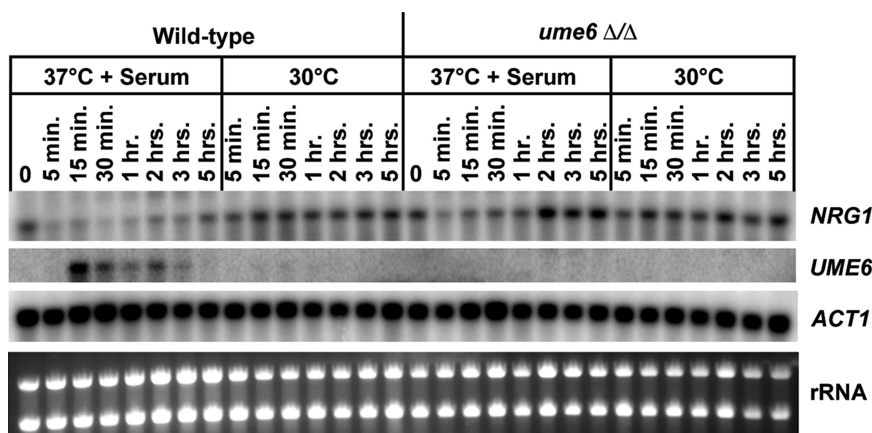
Figure 6. Kinetics of *NRG1* down-regulation and *UME6* induction in response to serum at 37°C. Wild-type and *ume6*Δ/Δ strains were grown under non–filament-inducing conditions (YEPD medium at 30°C) and diluted into pre-warmed YEPD medium at 30°C or YEPD medium + 10% FCS at 37°C. Cells were harvested at the zero time point (immediately before induction) and at the indicated time points after induction for total RNA preparation. Northern analysis was carried out using 3 μg of RNA from each sample to assess transcript levels of the indicated genes. The *ACT1* transcript, as well as ribosomal RNA (rRNA) are shown as loading controls.

UME6 Controls the Level and Duration of NRG1 Down-Regulation in Response to Serum at 37°C

To further define the functional relationship between *UME6* and *NRG1*, we carried out an experiment to determine the kinetics of *UME6* induction and *NRG1* down-regulation under inducing conditions. Both wild-type and *ume6*Δ/*ume6*Δ strains were induced to form filaments in response to serum at 37°C, and RNA was prepared from cells harvested at very early time points up to 5 h. We observed strong *NRG1* down-regulation as early as our first time point, 5 min (Figure 6). *NRG1* levels remained very low (about 5-fold repressed) through the 1-h time point and then began to rise from the 2- to 5-h time points. In contrast, in the *ume6*Δ/*ume6*Δ mutant, *NRG1* was significantly down-regulated only at the 5-min time point and showed a clear reduction in down-regulation at the 15-min through 1-h time points; by 2 h after serum and temperature induction *NRG1* levels in the *ume6*Δ/*ume6*Δ mutant were equivalent or even slightly greater than those under non-inducing conditions (Figure 6). These results suggest that Ume6 functions as negative regulator of *NRG1* and plays an important role in controlling the level and duration of *NRG1* down-regulation in response to serum at 37°C. We first observed induction of *UME6* (~40-fold) at the 15-min time point. In general, *UME6* transcript levels gradually decline over the time course, although we did consistently observe a slight increase between the 1- and 2-h time points (Figure 6). Our results indicate that down-regulation of *NRG1* precedes induction of *UME6* in the series of regulatory events that mediate the filamentation response to serum at 37°C.

Epistatic Relationships between UME6 and RFG1, NRG1, and TUP1

The results described above suggest a functional relationship between *UME6* and the *NRG1-TUP1* filamentous growth regulatory pathway. To define this relationship more precisely and to investigate whether *UME6* also functions in the *RFG1-TUP1* pathway, we generated *rfg1*Δ/*rfg1*Δ *ume6*Δ/*ume6*Δ, *nrg1*Δ/*nrg1*Δ *ume6*Δ/*ume6*Δ, and *tup1*Δ/*tup1*Δ *ume6*Δ/*ume6*Δ double mutant strains and examined both colony and cell morphologies (compared with the individual single mutants) when cells were grown in the absence of filament-inducing conditions. Under non–filament-inducing conditions the *ume6*Δ/*ume6*Δ mutant grows as blastospores, the *rfg1*Δ/*rfg1*Δ mutant is mildly filamentous and *nrg1*Δ/*nrg1*Δ and *tup1*Δ/*tup1*Δ mutants are highly filamentous. We observed that both *rfg1*Δ/*rfg1*Δ *ume6*Δ/*ume6*Δ and *tup1*Δ/*tup1*Δ *ume6*Δ/*ume6*Δ double mutants grew as smooth colo-

nies on solid non–filament-inducing medium and that *tup1*Δ/*tup1*Δ *ume6*Δ/*ume6*Δ colonies were mildly defective for growth (Figure 7). The *nrg1*Δ/*nrg1*Δ *ume6*Δ/*ume6*Δ double mutant showed a wrinkled colony morphology, although this strain did not appear as wrinkled or as adherent to the solid agar as the *nrg1*Δ/*nrg1*Δ single mutant. In liquid non–filament-inducing medium (YEPD) the *rfg1*Δ/*rfg1*Δ *ume6*Δ/*ume6*Δ mutant grew as a combination of blastospores and short filaments (Figure 8A). Under these conditions both *nrg1*Δ/*nrg1*Δ *ume6*Δ/*ume6*Δ and *tup1*Δ/*tup1*Δ *ume6*Δ/*ume6*Δ double mutants were still mostly filamentous but lacked the extended filaments that are normally observed in the *nrg1*Δ/*nrg1*Δ and *tup1*Δ/*tup1*Δ single mutants. This defect was significantly more pronounced in the *tup1*Δ/*tup1*Δ *ume6*Δ/*ume6*Δ mutant and a significant number of individual cells of this strain also appeared to grow in an elongated oval-like morphology. These findings suggest that while the *ume6*Δ/*ume6*Δ mutation is not completely epistatic to the *rfg1*Δ/*rfg1*Δ, *nrg1*Δ/*nrg1*Δ, or *tup1*Δ/*tup1*Δ mutations with respect to filamentation, *UME6* plays a specific important role in the ability of these mutants to generate extended filaments.

We also examined epistatic relationships between *UME6* and *RFG1*, *NRG1*, *TUP1* at the transcriptional level. Northern analysis was carried out using total RNA prepared from all the single and double mutant strains described above as
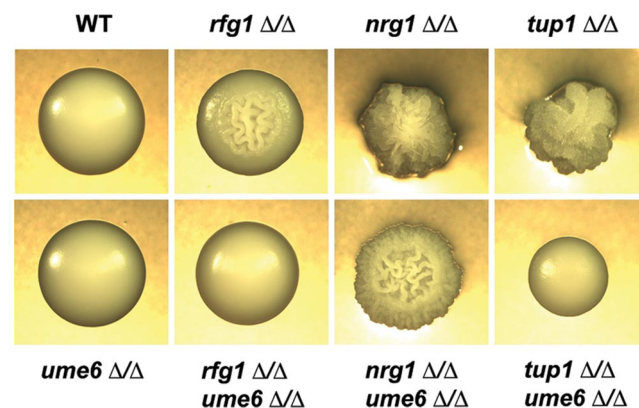

Figure 7. Colony morphologies of *rfg1*Δ/Δ *ume6*Δ/Δ, *nrg1*Δ/Δ *ume6*Δ/Δ, and *tup1*Δ/Δ *ume6* Δ/Δ double mutants and respective single mutants. Colony morphologies of the indicated strains are shown after growth on solid YEPD medium at 30°C (non–filament-inducing conditions) for 3 d. Colonies were visualized by light microscopy and photographed at ~×20 magnification.
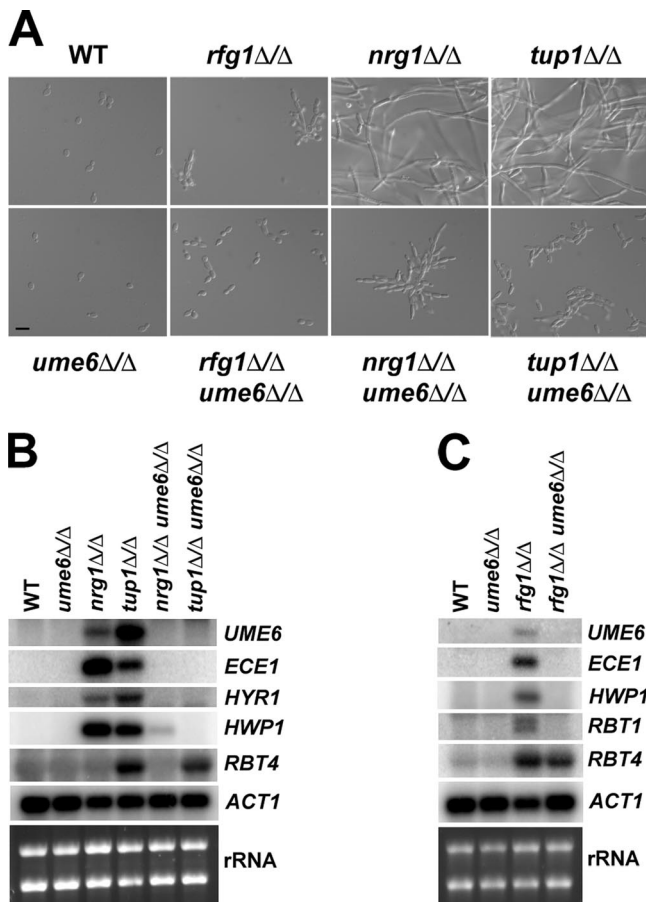
Figure 8. Cell morphologies and filament-specific gene expression in *rfg1Δ/Δ ume6Δ/Δ*, *nrg1Δ/Δ ume6Δ/Δ*, and *tup1Δ/Δ ume6 Δ/Δ* double mutants and respective single mutants. (A) Cell morphologies of the indicated strains are shown after growth in liquid YEPD medium at 30°C to an $OD_{600} \sim 1.0$. Aliquots of cells were fixed in 4.5% formaldehyde, washed twice in 1× PBS, and then visualized by Nomarski/DIC optics (Bar, 10 μm). (B and C) Total RNA was prepared from the strains shown in A, and Northern analysis was carried using 3 μg RNA and probes to the indicated filament-specific transcripts. The *ACT1* transcript and ribosomal RNA (rRNA) are included as loading controls.

well as a wild-type control strain grown under non–filament-inducing conditions. We examined several strongly induced filament-specific transcripts that have previously been shown to be derepressed in *rfg1Δ/rfg1Δ*, *nrg1Δ/nrg1Δ*, and/or *tup1Δ/tup1Δ* mutants (Braun and Johnson, 2000; Braun *et al.*, 2001; Kadosh and Johnson, 2001, 2005; Murad *et al.*, 2001). The majority of these transcripts failed to show derepression in the *rfg1Δ/rfg1Δ ume6Δ/ume6Δ*, *nrg1Δ/nrg1Δ ume6Δ/ume6Δ*, and *tup1Δ/tup1Δ ume6Δ/ume6Δ* double mutant strains under non–filament-inducing conditions (Figures 8, B and C). These results indicate that with respect to the transcriptional regulation of several filament-specific genes the *ume6Δ/ume6Δ* mutation is epistatic to *rfg1Δ/rfg1Δ*, *nrg1Δ/nrg1Δ*, and *tup1Δ/tup1Δ* mutations and suggest that Ume6 functions as a downstream component of the Rfg1, Nrg1, and Tup1 pathways. Interestingly, the *HWP1* transcript was mildly derepressed in the *nrg1Δ/nrg1Δ ume6Δ/ume6Δ* double mutant and derepression of the *RBT4* transcript did not appear to be affected to a significant degree in the *rfg1Δ/rfg1Δ ume6Δ/ume6Δ* and *tup1Δ/tup1Δ ume6Δ/ume6Δ* mutants. These results suggest that in the case of a

few specific genes Rfg1, Nrg1, and Tup1 may direct repression to some extent by a Ume6-independent pathway.

In confirmation of our previous findings by DNA microarray analysis (Kadosh and Johnson, 2005), the *UME6* transcript was derepressed in both the *nrg1Δ/nrg1Δ* and *tup1Δ/tup1Δ* mutant strains in the absence of filament-inducing conditions (Figure 8B). The level of *UME6* derepression was greater in the *tup1Δ/tup1Δ* mutant compared with that in the *nrg1Δ/nrg1Δ* mutant, suggesting that *UME6* is also regulated by a Nrg1-independent Tup1 repression pathway(s). Consistent with this finding, we observed that *UME6* is mildly derepressed in the *rfg1Δ/rfg1Δ* mutant, indicating that *UME6* is also under negative control by the Rfg1-Tup1 repression pathway (Figure 8C). We also note that under the growth conditions used in our experiment the *ECE1* transcript appears to show greater derepression in the *nrg1Δ/nrg1Δ* versus *tup1Δ/tup1Δ* mutant (Figure 8B), suggesting that this gene may partially be under the control of a Tup1-independent Nrg1 repression pathway.

DISCUSSION

A Novel Transcriptional Regulator Specifically Controls Hyphal Filament Extension and Virulence

A wide variety of both pathogenic and nonpathogenic fungal species are capable of growing in a filamentous form. Extension of initial short filaments (germ tubes) to elongated hyphae is critical for a diverse array of processes including nutrient scavenging, mating, thigmotropism, and virulence (Odds, 1988; Collier *et al.*, 1998; Madden and Snyder, 1998; Kumamoto and Vinces, 2005a). In pathogenic fungi hyphal extension has been shown to be specifically important for the virulence-related processes of tissue invasion (particularly invasion of mucosal epithelial cells), angioinvasion, breaching of endothelial cells and the lysis of macrophages and neutrophils (Lo *et al.*, 1997; Korting *et al.*, 2003; Kumamoto and Vinces, 2005b; Filler and Sheppard, 2006).

Despite the widespread importance of hyphal filament extension for a variety of fungal species, little is known about the regulatory mechanisms that control this process. In *Neurospora crassa*, maintenance of a Ca^{2+} gradient at the hyphal tip as well as interaction between two kinases (*POD6* and *COT1*) is important for the extension of hyphal filaments (Silverman-Gavrila and Lew, 2003; Seiler *et al.*, 2006). *C. albicans* also appears to utilize a Ca^{2+}-dependent mechanism to orient hyphal growth (Brand *et al.*, 2007), and calcineurin has been shown to be important for hyphal elongation in both *A. fumigatus* and *Cryptococcus neoformans* (Cruz *et al.*, 2001; Steinbach *et al.*, 2006). In the corn smut fungus *Ustilago maydis*, deletion of a kinesin affects both hyphal extension as well as pathogenesis and several kinesins are up-regulated in hyphae (Lehmler *et al.*, 1997; Schuchardt *et al.*, 2005).

The identification of *UME6* represents a significant advance in our understanding of the molecular mechanisms that regulate hyphal filament extension. Because *UME6* encodes a transcriptional regulator, it appears that expression of many of the components required for hyphal extension (such as those described above) is controlled in a coordinated manner by a central regulatory mechanism. There are several explanations for our finding that the *ume6Δ/ume6Δ* mutant is specifically defective for hyphal extension, but not germ tube formation. One possibility is that germ tube formation and hyphal extension represent distinct processes that are controlled by separate gene sets. A second more likely possibility, supported by our finding that filament-

specific gene expression is still induced (albeit at lower levels) during germ tube formation in the *ume6Δ/ume6Δ* mutant, is that a common, or very similar, set of genes functions to direct both processes.

These studies also allow us to gain insight into the role of hyphal filament extension in virulence. Previous reports have shown that *C. albicans* strains locked in the blastospore form, such as the *efg1Δ/efg1Δ* mutant or a strain expressing high constitutive levels of *NRG1*, are completely attenuated for virulence in a mouse model of systemic candidiasis (Lo *et al.*, 1997; Saville *et al.*, 2003). Interestingly, although the *ume6Δ/ume6Δ* mutant, which can form filaments but is defective for hyphal extension both in vitro and during infection in vivo, is clearly attenuated for virulence, most of the mice eventually succumb to infection and die (albeit after a significantly longer time period than mice infected with a wild-type strain). Our results suggest that the attenuated virulence of this mutant can be at least partially ascribed to a defect in hyphal filament extension causing reduced and/or delayed tissue invasion and damage. Hyphal extension may therefore be important, but not absolutely required, for virulence in this infection model. Although our findings are consistent with previous studies implicating hyphal extension in a variety of virulence-related processes (Scherwitz, 1982; Ray and Payne, 1988; Zink *et al.*, 1996; Lo *et al.*, 1997; Jong *et al.*, 2001; Kumamoto and Vinces, 2005b; Filler and Sheppard, 2006), we cannot exclude the possibility that the *ume6Δ/ume6Δ* mutant is attenuated for virulence as a result of the reduced expression of virulence factors that are not involved in filamentation per se.

Correlation of Hyphal Extension with the Level and Duration of Expression the C. albicans Filamentous Growth Program

An important observation is that *UME6*, itself a filament-induced transcript, plays a role in controlling the level and duration of expression of nearly all of the most highly induced genes in the *C. albicans* filamentous growth program. Consistent with this observation, the *ume6Δ/ume6Δ* defect in hyphal extension becomes most apparent toward the later time points (after 2 h) following serum and temperature induction. Interestingly, our results suggest that genes in the *C. albicans* filamentous growth program must be expressed at high levels for an extended period of time in order for cells to make the transition from germ tubes to extended hyphal filaments. It is important to note that our DNA microarray analysis did not detect the activation or repression of a novel set of genes that specifically correlates with the *ume6Δ/ume6Δ* hyphal extension defect. Thus, we hypothesize that hyphal filament extension is correlated with the level and duration of filament-specific gene expression rather than activation (or repression) of a separate gene set.

Do specific Ume6 target genes play key roles in carrying out the hyphal filament extension process? Although we do not yet have definitive evidence to answer this question, two hyphal-specific genes, encoding Eed1 and the Hgc1 cyclin-related protein, are transcriptionally regulated and have been shown to play important roles in hyphal extension (Zheng *et al.*, 2004; Zakikhany *et al.*, 2007). Sinha *et al.* (2007) have recently proposed a model for the initiation and maintenance of polarized hyphal growth that involves regulation of septin phosphorylation by Cdc28-cyclin complexes that can include Hgc1, and Zheng *et al.* (2007) have recently shown that phosphorylation of Rga2, a GTPase activating protein, by Cdc28/Hgc1 and localization of Rga2 away from the hyphal tip is important for extended hyphal cell development. These findings suggest that hyphal extension may be regulated not only at the transcriptional level but also at the level of posttranslational modification and intracellular protein localization.

Model for Control of Induction and Maintenance of Expression of the C. albicans Filamentous Growth Program by Rfg1, Nrg1, Tup1, and Ume6

Our results suggest a model in which Nrg1 and Ume6 function together by a negative feedback loop to control the induction and maintained expression of the *C. albicans* filamentous growth program in response to filament-inducing conditions such as serum at 37°C (Figure 9). Under non–filament-inducing conditions the *UME6* would not be expressed (or expressed at very low levels) as a consequence of strong repression by the Nrg1-Tup1 pathway. *NRG1* levels would be too high and *UME6* levels
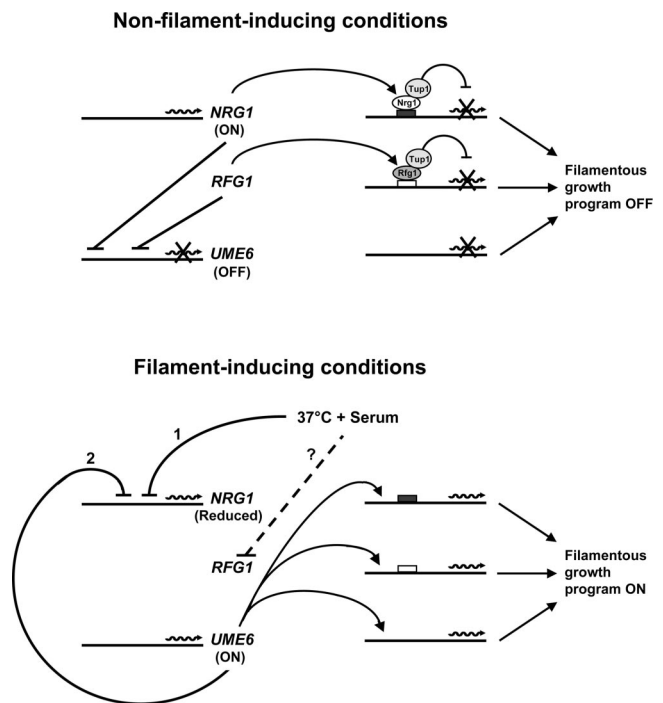

Figure 9. Model for control of induction and maintenance of expression of the *C. albicans* filamentous growth program by Rfg1, Nrg1, Tup1, and Ume6. Nrg1 and Ume6 are believed to repress each other in a negative feedback loop. Under steady-state non–filament-inducing conditions (top) Nrg1 levels are high, and *UME6* is not expressed (due to strong Nrg1-Tup1 repression). In turn, many filament-specific transcripts are not induced as a consequence of repression by the Nrg1-Tup1 pathway and/or lack of activation by Ume6. In addition, under non–filament-inducing conditions, *UME6* as well as several filament-specific transcripts, are repressed by the Rfg1-Tup1 pathway. In the presence of filament-inducing conditions such as serum at 37°C (bottom) the *NRG1* transcript undergoes a very rapid transient down-regulation (1) causing *UME6* levels to rise. As a consequence of repression by Ume6 (2), the *NRG1* transcript is maintained at a reduced level for a significantly longer time period. Filament-specific transcripts are subsequently induced as a result of relief of repression by the Nrg1-Tup1 and Rfg1-Tup1 pathways and/or activation by Ume6. Also, in the presence of serum at 37°C repression of *UME6* by the Rfg1-Tup1 pathway is believed to be relieved by a mechanism not yet determined (dashed line). Please note that it is unclear at this point as to whether Ume6 and Nrg1 repress each other by direct or indirect effects.

would be too low to cause expression of filament-specific target genes. Initially, within 5 min after induction by serum at 37°C, the *NRG1* transcript is strongly down-regulated; although the mechanism controlling *NRG1* down-regulation is not entirely clear, another key transcriptional regulator of filamentous growth, Efg1 (the target of a cAMP-PKA signaling pathway; Ernst, 2000), has been shown to be important for this process (Braun *et al.,* 2001). Rapid down-regulation of the *NRG1* transcript would lead to a strong induction (or derepression) of *UME6,* which is subsequently observed at the 15-min time point. We believe that a certain threshold level of Ume6 expression is required to maintain repression of the *NRG1* transcript. As the time course progresses Ume6 levels eventually fall below this threshold, causing *NRG1* transcript levels to increase starting at ~2 h after induction. The net result of this negative feedback loop would be a transient reduction of *NRG1* levels and elevation in *UME6* levels, which becomes most apparent at early time points. As a consequence of *NRG1* down-regulation, Nrg1-Tup1–controlled target genes in the *C. albicans* filamentous growth program would be derepressed. In addition Ume6, when expressed at sufficiently high levels, would function independently as a strong activator of filament- and virulence-specific target genes. Rfg1 also appears to function as a repressor of *UME6* under non–filament-inducing conditions, and we hypothesize that relief of Rfg1-Tup1–mediated repression, by an unknown mechanism, may also play a role in the induction of *UME6*. This model is supported by several lines of evidence: 1) *UME6* is repressed by both the Rfg1-Tup1 and Nrg1-Tup1 pathways under non–filament-inducing conditions, and epistasis analysis indicates that Ume6 functions as a downstream component of these pathways to control expression of several filament-specific transcripts, 2) the *NRG1* repressor is down-regulated by serum at 37°C starting at the 5-min time point, 3) transcription of *UME6* is induced by serum and 37°C at the 15-min time point, after down-regulation of *NRG1*, 4) *UME6* functions as a repressor of *NRG1* only under inducing conditions and is important for controlling the level and duration of *NRG1* down-regulation in response to serum and 37°C, 5) several genes in the filamentous growth program, which are not repressed by the Rfg1-Tup1 or Nrg1-Tup1 pathways (Kadosh and Johnson, 2005), show reduced serum and temperature induction in the *ume6Δ/ume6Δ* mutant (e.g., *USO5, NBP35, ERV46*), suggesting that Ume6 also functions independently as a strong activator of these transcripts, 6) both the level and duration of filament-specific gene induction are reduced in the *ume6Δ/ume6Δ* mutant, suggesting that a mechanism for amplifying the inducing signal (and consequently maintaining expression of filament-specific genes) is in place.

It is important to bear in mind that this model may not apply to all genes in the *C. albicans* filamentous growth program. *RBT4*, for example, appears to be controlled by the Rfg1-Tup1 pathway but not by Ume6 (to a significant degree). We also cannot exclude the possibility of alternative models in which *UME6* is induced by Rfg1-, Nrg1-, and Tup1-independent mechanisms (or by relief of repression by a Tup1 pathway that does not depend upon Rfg1 or Nrg1) or models in which down-regulation of *NRG1* is maintained by additional Ume6-independent mechanisms.

How can *UME6* simultaneously function as a repressor of *NRG1* and activator of filament-specific transcripts? It is possible that at least one of these events occurs as the result of an indirect effect. For example, rather than directly repressing the *NRG1* transcript, Ume6 may instead function to activate a strong repressor of *NRG1*. It is also unclear at this point whether Rfg1 represses *UME6* under non–filament-inducing conditions by a direct or indirect mechanism.

Interestingly, although *UME6* is most strongly induced at early time points after serum- and temperature-induction, this regulator plays an important role in promoting hyphal extension at later time points. We hypothesize that this "lag time" may be due to the continued presence of Ume6 protein levels after *UME6* transcript levels have decreased. Consistent with this hypothesis, filament-specific transcript levels are reduced most significantly at the later time points following serum- and temperature-induction in the *ume6Δ/ume6Δ* mutant, suggesting that in a wild-type strain the Ume6 protein is normally present at sufficiently high levels to cause activation of these genes (we are currently in the process of generating Ume6 antibodies to address this hypothesis as well as carry out additional planned Ume6 protein studies). Alternatively, because many filament-specific transcripts are initially expressed at lower levels in the *ume6Δ/ume6Δ* mutant, these transcripts may sooner decline (due to degradation over the time course) below the threshold required to maintain extended hyphal growth.

The work presented in this article indicates that *UME6* functions as an important regulator of *C. albicans* hyphal filament extension and virulence as well as a key downstream component of the Rfg1, Nrg1, and Tup1 filamentous growth regulatory pathways. Our observation that *UME6* is induced by multiple filament-inducing conditions (in addition to serum and 37°C) suggests that a variety of filamentous growth signaling and regulatory pathways may utilize this mechanism to promote hyphal filament extension. Future studies on Ume6 should provide significantly greater insight into the molecular basis for this poorly understood virulence trait.

ACKNOWLEDGMENTS

REFERENCES

Arnaud, M. B., Costanzo, M. C., Skrzypek, M. S., Binkley, G., Lane, C., Miyasato, S. R., and Sherlock, G. (2005). The *Candida* Genome Database (CGD), a community resource for *Candida albicans* gene and protein information. Nucleic Acids Res. *33*, D358–D363.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds.) (1992). Current Protocols in Molecular Biology, New York: Greene Publishing Associates and Wiley-Interscience.

Bailey, D. A., Feldmann, P. J., Bovey, M., Gow, N. A., and Brown, A. J. (1996). The *Candida albicans HYR1* gene, which is activated in response to hyphal development, belongs to a gene family encoding yeast cell wall proteins. J. Bacteriol. *178*, 5353–5360.

Bartie, K. L., Williams, D. W., Wilson, M. J., Potts, A. J., and Lewis, M. A. (2004). Differential invasion of *Candida albicans* isolates in an in vitro model of oral candidosis. Oral Microbiol. Immunol. *19*, 293–296.

Bennett, R. J., Uhl, M. A., Miller, M. G., and Johnson, A. D. (2003). Identification and characterization of a *Candida albicans* mating pheromone. Mol. Cell. Biol. *23*, 8189–8201.

Blankenship, J. R., and Mitchell, A. P. (2006). How to build a biofilm: a fungal perspective. Curr. Opin. Microbiol. *9*, 588–594.

Bowdish, K. S., Yuan, H. E., and Mitchell, A. P. (1995). Positive control of yeast meiotic genes by the negative regulator *UME6*. Mol. Cell. Biol. *15*, 2955–2961.

Brand, A., Shanks, S., Duncan, V. M., Yang, M., Mackenzie, K., and Gow, N. A. (2007). Hyphal orientation of *Candida albicans* is regulated by a calcium-dependent mechanism. Curr. Biol. *17*, 347–352.

Braun, B. R., Head, W. S., Wang, M. X., and Johnson, A. D. (2000). Identification and characterization of *TUP1*-regulated genes in *Candida albicans.* Genetics *156*, 31–44.

Braun, B. R., and Johnson, A. D. (1997). Control of filament formation in *Candida albicans* by the transcriptional repressor *TUP1*. Science *277*, 105–109.

Braun, B. R., and Johnson, A. D. (2000). *TUP1, CPH1* and *EFG1* make independent contributions to filamentation in *Candida albicans*. Genetics *155*, 57–67.

Braun, B. R., Kadosh, D., and Johnson, A. D. (2001). *NRG1*, a repressor of filamentous growth in *C. albicans*, is down-regulated during filament induction. EMBO J. *20*, 4753–4761.

Braun, B. R., *et al.* (2005). A human-curated annotation of the *Candida albicans* genome. PLoS Genet. *1*, 36–57.

Brown, A. J. (2002). Morphogenetic signaling pathways in *Candida albicans.* In: *Candida* and Candidiasis, ed. R. A. Calderone, Washington, DC: ASM Press, 95–106.

Calderone, R. A., and Gow, N. A. (2002). Host recognition by *Candida* species. In: *Candida* and Candidiasis, ed. R. A. Calderone, Washington, DC: ASM Press, 67–86.

Church, G. M., and Gilbert, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA *81*, 1991–1995.

Collier, L., Balons, A., and Sussman, M. (eds.) (1998). Microbiology and Microbial Infections, New York: Oxford University Press.

Cruz, M. C., Fox, D. S., and Heitman, J. (2001). Calcineurin is required for hyphal elongation during mating and haploid fruiting in *Cryptococcus neoformans.* EMBO J. *20*, 1020–1032.

Deckert, J., Rodriguez Torres, A. M., Simon, J. T., and Zitomer, R. S. (1995). Mutational analysis of Rox1, a DNA-bending repressor of hypoxic genes in *Saccharomyces cerevisiae.* Mol. Cell. Biol. *15*, 6109–6117.

Dupont, P. F. (1995). *Candida albicans,* the opportunist. A cellular and molecular perspective. J. Am. Podiatr. Med. Assoc. *85*, 104–115.

Edmond, M. B., Wallace, S. E., McClish, D. K., Pfaller, M. A., Jones, R. N., and Wenzel, R. P. (1999). Nosocomial bloodstream infections in United States hospitals: a three-year analysis. Clin. Infect. Dis. *29*, 239–244.

Einerhand, A. W., Kos, W., Smart, W. C., Kal, A. J., Tabak, H. F., and Cooper, T. G. (1995). The upstream region of the *FOX3* gene encoding peroxisomal 3-oxoacyl-coenzyme A thiolase in *Saccharomyces cerevisiae* contains ABF1- and replication protein A-binding sites that participate in its regulation by glucose repression. Mol. Cell. Biol. *15*, 3405–3414.

Ernst, J. F. (2000). Transcription factors in *Candida albicans*—environmental control of morphogenesis. Microbiology *146*, 1763–1774.

Filler, S. G., and Sheppard, D. C. (2006). Fungal invasion of normally non-phagocytic host cells. PLoS Pathog. *2*, e129.

Fonzi, W. A., and Irwin, M. Y. (1993). Isogenic strain construction and gene mapping in *Candida albicans.* Genetics *134*, 717–728.

Guthrie, C., and Fink, G. R. (1991). Guide to Yeast Genetics and Molecular Biology, San Diego: Academic Press.

Jong, A. Y., Stins, M. F., Huang, S. H., Chen, S. H., and Kim, K. S. (2001). Traversal of *Candida albicans* across human blood-brain barrier in vitro. Infect. Immun. *69*, 4536–4544.

Kadosh, D., and Johnson, A. D. (2001). Rfg1, a protein related to the *Saccharomyces cerevisiae* hypoxic regulator Rox1, controls filamentous growth and virulence in *Candida albicans.* Mol. Cell. Biol. *21*, 2496–2505.

Kadosh, D., and Johnson, A. D. (2005). Induction of the *Candida albicans* filamentous growth program by relief of transcriptional repression: a genome-wide analysis. Mol. Biol. Cell *16*, 2903–2912.

Keleher, C. A., Redd, M. J., Schultz, J., Carlson, M., and Johnson, A. D. (1992). Ssn6-Tup1 is a general repressor of transcription in yeast. Cell *68*, 709–719.

Khalaf, R. A., and Zitomer, R. S. (2001). The DNA binding protein Rfg1 is a repressor of filamentation in *Candida albicans.* Genetics *157*, 1503–1512.

Korting, H. C., Hube, B., Oberbauer, S., Januschke, E., Hamm, G., Albrecht, A., Borelli, C., and Schaller, M. (2003). Reduced expression of the hyphal-independent *Candida albicans* proteinase genes *SAP1* and *SAP3* in the *efg1* mutant is associated with attenuated virulence during infection of oral epithelium. J. Med. Microbiol. *52*, 623–632.

Kumamoto, C. A., and Vinces, M. D. (2005a). Alternative *Candida albicans* lifestyles: growth on surfaces. Annu. Rev. Microbiol. *59*, 113–133.

Kumamoto, C. A., and Vinces, M. D. (2005b). Contributions of hyphae and hypha-co-regulated genes to *Candida albicans* virulence. Cell Microbiol. *7*, 1546–1554.

Lee, K. L., Buckley, H. R., and Campbell, C. C. (1975). An amino acid liquid synthetic medium for the development of mycelial and yeast forms of *Candida albicans.* Sabouraudia *13*, 148–153.

Lehmler, C., Steinberg, G., Snetselaar, K. M., Schliwa, M., Kahmann, R., and Bolker, M. (1997). Identification of a motor protein required for filamentous growth in *Ustilago maydis.* EMBO J. *16*, 3464–3473.

Liu, H., Kohler, J., and Fink, G. R. (1994). Suppression of hyphal formation in *Candida albicans* by mutation of a *STE12* homolog. Science *266*, 1723–1726.

Lo, H. J., Kohler, J. R., DiDomenico, B., Loebenberg, D., Cacciapuoti, A., and Fink, G. R. (1997). Nonfilamentous *C. albicans* mutants are avirulent. Cell *90*, 939–949.

Lotz, H., Sohn, K., Brunner, H., Muhlschlegel, F. A., and Rupp, S. (2004). *RBR1*, a novel pH-regulated cell wall gene of *Candida albicans*, is repressed by *RIM101* and activated by *NRG1*. Eukaryot. Cell *3*, 776–784.

Madden, K., and Snyder, M. (1998). Cell polarity and morphogenesis in budding yeast. Annu. Rev. Microbiol. *52*, 687–744.

Miller, L. G., Hajjeh, R. A., and Edwards, J. E., Jr. (2001). Estimating the cost of nosocomial candidemia in the United States. Clin. Infect. Dis. *32*, 1110

Mitchell, A. P. (1998). Dimorphism and virulence in *Candida albicans.* Curr. Opin. Microbiol. *1*, 687–692.

Murad, A.M.A. *et al.* (2001). *NRG1* represses yeast-hypha morphogenesis and hypha-specific gene expression in *Candida albicans.* EMBO J. 4742–4752.

Noble, S. M., and Johnson, A. D. (2005). Strains and strategies for large-scale gene deletion studies of the diploid human fungal pathogen *Candida albicans.* Eukaryot. Cell *4*, 298–309.

Odds, F. C. (1988). *Candida* and Candidosis, London: Baillière Tindall.

Park, S. H., Koh, S. S., Chun, J. H., Hwang, H. J., and Kang, H. S. (1999). Nrg1 is a transcriptional repressor for glucose repression in *STA1* gene expression in *Saccharomyces cerevisiae.* Mol. Cell. Biol. *19*, 2044–2050.

Ramage, G., Saville, S. P., Thomas, D. P., and Lopez-Ribot, J. L. (2005). *Candida* biofilms: an update. Eukaryot. Cell *4*, 633–638.

Ray, T. L., and Payne, C. D. (1988). Scanning electron microscopy of epidermal adherence and cavitation in murine candidiasis: a role for *Candida* acid proteinase. Infect. Immun. *56*, 1942–1949.

Reuss, O., Vik, A., Kolter, R., and Morschhauser, J. (2004). The *SAT1* flipper, an optimized tool for gene disruption in *Candida albicans.* Gene *341*, 119–127.

Sanglard, D., Hube, B., Monod, M., Odds, F. C., and Gow, N. A. (1997). A triple deletion of the secreted aspartyl proteinase genes *SAP4, SAP5,* and *SAP6* of *Candida albicans* causes attenuated virulence. Infect. Immun. *65*, 3539–3546.

Saville, S. P., Lazzell, A. L., Monteagudo, C., and Lopez-Ribot, J. L. (2003). Engineered control of cell morphology *in vivo* reveals distinct roles for yeast and filamentous forms of *Candida albicans* during infection. Eukaryot. Cell *2*, 1053–1060.

Schaller, M., Korting, H. C., Schafer, W., Bastert, J., Chen, W., and Hube, B. (1999). Secreted aspartic proteinase (Sap) activity contributes to tissue damage in a model of human oral candidosis. Mol. Microbiol. *34*, 169–180.

Scherwitz, C. (1982). Ultrastructure of human cutaneous candidosis. J. Invest. Dermatol. *78*, 200–205.

Schuchardt, I., Assmann, D., Thines, E., Schuberth, C., and Steinberg, G. (2005). Myosin-V, Kinesin-1, and Kinesin-3 cooperate in hyphal growth of the fungus *Ustilago maydis.* Mol. Biol. Cell *16*, 5191–5201.

Seiler, S., Vogt, N., Ziv, C., Gorovits, R., and Yarden, O. (2006). The *STE20/* germinal center kinase *POD6* interacts with the NDR kinase *COT1* and is involved in polar tip extension in *Neurospora crassa.* Mol. Biol. Cell *17*, 4080–4092.

Shepherd, M. G., Poulter, R. T., and Sullivan, P. A. (1985). *Candida albicans*: biology, genetics, and pathogenicity. Annu. Rev. Microbiol. *39*, 579–614.

Silverman-Gavrila, L. B., and Lew, R. R. (2003). Calcium gradient dependence of *Neurospora crassa* hyphal growth. Microbiology *149*, 2475–2485.

Sinha, I., Wang, Y. M., Philp, R., Li, C. R., Yap, W. H., and Wang, Y. (2007). Cyclin-dependent kinases control septin phosphorylation in *Candida albicans* hyphal development. Dev. Cell *13*, 421–432.

Smith, R. L., and Johnson, A. D. (2000). Turning genes off by Ssn6-Tup1, a conserved system of transcriptional repression in eukaryotes. Trends Biochem. Sci. *25*, 325–330.

Steber, C. M., and Esposito, R. E. (1995). *UME6* is a central component of a developmental regulatory switch controlling meiosis-specific gene expression. Proc. Natl. Acad. Sci. USA *92*, 12490–12494.

Steinbach, W. J. *et al.* (2006). Calcineurin controls growth, morphology, and pathogenicity in *Aspergillus fumigatus.* Eukaryot. Cell *5*, 1091–1103.

Strich, R., Surosky, R. T., Steber, C., Dubois, E., Messenguy, F., and Esposito, R. E. (1994). *UME6* is a key regulator of nitrogen repression and meiotic development. Genes Dev. *8*, 796–810.

Sudbery, P., Gow, N., and Berman, J. (2004). The distinct morphogenic states of *Candida albicans.* Trends Microbiol. *12*, 317–324.

Sweet, D. H., Jang, Y. K., and Sancar, G. B. (1997). Role of *UME6* in transcriptional regulation of a DNA repair gene in *Saccharomyces cerevisiae.* Mol. Cell. Biol. *17*, 6223–6235.

Tzamarias, D., and Struhl, K. (1995). Distinct TPR motifs of Cyc8 are involved in recruiting the Cyc8-Tup1 corepressor complex to differentially regulated promoters. Genes Dev. *9*, 821–831.

Weig, M., Gross, U., and Muhlschlegel, F. (1998). Clinical aspects and pathogenesis of *Candida* infection. Trends Microbiol. *6*, 468–470.

Zakikhany, K., Naglik, J. R., Schmidt-Westhausen, A., Holland, G., Schaller, M., and Hube, B. (2007). In vivo transcript profiling of *Candida albicans* identifies a gene essential for interepithelial dissemination. Cell Microbiol. *9*, 2938–2954.

Zhao, X., Oh, S. H., Cheng, G., Green, C. B., Nuessen, J. A., Yeater, K., Leng, R. P., Brown, A. J., and Hoyer, L. L. (2004). *ALS3* and *ALS8* represent a single locus that encodes a *Candida albicans* adhesin; functional comparisons between Als3p and Als1p. Microbiology *150*, 2415–2428.

Zheng, X., Wang, Y., and Wang, Y. (2004). Hgc1, a novel hypha-specific G1 cyclin-related protein regulates *Candida albicans* hyphal morphogenesis. EMBO J. *23*, 1845–1856.

Zheng, X. D., Lee, R. T., Wang, Y. M., Lin, Q. S., and Wang, Y. (2007). Phosphorylation of Rga2, a Cdc42 GAP, by CDK/Hgc1 is crucial for *Candida albicans* hyphal growth. EMBO J. *26*, 3760–3769.

Zink, S., Nass, T., Rosen, P., and Ernst, J. F. (1996). Migration of the fungal pathogen *Candida albicans* across endothelial monolayers. Infect. Immun. *64*, 5085–5091.