Candida albicans Ume6, a Filament-Specific Transcriptional Regulator, Directs Hyphal Growth via a Pathway Involving Hgc1 Cyclin-Related Protein

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The ability of Candida albicans, the most common human fungal pathogen, to transition from yeast to hyphae is essential for pathogenicity. While a variety of transcription factors important for filamentation have been identified and characterized, links between transcriptional regulators of C. albicans morphogenesis and molecular mechanisms that drive hyphal growth are not well defined. We have previously observed that constitutive expression of UME6, which encodes a filament-specific transcriptional regulator, is sufficient to direct hyphal growth in the absence of filament-inducing conditions. Here we show that HGC1, encoding a cyclin-related protein necessary for hyphal growth under filament-inducing conditions, is specifically important for agar invasion, hyphal extension, and formation of true septa in response to constitutive UME6 expression under non-filament-inducing conditions. HGC1-dependent inactivation of Rga2, a Cdc42 GTPase activating protein (GAP), also appears to be important for these processes. In response to filament-inducing conditions, HGC1 is induced prior to UME6 although UME6 controls the level and duration of HGC1 expression, which are likely to be important for hyphal extension. Interestingly, an epistasis analysis suggests that UME6 and HGC1 play distinct roles during early filament formation. These findings establish a link between a key regulator of filamentation and a downstream mechanism important for hyphal formation. In addition, this study demonstrates that a strain expressing constitutive high levels of UME6 provides a powerful strategy to specifically dissect downstream mechanisms important for hyphal development in the absence of complex filament-inducing conditions.

Candida albicans, the most frequently isolated human fungal pathogen, can cause a variety of infections, especially in immunocompromised individuals (12, 39). These infections can occur systemically and superficially, as well as on mucosal surfaces such as the mouth and vagina. Candida species are now the fourth leading cause of hospital-acquired bloodstream infections in the United States. There is an ~40% mortality rate associated with systemic candidiasis, which translates to over 10,000 deaths per year (13). These infections are becoming increasingly difficult to treat due to the fact that there are a limited number of antifungal drugs available and many cases of resistant candidiasis (10, 11). Currently, the United States spends approximately $1 billion per year for antifungal therapies to treat systemic candidiasis (19).

One of the most intensely studied virulence traits of C. albicans is the ability to undergo a reversible transition between yeast and filamentous morphologies. Filaments can occur in either pseudohyphal (ellipsoid-shaped cells, attached end to end) or hyphal (attached cells with parallel sides and no septal constrictions) form (32). Hyphal filaments are known to play an important role in a variety of virulence-related processes, including tissue invasion, breaching of endothelial cells, and lysis of macrophages and neutrophils (17). Several previous studies have indicated that C. albicans strains defective for hyphal formation are highly attenuated for virulence in a mouse model of systemic candidiasis (2, 18, 27, 28, 31, 41), providing strong evidence that the hyphal morphology and/or hypha-associated gene expression is important for C. albicans pathogenicity.

The C. albicans yeast-to-filament transition occurs in response to a variety of environmental conditions present in host tissues, such as body temperature (37°C), serum, neutral pH, and hormones (7, 18, 23, 28). Exposure to these filament-inducing conditions results in the activation of signaling cascades within a yeast cell and causes hypha-specific genes to be induced (4, 6, 7, 16, 21). In addition to this transcriptional response, posttranslational modification of a variety of proteins present in yeast cells also contributes to hyphal growth. Numerous signaling pathways which respond to filament-inducing conditions have been identified in C. albicans, including a mitogen-activated protein (MAP) kinase pathway, the cyclic AMP (cAMP)/protein kinase A (PKA) pathway, and a pH response pathway.

The signaling pathways described above generally drive filamentous growth by activation of specific transcription factors (3, 4, 7, 14, 20). Our work has focused on one such transcription factor, Ume6, which was recently shown to play an important role in hyphal filament extension (2, 40). ume6ΔΔ mutants are attenuated for virulence and defective for hyphal extension both in vitro and during infection in vivo. We have previously observed that high-level constitutive UME6 expres-
sion promotes tissue invasion and virulence in a mouse model of systemic candidiasis and is sufficient to generate a nearly complete hyphal population in the absence of filament-inducing conditions (8). However, little is known about the downstream mechanism(s) by which UME6 drives hyphal growth and promotes virulence.

One potential mechanism for Ume6-driven hyphal growth may be a pathway involving Hgc1 cyclin-related protein. Both hgc1Δ/Δ and ume6Δ/Δ mutants are defective for hyphal extension in response to filament-inducing conditions, and it has been previously shown that expression of UME6 is sufficient to induce HGC1 under non-filament-inducing conditions (2, 8, 40, 41). HGC1 is a hypha-specific gene which is specifically important for hyphal growth (41). The Hgc1 protein forms a complex with Cdc28, a cyclin-dependent kinase, which is important for phosphorylation of Cdc11, a septin protein, as well as Rga2, a Cdc42 GTPase activating protein (GAP) (29, 38, 41, 42). Phosphorylation of Cdc11 is required for the proper formation of true septa in hyphae. Rga2 negatively controls Cdc42, a master polarity regulator; activated Cdc42 directs polarized growth by promoting septin ring organization, secretory vesicle transport, and Spitzenkörper formation, all of which are essential for hyphal formation (24). Phosphorylation of Rga2 by Hgc1/Cdc28 causes Rga2 to localize away from the hyphal tip, thereby blocking Rga2-dependent inactivation of Cdc42 (9, 42). The Hgc1/Cdc28 complex is also known to promote hyphal growth by phosphorylating Sep7 septin and the Efg1 transcription factor, causing inhibition of cell separation (15, 37).

In this paper we use a variety of approaches to demonstrate a functional relationship between the filament-specific transcriptional regulator Ume6 and a pathway involving Hgc1 cyclin-related protein. This relationship appears to be critical for proper C. albicans hyphal growth, which, in turn, is necessary for virulence as well as a variety of virulence-related processes.

### MATERIALS AND METHODS

#### Strain and plasmid constructions.

The genotypes for all strains used in this study are listed in Table 1. ume6Δ/Δ and tetO-UME6 strains have been previously described (2, 8, 22). DK318 (2) was used as the wild-type (WT) strain in all experiments except the agar invasion assay (see Fig. 2). PCY87 was used as the wild-type strain in the agar invasion assay. PCY87 was generated by linearizing plasmid Clp10 (20a) with SfiI and integrating at the RPS1 locus of strain TCE1 (20b). In order to construct all strains deleted for HGC1, primer pair 1/2 was used in a PCR to generate a 5′ flank immediately upstream of the HGC1 coding region and primer pair 3/4 was used to generate a 3′ flank (see Table S1 in the supplemental material for a listing of primers used in this study). The 5′ HGC1 flank was digested with KpnI and XhoI, and the 3′ flank was digested with NotI and SacII. These flanks were then cloned stepwise into plasmid pSF12 (26). The rga2Δ::SAT1 construct was generated in the same manner, using primer pair 5/6 and primer pair 7/8 to generate the 5′ and 3′ flanks, respectively. The resulting plasmids were digested with KpnI and SacII to release hgc1Δ::SAT1 and rga2Δ::SAT1 fragments, which were used to transform WT (DK318), ume6Δ/Δ mutant, and tetO-UME6 strains. Homozygous deletion mutations were generated using the SAT flipper method as described by Reiss et al. (26). Whole-cell PCR was used to verify correct integration of the deletion constructs over the 5′ and 3′ disruption junctions and absence of the open reading frame (ORF) in homozygous deletion mutants.

#### Media and growth conditions.

Strains constructed in the tetO-UME6 background were grown in liquid culture yeast extract-peptone-dextrose (YPD) or on solid agar YEPD at 30°C in the presence or absence of 20 μg/ml doxycycline (DOX) (Sigma-Aldrich, St. Louis, MO); cells grown in liquid culture were harvested at an optical density at 600 nm (OD600) of ~1.0 for microscopy and RNA extraction. Growth for filament induction took place at 37°C in YEPD (liquid and solid media) plus 10% fetal bovine serum (FBS). For microscopy, induction was carried out by growing the strains overnight to an OD600 of ~0.5 and then inoculating them into prewarmed media as described previously (2). Induction for Northern analysis was carried out as described by Banerjee et al. (2).

#### Invasion assays.

The invasion assay was performed by growing strains overnight at 30°C in YEPD medium. Saturated cultures were diluted to an OD600 of ~1.0, and four subsequent 1:10 serial dilutions were made. Three microliters of each dilution was spotted on YEPD agar plates in the presence or absence of 20 μg/ml DOX (a dilution containing 2 × 10⁶ cells/ml is shown in Fig. 2). Cells were grown for 24 h, and images were taken before and after washing with double-distilled water (ddH₂O).

| Strain | Genotype | Source or reference |
|--------|----------|---------------------|
| DK318 (WT) | ade2::HisG/ade2::his2G ura3::imm434/ura3::imm434 ENO1/eno1::ENO1-tetR-SchHAP4AD-3×HA-ADE2 rga2Δ::URA3/URA3 RPS1 | 8 |
| DK312 (ume6Δ/Δ) | ade2::his2G/ade2::his2G ura3::imm434/ura3::imm434 ENO1/eno1::ENO1-tetR-SchHAP4AD-3×HA-ADE2 tetO-UME6::URA3/UME6 | This study |
| PCY93 (hgc1Δ/Δ) | ade2::his2G/ade2::his2G ura3::imm434/ura3::imm434 ENO1/eno1::ENO1-tetR-SchHAP4AD-3×HA-ADE2 hgc1Δ::UAS1-Δ::tandem::tetO::UAS1-Δ::tandem::URA3/URA3 RPS1 | This study |
| PCY179 (rga2Δ/Δ) | ade2::his2G/ade2::his2G ura3::imm434/ura3::imm434 ENO1/eno1::ENO1-tetR-SchHAP4AD-3×HA-ADE2 hgc1Δ::UAS1-Δ::tandem::tetO::UAS1-Δ::tandem::URA3/URA3 RPS1 | This study |
| PCY95 (ume6Δ/Δ hgc1Δ/Δ) | ade2::his2G/ade2::his2G ura3::imm434/ura3::imm434 ENO1/eno1::ENO1-tetR-SchHAP4AD-3×HA-ADE2 hgc1Δ::UAS1-Δ::tandem::tetO::UAS1-Δ::tandem::URA3/URA3 RPS1 | This study |
| PCY9144 (ume6Δ/Δ rga2Δ/Δ) | ade2::his2G/ade2::his2G ura3::imm434/ura3::imm434 ENO1/eno1::ENO1-tetR-SchHAP4AD-3×HA-ADE2 hgc1Δ::UAS1-Δ::tandem::tetO::UAS1-Δ::tandem::URA3/URA3 RPS1 | This study |
| PCY87 (WT) | ade2::his2G/ade2::his2G ura3::imm434/ura3::imm434 ENO1/eno1::ENO1-tetR-SchHAP4AD-3×HA-ADE2 rga2Δ::URA3/URA3 RPS1 | This study |
| MBY38 (tetO-UME6) | ade2::his2G/ade2::his2G ura3::imm434/ura3::imm434 ENO1/eno1::ENO1-tetR-SchHAP4AD-3×HA-ADE2 tetO-UME6::URA3/UME6 | This study |
| PCY50 (tetO-UME6 hgc1Δ/Δ) | ade2::his2G/ade2::his2G ura3::imm434/ura3::imm434 ENO1/eno1::ENO1-tetR-SchHAP4AD-3×HA-ADE2 hgc1Δ::UAS1-Δ::tandem::tetO::UAS1-Δ::tandem::URA3/URA3 RPS1 | This study |
| PCY181 (tetO-UME6 hgc1Δ/Δ rga2Δ/Δ) | ade2::his2G/ade2::his2G ura3::imm434/ura3::imm434 ENO1/eno1::ENO1-tetR-SchHAP4AD-3×HA-ADE2 hgc1Δ::UAS1-Δ::tandem::tetO::UAS1-Δ::tandem::URA3/URA3 RPS1 | This study |
| PCY197 (tetO-UME6 rga2Δ/Δ) | ade2::his2G/ade2::his2G ura3::imm434/ura3::imm434 ENO1/eno1::ENO1-tetR-SchHAP4AD-3×HA-ADE2 rga2Δ::UAS1-Δ::tandem::tetO::UAS1-Δ::tandem::URA3/URA3 RPS1 | This study |

*CD, Candida dubliniensis; Cm, Candida maltosa;Sc, Saccharomyces cerevisiae.*
RNA preparation and analysis. RNA was extracted using the hot acid phenol protocol (1). Northern analysis was performed and images were visualized as described previously (2). Samples were normalized using both ACT1 and rRNA as loading controls.

Microscopy. Liquid culture images were captured by differential interference contrast (DIC) microscopy using a Zeiss Axiovision Imager Z1. Colony images were captured using a Leica S6D light microscope and digital camera. The invasion assay images were taken using an Alphaimager multi-image light cabinet.

RESULTS

Constitutive UME6 expression drives hyphal growth via an HGC1-dependent mechanism. In order to investigate the possibility that HGC1 is important for hyphal growth resulting from constitutive high-level UME6 expression, we generated a homozygous hgc1 deletion mutation in a strain in which one allele of UME6 was driven by a tetracycline-regulatable promoter (tetO-UME6 strain) (8). In the absence of doxycycline (DOX), a tetracycline derivative, UME6 is expressed at high constitutive levels, and in the presence of DOX UME6 expression is shut off. In the presence of DOX both tetO-UME6 and tetO-UME6 hgc1Δ/Δ strains grew in the yeast form as expected (data not shown). We also compared the phenotypes of these strains on solid medium and in liquid culture in the absence of DOX. On YEPD solid agar, colonies of the tetO-UME6 hgc1Δ/Δ mutant display a lacy wrinkled phenotype, which differs from the highly filamentous fuzzy colonies generated by the parental tetO-UME6 strain, suggesting a change in cell morphology (Fig. 1A). Interestingly, in liquid culture, the tetO-UME6 hgc1Δ/Δ mutant grew as very short filaments that generally had parallel sides although septal constrictions were visible (Fig. 1B). In contrast, the tetO-UME6 parent strain grew as long, extended hyphal filaments as previously observed (8). This result suggests that HGC1 is required for proper formation of the septal junction between hyphal cells and plays an important role in hyphal filament extension. These findings are consistent with previous studies demonstrating that HGC1 is important for both hyphal extension and maintenance of septin phosphorylation (important for proper septum formation) during growth in response to filament-inducing conditions (29, 41, 42).

In addition to septin phosphorylation, another previously described function of the Hgc1/Cdc28 complex is phosphorylation of Rga2, a Cdc42 GAP (42). When Rga2 is phosphorylated in response to filament-inducing conditions, it can no longer inactivate the Cdc42 master polarity regulator and hyphal growth is promoted. Zheng et al. have shown that the rga2Δ/Δ mutation can rescue the hgc1Δ/Δ hyphal extension defect during filament induction (42). Based on this informa-
adheres to agar in the absence, but not the presence, of DOX. Deletion of RGA2 alone did not affect this process. However, we observed that the tetO-UME6 hgc1Δ/Δ and tetO-UME6 hgc1Δ/Δ rga2Δ/Δ strains showed significantly reduced agar invasion in the absence of DOX, suggesting that HGC1 plays an important role in invasion directed by UME6-driven hyphal growth (Fig. 2). Interestingly, invasion by the tetO-UME6 hgc1Δ/Δ rga2Δ/Δ mutant is partially restored at the edge of the cell spot. We hypothesize that deletion of RGA2 provides a weak filamentation signal and that cells at the edge of the spot are more likely to filament because they may also be responding to weak nutrient scavenging signals. These results suggest that inactivation of Rga2 is a function of Hgc1 that is important for invasion and are consistent with our finding that the tetO-UME6 hgc1Δ/Δ hyphal extension defect can be partially rescued by deletion of RGA2 (Fig. 1B).

HGC1 and RGA2 are not required for induction of filament-specific genes in response to constitutive high-level UME6 expression. It has previously been shown that deletion of HGC1 does not affect the expression of several hypha-specific genes during filament induction (41). In order to determine whether HGC1 and RGA2 are important for induction of filament-specific genes in response to constitutive high-level UME6 expression, Northern analysis was carried out using RNA isolated from tetO-UME6, tetO-UME6 rga2Δ/Δ, tetO-UME6 hgc1Δ/Δ, and tetO-UME6 hgc1Δ/Δ rga2Δ/Δ strains grown in the presence and absence of DOX. As shown in Fig. 3A, in the absence of DOX, three known filament-specific transcripts (4, 6, 14), HWP1, ECE1, and PHR1, are expressed at equivalent levels in all four strains. These transcripts were expressed at higher levels in response to serum at 37°C (ECE1 and HWP1 are shown as examples in Fig. 3B), which is not unexpected given that multiple signaling pathways and transcriptional regulators, in addition to UME6, are known to activate hyphal genes under filament-inducing conditions (4, 6, 7, 14). The data in Fig. 3A indicate that HGC1 and RGA2 do not play a role in the transcriptional induction of several known filament-specific UME6 target genes, suggesting that the defects in hyphal extension and septum formation observed in the tetO-UME6 hgc1Δ/Δ mutant are most likely due to direct loss of a functional Hgc1 cyclin/Cdk complex. Induction of HGC1 was not affected by deletion of RGA2. We observed that the RGA2 transcript was mildly induced in the tetO-UME6 strain in the absence of DOX (Fig. 3A). This result was unexpected since RGA2 has not been reported to be transcriptionally induced under filament-inducing conditions and we have specifically observed that the expression pattern of RGA2 is not significantly affected by serum at 37°C (Fig. 4A). Our findings suggest that RGA2 may be a very weak UME6 target gene whose induction by Ume6 is detectable only upon constitutive UME6 expression; under normal filament-inducing conditions UME6 is not constitutively expressed (Fig. 4B) (8, 40).

HGC1 is induced prior to UME6, which, in turn, controls the level and duration of HGC1 expression in response to filament-inducing conditions. The experiments described above examine HGC1 expression in response to constitutive high levels of UME6. In order to determine whether UME6 is important for induction of HGC1 under filament-inducing conditions, we carried out a Northern analysis using RNA samples prepared from both wild-type and um6ΔΔ strains grown in

![FIG. 3. Constitutive UME6 expression causes induction of filament-specific genes in an HGC1- and RGA2-independent manner. (A) The tetO-UME6 strain and indicated mutants were grown as described for Fig. 1B. Cells were harvested, total RNA was isolated, and Northern analysis was carried out using probes to the indicated transcripts. (B) A wild-type strain was grown at 30°C in YEPD and diluted into filament-inducing (YEPD plus 10% serum at 37°C) and non-filament-inducing (YEPD at 30°C) media. Cells were harvested at the indicated time points, total RNA was isolated, and Northern analysis was performed using the indicated probes. Three micrograms of RNA was used for each sample. ACT1 and rRNA are included as loading controls.]
the presence or absence of serum at 37°C. As shown in Fig. 4A, in both strains HGC1 is initially induced very early, at the 5-min postinduction time point. We observed in the wild-type strain that HGC1 continues to be expressed through the 5-hour time point, whereas in the um6ΔΔ mutant the HGC1 transcript decreases after 30 min and does not appear to be significantly expressed by the 3-hour postinduction time point. This result indicates that UME6 is important for controlling both the level and duration of HGC1 induction in response to filament-inducing conditions. These findings are consistent with a previous observation that UME6 is important for HGC1 expression (40) as well as our DNA microarray analysis, which showed that UME6 controls the level and duration of induction of a variety of filament-specific transcripts (2). The RGA2 transcript showed reduced levels during the early periods of the time course (Fig. 4A). However, unlike that of HGC1, the expression pattern of RGA2 was not significantly affected by the presence or absence of filament-inducing conditions; we hypothesize that RGA2 transcript levels may be influenced by cell density. The observed decrease in RGA2 transcript levels is not likely to affect C. albicans morphology since Zheng et al. have previously shown that the Rga2 protein is expressed at all stages during serum and temperature induction and that Rga2 activity is regulated at the posttranslational level by phosphorylation (42).

We also examined the effect of hgc1ΔΔ and rga2ΔΔ mutations on UME6 induction in response to serum at 37°C (Fig. 4B). We observed that induction of UME6 was not affected in the rga2ΔΔ mutant. Interestingly, however, there was a slight decrease in the duration of UME6 induction in the hgc1ΔΔ mutant. Importantly, the results in Fig. 4 allow us to compare the timing of HGC1 expression with that of UME6. While HGC1 is induced at the 5-min postinduction time point (in both wild-type and um6ΔΔ strains) (Fig. 4A), UME6 is first detected at 15 min after induction by serum at 37°C (Fig. 4B) (2); both transcripts peaked at the 15-min time point. Taken together, these findings suggest that, while UME6 controls the level and duration of HGC1 induction, HGC1 is initially induced by an UME6-independent mechanism(s).

Genetic relationships among UME6, HGC1, and RGA2. In order to further investigate the genetic relationship between UME6 and HGC1, we generated a um6ΔΔ hgc1ΔΔ homozygous double deletion mutant and compared its phenotype with those of the respective single mutants in response to filament induction. As expected, on solid filament-inducing medium (YEPD plus serum at 37°C) colonies of the wild-type strain showed significant wrinkling, an indication of strong filamentation (Fig. 5). Under these conditions, um6ΔΔ and hgc1ΔΔ colonies appeared nearly identical, growing with smooth edges and wrinkled centers (an indication of a filamentous growth defect). However, the um6ΔΔ hgc1ΔΔ double mutant was more defective for filamentation than either single mutant since colonies of this strain appeared completely smooth, suggesting that the large majority of cells grew in the yeast form. As previously observed (2, 40, 41), in response to induction

FIG. 4. UME6 is important for determining the level and duration of HGC1 induction in response to serum at 37°C. The indicated strains were grown as described for Fig. 3B. Cells were harvested at the indicated time points, total RNA was isolated, and Northern analysis was performed using probes to the HGC1 and RGA2 (A) and UME6 (B) transcripts. Three micrograms of RNA was used for each sample. ACT1 and rRNA are used as loading controls.

FIG. 5. Colony morphologies of um6ΔΔ, hgc1ΔΔ, and rga2ΔΔ single and double deletion mutants. The indicated strains were grown on solid YEPD medium at 30°C or at 37°C in the presence of 10% serum for 3 days. Images were taken at ×20 magnification.
and hgc1/H9004/H9004 mutants are capable of forming short filaments, although both strains are defective for hyphal filament extension (Fig. 6). However, unlike wild-type and ume6/H9004/H9004 strains, which initially formed filaments at the 1-hour time point, most of the hgc1/H9004/H9004 cells showed initial filament formation at the 2-hour time point. The ume6/H9004/H9004 hgc1/H9004/H9004 double mutant grew as slightly elongated yeast cells between the 2- and 5-hour time points and was not capable of extending short filaments (Fig. 6), indicating that this strain had a more severe filamentation defect than either single mutant. These results are consistent with the colony phenotypes shown in Fig. 5. Interestingly, while constitutive UME6 expression directs extended hyphal growth via an HGC1-dependent pathway, our finding that ume6/H9004/H9004 and hgc1/H9004/H9004 mutations have an additive effect suggests that UME6 and HGC1 make independent contributions to early filament formation in response to inducing conditions.

In order to examine the genetic relationship between UME6 and RGA2, we carried out a similar analysis using a ume6/H9004/rga2/H9004 double mutant. Consistent with previous findings (42), deletion of RGA2 alone did not appear to affect filamentation in either solid or liquid inducing medium. Colonies of the ume6/H9004/rga2/H9004 double mutant appeared to have the same phenotype (smooth edges with a wrinkled center) as that of the
ume6ΔΔ single mutant in response to serum at 37°C on solid YEPD medium (Fig. 5). However, closer examination of cell morphology in a liquid serum and temperature induction experiment (Fig. 6) revealed that the ume6ΔΔ rga2ΔΔ double mutant generated filaments slightly longer than those of the ume6ΔΔ single mutant between the 3- and 5-hour time points. This result indicates that deletion of RGA2 can partially rescue the hyphal extension defect of the ume6ΔΔ mutant in response to filament-inducing conditions and provides additional evidence that UME6 directs extended hyphal growth via a pathway involving HGC1 and RGA2.

DISCUSSION

The morphological transition from yeast to hyphae in C. albicans has been an area of intense interest due to the importance of hyphal filaments for pathogenicity (17, 23, 32). In addition, pathways and mechanisms that drive this transition in C. albicans are likely to be conserved in other dimorphic fungal pathogens. While many transcriptional regulators of C. albicans filamentation have been identified (4, 14), the downstream mechanisms by which these regulators control filamentous growth are poorly understood. In this paper we identify at least one mechanism by which Ume6, a key transcriptional regulator of filamentous growth, directs hyphal filament extension.

A key regulator of C. albicans hyphal extension functions via the Hgc1 pathway. Ume6 has previously been shown to play a critical role in the process of C. albicans hyphal filament extension, which is important for both tissue invasion and virulence (2, 40). In this paper we have presented multiple lines of evidence to suggest that Ume6 directs hyphal extension via a pathway involving Hgc1 cyclin-related protein. The Hgc1/Cdc28 cyclin/Cdk complex is known to promote hyphal development by a number of mechanisms. First Hgc1/Cdc28 phosphorylates Rga2, a Cdc42 GAP, leading to Cdc42 activation, which, in turn, promotes septin ring organization, actin polymerization, and transport of secretory vesicles to the Spitzenkörper at the hyphal tip (24, 42). Ume6 appears to drive hyphal development via this mechanism since the tetO-UME6 strain is defective for hyphal extension, true septum formation, and agar invasion when deletion of HGC1 is deleted and since these defects can be partially rescued upon deletion of RGA2. Our observation that deletion of RGA2 is sufficient to partially rescue the ume6ΔΔ mutant hyphal extension defect supports this conclusion as well. These results are also consistent with previous findings that deletion of RGA2 rescues the hgc1ΔΔ mutant hyphal extension defect and that the Hgc1/Cdc28 complex is important for maintaining phosphorylation of Cdc11 septin, which, in turn, promotes the establishment and maintenance of hyphal growth (29, 42). In addition, our observation that there is a significant decline in HGC1 levels at the later serum and temperature induction time points in the ume6ΔΔ mutant and that this decline correlates well with the appearance of a hyphal extension defect (2) strongly suggests that UME6 drives hyphal extension via activation of the Hgc1 pathway. Finally, we have previously shown that constitutive high-level expression of UME6 in the absence of inducing conditions is sufficient to drive nearly complete hyphal growth and cause induction of the HGC1 transcript (8). Zeidler et al. have also observed that expression of UME6 caused germ tube formation in a small fraction of cells and led to a modest increase in HGC1 levels (40). We believe it is likely, however, that the tetO-UME6 expression system they were using did not generate sufficiently high levels of UME6 to observe identical effects (in their system UME6 was induced by the addition, rather than removal, of DOX [25]).

The Hgc1/Cdc28 complex is also known to promote hyphal growth by phosphorylation of both the Sep7 septin and the transcription factor Efg1 (15, 37). Both of these phosphorylation events prevent cell separation after cytokinesis. Sep7 phosphorylation is necessary for the assembly of hypha-specific septin rings, and phosphorylation of Efg1 is critical for down-regulation of cell separation genes. Since UME6 is important for HGC1 induction, our findings therefore suggest that Efg1 may indirectly function as a downstream target of Ume6. Interestingly, however, previous studies have shown that Efg1 is also important for transcriptional induction of both UME6 and HGC1 in response to filament-inducing conditions (40, 41). Efg1 is known to function as a downstream target of a protein kinase A (PKA) filamentous growth signaling pathway (5, 30, 33). Cdc42 is also thought to promote hyphal growth via activation of both Efg1 and the mitogen-activated protein (MAP) kinase signaling pathway (34, 35). Our results may therefore suggest a positive-feedback loop in which UME6 is first induced in response to environmental conditions in an Efg1-dependent manner via the PKA pathway. As a consequence, HGC1 is expressed for a longer period of time, leading to sustained activation of Cdc42 via inhibition of the Rga2 GAP. Finally, activated Cdc42 would function to maintain UME6 expression via activation of Efg1, therefore promoting hyphal extension. Consistent with this hypothesis, we have observed a mild decrease in the level and duration of UME6 induction in response to serum at 37°C in the hgc1ΔΔ mutant background. While activation of the HGC1-RGA2 pathway appears to be an important mechanism by which Ume6 drives hyphal extension, our finding that the hyphal extension defect of the ume6ΔΔ mutant is only partially rescued by deletion of RGA2, in contrast to the hgc1ΔΔ defect, which is fully rescued (42), suggests that Ume6 targets additional pathways as well.

Relationship between UME6 and HGC1 during early and late stages of C. albicans filament induction. Several lines of evidence suggest that Ume6 and Hgc1 may function via distinct mechanisms during the early stages of filament formation. First, while both ume6ΔΔ and hgc1ΔΔ mutants are capable of forming short filaments in response to serum at 37°C, the double mutant grows as elongated yeast cells, displaying a more severe filamentation defect. Second, we observed that during serum and temperature induction both wild-type and ume6ΔΔ strains extend short filaments by the 1-hour time point, whereas in the hgc1ΔΔ mutant short-filament formation occurs later, around the 2-hour time point. This result suggests that Hgc1 plays a Ume6-independent role in early filament formation. Finally, and most importantly, the HGC1 transcript is induced by 5 min after the cell receives a filament-inducing signal, whereas the UME6 transcript is first detected at the 15-min time point (Fig. 4) (2). This last observation suggests that HGC1 is initially induced by a UME6-independent mechanism. At later postinduction time points, however, UME6 is required for maintaining the level and duration of HGC1 expression, as well as the expression of many other filament-
induced (2), is important for maintaining both the level and duration of HGC1 transcriptional induction of Hgc1 pathway. An upstream filament-inducing signal(s) initially causes vesicle transport, and Spitzenkörper formation (24, 42). The Hgc1/Cdc28 complex also drives hyphal development by promoting septin phosphorylation and inhibiting cell separation (15, 29, 37). Additional HGC1-independent mechanisms are also known, or likely, to play an important role in these processes (9, 29, 38). Aside from driving septum formation and hyphal extension via the HGC1 pathway, UME6 is likely to promote additional aspects of hyphal development via activation of other target genes.

Specific genes (2). This observation, combined with our finding that HGC1 is necessary for hyphal development of the tetO-UME6 strain, clearly establishes a requirement for HGC1 in UME6-mediated hyphal extension. Therefore, the ume6ΔΔ hgc1ΔΔ mutant lacks essential components required to both initiate early filament formation and maintain hyphal growth and as a consequence exhibits a very severe defect in filamentation. Interestingly, Rga2 does not appear to play a role in early filament formation since rga2ΔΔ mutants are not defective for filamentous growth in response to serum at 37°C and deletion of RGA2 specifically improves the ability of the ume6ΔΔ mutant to extend filaments at later time points.

The results described above suggest a model in which Ume6 and Hgc1 function together to drive hyphal development (Fig. 7). First, HGC1 is initially induced in response to a filament-inducing signal(s) by an unknown UME6-independent mechanism. Because HGC1 induction occurs very rapidly, we hypothesize that this event may be caused by activation of a transcription factor that is already present in the cell. Next, UME6 is induced, most likely by Efg1, which has been activated by the cAMP-PKA signaling pathway; additional activators of filamentous growth are likely to target UME6 as well (40). Both UME6 and HGC1 have been shown to be under negative control by NRG1 and TUP1 (2, 40, 41), and induction of UME6 could also occur as a result of relief of transcriptional repression by the Nrg1-Tup1 pathway. Once induced, Ume6 is important for maintaining both the level and duration of HGC1 expression. Because expression of UME6 is sufficient to cause induction of HGC1 in the absence of filament-inducing conditions (8, 40), we hypothesize that Ume6 binds directly to the HGC1 promoter and directs transcriptional activation, although we cannot exclude the possibility that UME6 may also function indirectly to cause stabilization of the HGC1 message. Hgc1, in a complex with Cdc28, maintains phosphorylation of Cdc11 (29) and hyperphosphorylates Rga2 (42), causing activation of the master polarity regulator Cdc42 and subsequent hyphal extension. In addition, Hgc1/Cdc28 functions to phosphorylate both Sep7 septin and Efg1 to prevent cell separation (15, 37). Maintenance of HGC1 expression by UME6 appears to be critical since a previous study has shown that the Hgc1 protein is degraded very rapidly (36). We are planning to address this model more directly by examining the complex relationship between Hgc1 and Ume6 at the protein level. Because the hgc1ΔΔ mutation does not completely abolish UME6-driven hyphal growth (only septum formation and cell elongation are affected), Ume6 is also likely to promote other aspects of hyphal development by additional HGC1-independent mechanisms. Also, at this point it is unclear whether Hgc1/Cdc28 or additional, as yet uncharacterized, Hgc1/Cdk complexes play a role in the ability of Hgc1 to promote short filament formation independently of Ume6 during the early stages of filament induction.

Finally, this study demonstrates that the tetO-UME6 strain provides a powerful strategy to effectively tease apart the precise function of hyphal machinery components without the complexities of filament-inducing conditions. Because constitutive UME6 expression drives nearly complete hyphal growth, deletion of genes, such as HGC1, in the tetO-UME6 background allows for a more direct assessment of their role in extended hyphal formation. In the future this system is likely to provide new insight into the functions of additional known and potential hyphal machinery components.

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