Expression of **UME6**, a Key Regulator of *Candida albicans* Hyphal Development, Enhances Biofilm Formation via Hgc1- and Sun41-Dependent Mechanisms

Mohua Banerjee,a Priya Uppuluri,b Xiang R. Zhao,c Patricia L. Carlisle,a Geethanjali Vipulanandan,a Cristina C. Villar,c José L. López-Ribot,b David Kadosh,a

Department of Microbiology and Immunology, University of Texas Health Science Center at San Antonio, San Antonio, Texas, USAa; Department of Biology and South Texas Center for Emerging Infectious Diseases, The University of Texas at San Antonio, San Antonio, Texas, USAe; Department of Periodontics, University of Texas Health Science Center at San Antonio, San Antonio, Texas, USAe

Biofilm formation is associated with the ability of *Candida albicans*, the major human fungal pathogen, to resist antifungal therapies and grow on tissues, catheters, and medical devices. In order to better understand the relationship between *C. albicans* morphology and biofilm formation, we examined biofilms generated in response to expression of **UME6**, a key filament-specific transcriptional regulator. As **UME6** levels rise, *C. albicans* cells are known to transition from yeast to hyphae, and we also observed a corresponding increase in the level of biofilm formation in vitro. In addition to forming a biofilm, we observed that a *C. albicans* strain expressing constitutive high levels of **UME6** promoted tissue invasion in a reconstituted human three-dimensional model of oropharyngeal candidiasis. Confocal microscopy indicated that both the top and bottom layers of the biofilm generated upon high-level constitutive **UME6** expression consist primarily of hyphal cells. **UME6**-driven biofilm formation was reduced upon deletion of Hgc1, a cyclin-related protein important for hyphal development, as well as Sun41, a putative cell wall glycosidase. Constitutive high-level **UME6** expression was also able to completely bypass both the filamentation and biofilm defects of a strain deleted for Efg1, a key transcriptional regulator of these processes. Finally, we show that both Sun41 and Efg1 affect the ability of **UME6** to induce certain filament-specific transcripts. Overall, these findings indicate a strong correlation between increased *C. albicans* hyphal growth and enhanced biofilm formation and also suggest functional relationships between **UME6** and other regulators of biofilm development.

*Candida albicans* is a major human fungal pathogen responsible for a wide variety of both systemic and mucosal infections (1). *Candida* species are the fourth leading cause of nosocomial infections in the United States, with a mortality rate of 30 to 50% (2–5). Immunocompromised individuals, such as AIDS patients, cancer patients on chemotherapy, and organ transplant recipients, are especially susceptible to infections (6–10). Many of these infections are associated with the formation of biofilms on tissues, catheters, and implanted medical devices. *C. albicans* biofilms are known to provide protection from host immune defenses and are also extremely resistant to antifungal treatments. In addition, these biofilms can serve as reservoirs for infection, since cells dispersed from the biofilm can traverse the bloodstream and eventually establish secondary sites of infection (11–14).

In *C. albicans*, biofilm formation involves adhesion of single cells to a surface (biotic or abiotic), proliferation, hyphal development, and generation of exopolymeric material (14–18). A variety of adhesins, including the glycoprophosphatidylinositol (GPI)-linked cell wall protein Eap1 and the agglutinin-like protein Als1, appear to play important roles in the initial attachment of *C. albicans* cells to surfaces (16, 19–21). Als3 and Hwp1, a mammalian transglutaminase substrate mimic, also function as complementory adhesins and are most likely involved in cell-cell and cell-surface interactions of hyphae in biofilms (16, 22–24). Several additional cell surface proteins have been implicated in *C. albicans* biofilm formation, the most notable of which is Sun41, a putative glycosidase. **sun41ΔΔ** mutants have defects in cytokinesis, cell wall biogenesis, and adhesion to host cells and are highly attenuated for virulence in mouse models of disseminated and oropharyngeal candidiasis (25–27). Most of these phenotypes are believed to be attributed to a cell wall defect since the **sun41ΔΔ** mutant is very sensitive to cell wall inhibitors, such as Congo red, and shows altered expression of several cell wall damage repair genes (25–27). Interestingly, this mutant shows defective hypha formation on solid medium, and one group reported that the **sun41ΔΔ** strain forms aberrant hyphae in liquid medium as well (25, 26). Although Sun41 has been extensively characterized at the phenotypic level, very little information is available to link this cell wall protein to known biofilm development pathways.

The ability to form hyphae appears to be particularly important for biofilm formation, since a strain genetically manipulated to grow exclusively in the yeast form is highly defective in generating biofilms, and a variety of *C. albicans* mutants defective for hypha formation also show biofilm defects (16, 28, 29). One such mutant strain bears a homozygous deletion of Efg1, a major transcriptional regulator of *C. albicans* filamentous growth (30). The **efg1ΔΔ** mutant grows as elongated yeast cells under most conditions and is highly defective for biofilm formation in vitro (28).
Recently, this mutant was also observed to be defective for biofilm formation in both rat catheter and denture in vivo models, and Efg1 was shown to function as a component of a master transcriptional network that controls C. albicans biofilm formation (31). Although many direct targets for Efg1 were identified by this analysis, few downstream genes have specifically been shown to be important for the ability of Efg1 to promote biofilm formation.

While a number of transcription factors have been identified which, like Efg1, are required to generate C. albicans biofilms, considerably less is known about regulators whose expression enhances biofilm formation. A recent screen of a C. albicans overexpression library identified four such regulators: GAT2, TEC1, CPH1, and UME6 (32). Our studies have focused on UME6, which encodes a key filament-specific transcriptional regulator of C. albicans hyphal development and virulence (33). UME6 is a downstream target of multiple filamentous growth signaling pathways, and we have previously shown that constitutive high-level expression of UME6 is sufficient to drive complete hypha formation in the absence of filament-inducing conditions (34, 35). Interestingly, as UME6 levels rise, cells sequentially transition from yeast cells to pseudohyphae to hyphae, and there is a corresponding increase in the number of filament-specific genes expressed as well as their levels of expression. 

$mex6^\Delta$ mutants are attenuated for virulence, are defective for hyphal extension, and also show a defect in biofilm formation (33). In addition, we have demonstrated that a strain expressing constitutive high levels of UME6 generates a very filamentous biofilm and is highly defective for biofilm dispersion (36).

We have previously shown that UME6 drives hyphal development via transcriptional induction of HGC1, which encodes a cyclin-related protein (37). Hgc1 is known to form a cyclin/Cdk complex with Cdc28 kinase, which, in turn, is important for septin phosphorylation, inhibition of cell separation, and activation of the Cdc42 master polarity regulator (involved in septin ring organization, vesicle transport to the hyphal tip, and actin polymerization) (38–44). Expression of UME6 in an hgc1$\Delta$ mutant strain results in shorter filaments with constrictions at septal junctions (37). Although Hgc1 is directly involved in a variety of mechanisms important for driving C. albicans hyphal growth, a role for Hgc1 in biofilm formation has not yet been reported.

Because biofilms generated by our UME6 expression strain contain a significantly high proportion of hyphal cells (36), this strain provides a powerful strategy to determine the specific role(s) of hyphae in biofilm formation. Here, we demonstrate that increased UME6 expression is correlated with enhanced biofilm formation. In order to gain a better understanding of the molecular mechanism(s) involved in UME6-driven increased biofilm formation, we examine the roles of a key biofilm transcriptional regulator (Efg1), a cyclin-related protein specifically important for the physical process of hyphal development (Hgc1), and a putative cell wall glycosidase (Sun41) in this process. Our results suggest that important functional relationships among these different proteins play a significant role in the ability of C. albicans hyphae to promote biofilm formation.

### MATERIALS AND METHODS

**Strain and plasmid constructions.** Genotypes for all strains used in this study are shown in Table 1. The wild-type tetR control strain (PCY87) as well as tet-O-UME6 (MBY38) and tet-O-UME6 hgc1$\Delta$ (PCY50) strains were described previously (34, 37). In order to construct the tet-O-UME6 efg1$\Delta$ strain (PCY21), primer pairs 1/2 and 3/4 (see Table S1 in the supplemental material) to generate PCR products corresponding to the 5’ and 3’ flanking regions (just outside the open reading frame), respectively, of EFG1. The 5’ flanking region was digested with KpnI and XhoI, and the 3’ flanking region was digested with KpnI and SacII. These fragments were then cloned stepwise into plasmid pSFS2 (46). The resulting construct was digested with KpnI and SacII to release an efg1$\Delta$:SAT1 fragment, which was used to transform the tet-O-UME6 strain (MBY38). Homozygous deletion mutants were generated by using the SAT flipper method (46), and whole-cell PCR was used to verify correct integration at the 5’ and 3’ disruption junctions as well as the absence of the open reading frame. A similar strategy was used to construct the tet-O-UME6 sun41$\Delta$ strain (MBY179), using primer pairs 5/6 and 7/8 (see Table S1 in the supplemental material) to generate PCR products corresponding to positions 650 to 122 (relative to the UME6 start ATG) and positions 45 to +443 (relative to the UME6 start ATG) were obtained by using primers 29/30 and 31/32, respectively (see Table S1 in the supplemental material). The UME6 fragment at positions 650 to 122 was digested with KpnI and Apal, and the UME6 fragment at positions 45 to +443 was digested with SacII and Ncol. These fragments were then cloned stepwise into plasmid pJK1000 (45). The resulting construct was digested with KpnI and Ncol to release an FLP-CaNAT1-tet-O-UME6 fragment, which was used to transform tetR parent strain JKC915. Whole-cell PCR was used to confirm the integration of the tetO cassette at the UME6 locus.

### TABLE 1 Strains used in this study

| Strain* | Genotype | Reference |
|---------|----------|-----------|
| PCY87 (WT) | ade2::hisG/ade2::hisG ura3::imm434/ura3::imm434 ENO1/eno1::EN01-tetR-ScHAP4AD-3::HA-ADE2 rps1::URA3/RPS1 tet-O-UME6::URA3/UME6 | 37 |
| MBY38 (tetO-UME6) | ade2::hisG/ade2::hisG ura3::imm434/ura3::imm434 ENO1/eno1::EN01-tetR-ScHAP4AD-3::HA-ADE2 rps1::URA3/RPS1 tet-O-UME6::URA3/UME6 | 34 |
| PCY50 (tetO-UME6 hgc1$\Delta$) | ade2::hisG/ade2::hisG ura3::imm434/ura3::imm434 ENO1/eno1::EN01-tetR-ScHAP4AD-3::HA-ADE2 rps1::URA3/RPS1 tet-O-UME6::URA3/UME6 | 37 |
| PCY21 (tet-O-UME6 efg1$\Delta$) | ade2::hisG/ade2::hisG ura3::imm434/ura3::imm434 ENO1/eno1::EN01-tetR-ScHAP4AD-3::HA-ADE2 rps1::URA3/RPS1 tet-O-UME6::URA3/UME6 | This study |
| MBY179 (tet-O-UME6 sun41$\Delta$) | ade2::hisG/ade2::hisG ura3::imm434/ura3::imm434 ENO1/eno1::EN01-tetR-ScHAP4AD-3::HA-ADE2 rps1::URA3/RPS1 tet-O-UME6::URA3/UME6 | This study |
| JKC915 (WT) | HIS1/ura3::FRT tetR | 45 |
| MBY208 (tet-O-UME6) | HIS1/ura3::FRT tetR UME6::FLP-CaNAT1 tetO-UME6 | This study |

*WT, wild type.
FIG 1 UME6 expression level is correlated with increased biofilm formation. Suspensions of $1 \times 10^6$ cells/ml of the indicated strains were allowed to form biofilms on 96-well polystyrene plates in Lee’s pH 6.8 medium at 30°C either in the absence of Dox or in the presence of the indicated Dox concentrations. Biofilm formation was assessed by using a standard colorimetric XTT reduction assay (18, 48). Error bars represent standard deviations ($n = 4$).

Media and growth conditions. Standard growth conditions for all strains included solid or liquid yeast extract-peptone-dextrose (YPEP) medium at 30°C (47) in the presence or absence of 20 μg/ml doxycycline (Dox) (Sigma-Aldrich, St. Louis, MO), unless otherwise indicated. Liquid cultures were grown overnight at 30°C and harvested at an optical density of 600 nm (OD$_{600}$) of ~1.0 for both differential interference contrast (DIC) microscopy and RNA preparation, as described previously (37). Cells for static biofilm formation assays were initially grown overnight in YEPD medium at 30°C in the presence of 20 μg/ml Dox. In these assays, either Lee’s medium, pH 6.8, or minimal medium (yeast nitrogen base [YNB] without amino acids plus 2% dextrose) was used for biofilm formation, as indicated. Biofilms used for confocal scanning laser microscopy (CSLM) were formed by using YNB with amino acids plus 2% dextrose medium.

Biofilm development assays. A standard 96-well assay was used to assess static biofilm formation, as described previously (48, 49). Briefly, cell suspensions grown overnight were washed twice in phosphate-buffered saline (PBS) and, based on OD$_{600}$ readings, diluted to a concentration of $1 \times 10^6$ cells/ml. One hundred microliters of each diluted cell suspension was added to single wells of a 96-well polystyrene plate containing the indicated medium and incubated at 30°C for 24 h. Each well was washed twice with 200 μl of PBS, and the level of biofilm formation was determined by using a standard semiquantitative colorimetric 2,3,5-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay, as reported previously (18, 48). Biofilms for CSLM were assessed by using a Zeiss LSM 510 upright confocal microscope with a Zeiss Axiosplan 40×, 0.8-W objective (excitation wavelength of 543 nm). Zeiss LSM Image Browser v.4.2 was used to assemble CSLM microscopy images.

Reconstituted three-dimensional model of the human oral mucosa. The three-dimensional model of the human oral mucosa used in this study was described previously (31–34). This system is composed of gingival fibroblasts embedded in a biomatrix of collagen type I and overlaid by a multilayer of oral epithelial cells. To study C. albicans invasion in this system, the three-dimensional model of the oral mucosa was challenged with $1 \times 10^6$ C. albicans yeast cells in 100 μl of airlift medium (keratinocyte serum-free medium [KSFM] containing 5% fetal bovine serum, 1.88 mM CaCl$_2$, and 0.025 mM dextrose) in the presence or absence of 20 μg/ml Dox (inoculum for the MBY208 tetO-UME6 strain was prepared from cells grown in the presence of 20 μg/ml Dox). Airlift medium with or without 20 μg/ml Dox was also added to the uninfected controls. At 24 h postinfection, the cultures were fixed with 10% formaldehyde–PBS and embedded in paraffin. Formalin-fixed paraffin-embedded sections (thickness, 5 μm) of three-dimensional oral mucosal cultures were stained with hematoxylin and eosin (H&E). Stained sections were visualized by using a Leica DM RB microscope connected to a digital camera.

RESULTS

Increased UME6 expression is correlated with enhanced C. albicans biofilm formation. In order to determine the effect of the UME6 expression level on C. albicans biofilm formation, we used a strain in which one allele of UME6 is placed under the control of the Escherichia coli tet operator (tetO) (34). This strain also expresses constitutive levels of an E. coli tetR DNA-binding domain–Saccharomyces cerevisiae HAP4 activation domain fusion protein (55). In the absence of Dox, the tetR-HAP4 transactivator binds as a dimer to the tet operator and directs high levels of UME6 expression. In the presence of Dox, the transactivator no longer dimerizes, and the UME6 allele is not expressed. Using this system, we have previously shown that when UME6 is not expressed in the presence of 20 μg/ml Dox, cells grow as yeast. As the Dox concentration is reduced and UME6 levels rise, cells gradually transition from yeast to pseudohyphae to hyphae (34). A similar experiment was performed to monitor biofilm formation in vitro as UME6 levels increased. As demonstrated in Fig. 1, the tetO-UME6 strain showed a level of biofilm formation equivalent to that of the wild-type control strain (which expresses the tetR-HAP4 transactivator but lacks a tet operator) at 20 μg/ml Dox. However, as Dox levels decreased, there was a gradual increase in the level of biofilm formation by the tetO-UME6 strain, whereas the wild-type control strain showed nearly constant biofilm levels and was not affected by Dox. Confocal microscopy indicated that cells in the bottom-most layer of the tetO-UME6 biofilm showed a significantly higher proportion of hyphae in the absence of Dox (−Dox) than in the presence of Dox (+Dox) and than cells of the wild-type control strain (+Dox or −Dox) (Fig. 2). Taken together, these results
Previously generated ability was assessed in the presence and absence of Dox. The tetO-UME6 strain was used to challenge the epithelial cell layer of this model in the presence or absence of Dox for a 24-h infection period. As indicated in Fig. 3, the level of biofilm formation appeared to be roughly equivalent on cell layers infected with the wild-type strain (+Dox and −Dox) and the tetO-UME6 strain in the presence of Dox. In the absence of Dox, the tetO-UME6 strain still formed a biofilm, and a significant proportion of C. albicans cells clearly appeared to invade the oral epithelium. Indeed, a large number of hyphal filaments completely penetrated the oral epithelium and reached the subepithelial layer of collagen-embedded fibroblasts. Increased invasion and penetration of the three-dimensional model of the human oral mucosa by the tetO-UME6 strain in the absence of Dox were also observed during a 36-h infection (data not shown). These results indicate that constitutive high-level UME6 expression alone is sufficient to promote tissue invasion in a reconstituted human model of oropharyngeal candidiasis and are consistent with our previous findings using a mouse systemic model (34).

Hgc1 and Sun41 are important for enhanced biofilm formation in response to UME6 expression. We next sought to determine the role of hypha-specific and cell wall components in mediating UME6-directed enhanced biofilm formation. Homozygous deletion mutations of Efg1 and Sun41 were generated in the tetO-UME6 strain background, and along with a previously generated tetO-hgc1Δ/Δ mutant (37), biofilm formation ability was assessed in the presence and absence of Dox. The tetO-UME6 efg1Δ/Δ strain showed a significant biofilm defect in the presence of Dox compared to the parent tetO-UME6 strain in the absence of Dox or the wild-type control strain in the presence or absence of Dox (Fig. 4A). However, the tetO-UME6 efg1Δ/Δ mutant continued to show enhanced biofilm formation, equivalent to that of the parent tetO-UME6 strain, upon high-level UME6 expression in the absence of Dox. This result indicates that expression of UME6 alone is sufficient to bypass the efg1Δ/Δ biofilm defect and suggests that UME6 functions downstream of the Efg1 regulator in C. albicans biofilm development. In the absence of Dox, the level of enhanced biofilm formation by the tetO-UME6 hgc1Δ/Δ mutant was clearly reduced, although not completely abolished, compared to that of the parent tetO-UME6 strain. In the presence of Dox, deletion of Hgc1 appeared to have very little, if any, effect on biofilm formation by the tetO-UME6 strain. Deletion of Sun41 caused an overall decrease in the level of tetO-UME6 biofilm formation (Fig. 4B). While the tetO-UME6 sun41Δ/Δ mutant still showed an increase in the level of biofilm formation in the absence versus the presence of Dox, the level of biofilm formation in the absence of Dox was equivalent to that of the wild-type control strain (+Dox or −Dox) and did not show an overall enhancement. As previously observed for the sun41Δ/Δ strain (26, 27), the tetO-UME6 sun41Δ/Δ mutant also showed a hypersensitivity to Congo red, strongly suggesting a cell wall defect (see Fig. S1 in the supplemental material). Taken together, these results indicate that both Hgc1 and Sun41 play important roles in the ability of UME6 expression to enhance overall C. albicans biofilm formation relative to that of a wild-type strain and suggest that both hypha-dependent and cell wall-dependent mechanisms are involved in this process.
Hgc1 and Sun41 are important for UME6-driven hyphal growth in biofilms. In order to specifically examine the effect of efg1Δ/Δ, sun41Δ/Δ, and hgc1Δ/Δ mutations on UME6-driven hyphal growth within biofilms, we used confocal microscopy. Interestingly, as shown in Fig. 5, the top layer of the tetO-UME6 efg1Δ/Δ biofilm showed increased hypha formation compared to that of the parent tetO-UME6 strain upon UME6 expression in the absence of Dox. In the presence of Dox, when UME6 was not expressed, this mutant showed a mostly yeast biofilm, as expected. The tetO-UME6 sun41Δ/Δ biofilm showed reduced hypha formation in the absence of Dox and appeared to have a substrate adherence defect in the presence of Dox, as previously observed for the sun41Δ/Δ mutant (27). Also consistent with a previous report (25), we observed a reduction in the hyphal compartment length of the tetO-UME6 strain upon deletion of Sun41 when this strain was grown as a biofilm in either the presence or absence of Dox (see Fig. S2 in the supplemental material). While the tetO-UME6 hgc1Δ/Δ mutant biofilm still formed hyphae upon UME6 expression in the absence of Dox, these filaments were shorter and not as prevalent as those observed in the parent tetO-UME6 strain biofilm (Fig. 5).

We also examined the effect of efg1Δ/Δ and sun41Δ/Δ mutations on UME6-driven filamentation under both solid and liquid non-filament-inducing conditions (we have previously shown that Hgc1 plays an important role in this process [37]). As indicated in Fig. 6A and B, constitutive high-level expression of UME6 was able to completely bypass the efg1Δ/Δ filamentation defect in either solid or liquid medium. Consistent with our biofilm results (Fig. 5), the tetO-UME6 sun41Δ/Δ mutant appeared to show a filamentation defect under solid growth conditions (Fig. 6A). Interestingly, however, under liquid growth conditions, this mutant appeared to show normal hyphal growth in response to UME6 expression in the absence of Dox (Fig. 6B), although we cannot exclude the possibility that the tetO-UME6 sun41Δ/Δ strain shows a reduction in the hyphal compartment length, as we have observed in biofilms (see Fig. S2 in the supplemental material) and as Firon et al. (25) observed previously in liquid medium. In the presence of Dox, this strain also showed a cell separation defect (Fig. 6B) characteristic of sun41Δ/Δ mutants growing in the yeast form (25, 26). Altogether, our results, combined with previous findings (37), indicate that Sun41 and Hgc1, but not Efg1, are important for UME6-driven filamentation in biofilms.

Sun41 and Efg1 affect the ability of Ume6 to induce certain filament-specific transcripts. Because Sun41 and Efg1 have different effects on UME6-driven hyphal growth, we next sought to determine the role that these proteins play in the ability of Ume6 to induce filament-specific transcripts (we have previously shown that an hgc1Δ/Δ mutation does not affect this process [37]). tetO-UME6 efg1Δ/Δ and tetO-UME6 sun41Δ/Δ mutants, as well as the tetO-UME6 parent strain, were grown under non-filament-inducing conditions in the presence and absence of Dox. Northern analysis was used to examine the levels of a variety of known filament-specific transcripts. As observed previously (34, 37), all of these transcripts were induced in the tetO-UME6 parent strain upon UME6 expression in the absence, but not the presence, of Dox (Fig. 7). Interestingly, five transcripts (HYR1, HWP1, RBT4, ECI1, and ALS3) showed significantly reduced expression levels in the tetO-UME6 efg1Δ/Δ mutant but significantly increased expression levels in the tetO-UME6 sun41Δ/Δ mutant. Levels of the RBT1 transcript showed a minor increase in the tetO-UME6 efg1Δ/Δ strain and a small decrease in the tetO-UME6 sun41Δ/Δ mutant. In contrast, induction of PHR1 and HGC1 by Ume6 did not appear to be significantly affected in the tetO-UME6 efg1Δ/Δ and tetO-UME6 sun41Δ/Δ mutant backgrounds. These results suggest, unexpectedly, that both Efg1 and Sun41 play important roles in the ability of Ume6 to induce certain filament-specific transcripts and that additional functional relationships may exist among these biofilm regulators.

**DISCUSSION**

**Correlation of increased hyphal growth with enhanced C. albicans biofilm formation.** While several previous studies identified regulators and pathways that are required or important for both C. albicans filamentation and biofilm formation (16, 28, 29, 33, 56–59), considerably less is known about mechanisms that pro-
As assays, our results suggest that following the initiation step, the yeast form (in the presence of Dox) prior to the start of biofilm formation. The indicated strains were grown as described in the legend of Fig. 6B. Cells were harvested, and total RNA was prepared for Northern analysis. Blots were probed for the indicated transcripts. Each lane was loaded with 3 μg of total RNA. ACT1 and rRNA are shown as loading controls.

The Sun41 cell wall putative glycosidase has been clearly shown to rescue the biofilm defect of a strain deleted for BCR1, an important transcriptional regulator of biofilm development (61, 62). Induction of ALS3 and HWP1 in response to UME6 expression could therefore possibly contribute to enhanced biofilm formation. However, based on our gene expression analysis (Fig. 7), neither adhesin may be solely required for this process (see below).

In addition to ALS3, HWP1, and other genes whose expression is associated with filamentous growth, UME6 is also known to control at least one gene, HGC1, important for the physical process of hyphal development (37). Hgc1 is known to promote hyphal development by septin phosphorylation, inhibition of cell separation, and activation of the Cdc42 master polarity regulator Hgc1 (38–44). We have previously demonstrated that UME6 directs hyphal growth via the Hgc1 pathway and that a tetO-UME6 strain bearing a homozygous deletion of HGC1 is defective for extended hyphal development and true septum formation (37). Our current finding that this mutant is also defective for biofilm formation is significant because it indicates that mechanisms specifically associated with the physical process of hypha formation play an important role in promoting biofilm development (Fig. 8).

The Sun41 cell wall putative glycosidase has been clearly shown to be important for C. albicans biofilm formation (25–27). Although we cannot exclude the possibility that the sun41ΔΔ biofilm defect is partially due to a filamentation defect, the available evidence suggests that this mutant shows reduced biofilm formation primarily as a consequence of a severe cell wall defect (27).
Our finding that enhanced biofilm formation in response to *UME6* expression is significantly reduced upon deletion of *SUN41* strongly suggests that general mechanisms important for cell wall integrity play an important role in this process (Fig. 8). Consistent with this hypothesis, the tetO-*UME6 sun41ΔΔ* mutant, like the *sun41ΔΔ* mutant, shows a clear cell wall defect, as indicated by hypersensitivity to Congo red. Interestingly, both our Northern analysis as well as a recent DNA microarray analysis (66) indicate that the *SUN41* transcript is very mildly induced upon *UME6* expression. In addition, we have observed that deletion of *Sun41* causes a slight decrease in both *UME6* and *EFG1* transcript levels in the tetO-*UME6* strain in the absence of Dox (although unlikely, we cannot exclude the possibility that these effects are due to a reduction in transactivator levels) (Fig. 7). These findings suggest that, at a transcriptional level, *UME6* and *SUN41* may function in a feedback loop as mildly positive regulators of each other; in addition, *SUN41* may also function as a slight positive regulator of *EFG1* expression when *UME6* is expressed at high constitutive levels (since *Sun41* is a cell wall component, transcriptional effects directed by this protein would most likely be indirect [see below]). However, the mild reduction in *UME6* and *EFG1* transcript levels in the tetO-*UME6 sun41ΔΔ* mutant generally did not appear to be sufficient to cause a decrease in *UME6* target gene expression levels; instead, most *UME6* target genes appeared to be induced at equivalent or higher levels in this strain. In addition, while the overall level of biofilm formation by the tetO-*UME6 sun41ΔΔ* mutant was not significantly enhanced compared to that of a wild-type control strain, it was still increased upon *UME6* expression. This observation suggests that additional mechanisms associated with *UME6* expression may still have a limited capacity to overcome the *sun41ΔΔ* biofilm defect.

In summary, both hypha-dependent and cell wall-dependent mechanisms, mediated via the Hgc1 cyclin-related protein and the Sun41 putative glycosidase, respectively, appear to play important roles in promoting *UME6*-driven enhanced biofilm formation (Fig. 8). At this point, we cannot exclude the possibility that an additional downstream mechanism(s), which has not yet been determined, may also be involved in this process.

Relationship between *EFG1* and *UME6* with respect to biofilm formation, filamentation, and filament-specific gene expression. *Efg1*, an important transcription factor which controls *C. albicans* filamentous growth, is also known to function as a key regulator of biofilm formation (28, 30). Our observation that constitutive-high-level *UME6* expression is sufficient to completely bypass the severe *efg1ΔΔ* mutant biofilm and filamentation defects is consistent with previous findings (35) and strongly suggests that *UME6* functions downstream of *EFG1* with respect to these processes (Fig. 8). These results are also consistent with previous findings that *Efg1* is important for transcriptional induction of *UME6* as well as downregulation of *NRG1*, which encodes a negative regulator of *UME6*, in response to serum at 37°C (33, 35, 63). Our finding that deletion of *EFG1* increases the density of hypha formation in the top layers of the tetO-*UME6* biofilm was unexpected but may be related to previous reports that *Efg1* functions as a negative regulator of *C. albicans* filamentation under embedded/matrix conditions (64) (as it is conceivable that the dense hyphal mat generated by constitutive high-level *UME6* expression may lead to similar microaerophilic conditions within the biofilms). Interestingly, despite the fact that the density of hyphal filaments was increased in the tetO-*UME6* strain upon deletion of *Efg1*, there was not a corresponding further increase in overall biofilm formation. This result suggests that while increased hyphal growth is generally correlated with enhanced biofilm formation, there may be a limit above which the density of hyphal filaments in a biofilm has no additional effect.

Given that *UME6* appears to function downstream of *Efg1* with respect to biofilm and filament formation, our finding that *Efg1* is also required for the ability of *UME6* to transcriptionally induce certain hypha-specific genes was also surprising. Of the five *UME6* target genes whose induction is affected by the *efg1ΔΔ* mutation, three (*HYR1, ALS3*, and *HWP1*) encode cell wall proteins, one is associated with cell elongation (*ECE1*), and one encodes a secreted virulence factor (*RBT4*). Interestingly, all five genes also show increased induction by *UME6* upon deletion of *SUN41* and no significant induction in the absence of *UME6* expression. These results suggest that perturbation of the *C. albicans* hyphal cell wall could indirectly trigger a compensatory increase in the expression level of cell wall/secreted proteins and are consistent with previous findings that deletion of *Sun41* causes altered expression of cell wall biogenesis genes and results in a compensatory regulation of other glycosidases (26, 27). *Efg1* is a major regulator of cell wall genes (65) and may also play an indirect role in this pathway, although deletion of *Efg1* does not appear to cause a cell wall defect (see Fig. S1 in the supplemental material). In either case, since the tetO-*UME6 efg1ΔΔ* mutant still showed increased biofilm formation and filamentation relative to that of a wild-type control strain, our gene expression results strongly suggest that *HYR1, HWP1, RBT4, ECE1*, and *ALS3* are largely dispensable for these processes; instead, additional target genes, which are induced by *UME6* in an Efg1-independent manner, are likely to function in *UME6*-driven enhanced biofilm formation and filamentous growth. Recently, Nobile et al. found that *Efg1* functions as a component of a large and complex regulatory network that controls *C. albicans* biofilm development (31). Chromatin immunoprecipitation with microarray technology (ChIP-chip) data from this study indicate that *UME6* appears to be a downstream target of many transcriptional regulators in the network, including *Efg1*. Perturbations in this network may also at

FIG 8 Model for roles of Efg1, Hgc1, and Sun41 in *UME6*-driven enhanced C. albicans biofilm formation. Ume6 functions downstream of Efg1 and upstream of both Hgc1 and Sun41 to promote biofilm development. *UME6* expression is known to cause transcriptional induction of the Hgc1 cyclin-related protein, which, in turn, directs hyphal development via septin phosphorylation, inhibition of cell separation genes, and activation of the Cdc42 master polarity regulator (38-44). *UME6* expression also appears to cause a slight increase in SUN41 transcript levels. SUN41, in turn, may function indirectly in a positive-feedback loop to increase *UME6* expression levels (not shown); however, the relevance of these transcriptional effects for biofilm formation has not yet been established. In either case, Sun41, a putative cell wall glycosidase, is known to be primarily involved in maintaining cell wall integrity (27). Both the physical process of hyphal development and Sun41-mediated cell wall integrity therefore appear to play important roles in *UME6*-driven enhanced biofilm formation. In addition, we cannot exclude the possibility that Sun41 at least partly contributes to *UME6*-driven biofilm growth by playing a role in hyphal development (dashed line). Finally, it is important to note that an additional mechanism(s), which at this point has not yet been determined, may also contribute to *UME6*-driven enhanced biofilm formation.
least partially explain the differential effects of efg1Δ/Δ and sun41Δ/Δ mutations on the induction of specific UME6 target genes.

While the complex pathways which control both C. albicans hyphal growth and biofilm formation have yet to be fully elucidated, these studies provide new information about the mechanistic relationship between these two processes. Future work in this area using strains (such as tetO-UME6) which can be genetically manipulated to alter morphology is likely to significantly expand our knowledge of and provide greater insight into this complex, but extremely important, relationship.

ACKNOWLEDGMENTS

We are grateful to Brian Wickes for useful advice and suggestions during the course of the experiments. P.L.C. was supported by a COSTAR fellowship (T32DE14318-07) and by a NRSA predoctoral fellowship from the National Institute of Dental and Craniofacial Research, or the National Institutes of Health.

REFERENCES

1. Odds FC. 1988. Candida and candidosis. Baillière Tindall, London, United Kingdom.
2. Beck-Sague C, Jarvis WR. 1993. Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980-1990. National Nosocomial Infections Surveillance System. J. Infect. Dis. 167:1247–1251.
3. Edmond MB, Wallace SE, McClish DK, Pfaller MA, Jones RN, Wenzel RN, Pfaller MA, Lockhart SR, Pujol C, Swails-Wenger JA, Messer SA, Breitenbach M, Bito A. 2009. Deep-seated candidal infections, p 341–348. In Calderone R (ed), Medical importance of biofilms in Candida albicans. 2nd ed. CRC Press.
4. Ramage G, Vanwalle K, Wickes BL, Lopez-Ribot JL. 2001. Charac- teristics of biofilm formation by Candida albicans. Rev. Iberoam. Micol. 18:163–170.
5. Hoyer LL. 2001. The ALS gene family of Candida albicans. Trends Micro- biol. 9:176–180.
6. LiF, Palecek SP. 2003. EAP1, a Candida albicans gene involved in binding human epithelial cells. Eukaryot. Cell 2:1266–1273.
7. Li F, Svarovsky MJ, Karlsson AJ, Wagner JP, Marchillo K, Oshel P, Andes D, Palecek SP. 2007. Eap1p, an adhesin that mediates Candida albicans biofilm formation in vitro and in vivo. Eukaryot Cell. 6:931–939.
8. Nobile CJ, Schneider HA, Nett JE, Sheppard DC, Filler SG, Andes DR, Mitchell AP. 2008. Complementary adhesion function in C. albicans biofilm formation. Curr. Biol. 18:1017–1024.
9. Maas CE, Panosian AN, Fisher LA, Daniels TB, Young JR. 2012. Roles of sun41 and sun42 genes are essential for cell separation in Candida albicans. Mol. Microbiol. 66:1256–1275.
10. Hiller E, Heine S, Brunner H, Rupp S. 2007. Candida albicans Sun41p, a putative glycosidase, is involved in morphogenesis, cell wall biogenesis, and biofilm formation. Eukaryot Cell. 6:2056–2065.
11. Norice CT, Smith FJ, Jr, Solis N, Filler SG, Mitchell AP. 2007. Require- ment for Candida albicans Sure1 in biofilm formation and virulence. Eukaryot Cell. 6:2046–2055.
12. Ramage G, VandeWalle K, Lopez-Ribot J, Wickes B. 2002. The filamentation pathway controlled by the Efg1 regulator protein is required for normal biofilm formation and development in Candida albicans. FEMS Microbiol. Lett. 214:95. doi:10.1111/j.1574-6968.2002.tb11330.x.
13. Uppuluri P, Pierce CG, Thomas DP, Bubeck SS, Saville SP, Lopez-Ribot JL. 2010. The transcriptional regulator Nrg1p controls Candida albicans biofilm formation and dispersion. Eukaryot Cell. 9:1531–1537.
14. Du H, Guan G, Xie J, Sun Y, Tong Z, Zhang I, Huang G. 2012. Roles of Candida albicans Gat2, a GATA-type zinc finger transcription factor, in biofilm formation, filamentous growth and virulence. PLoS One 7:e29707. doi:10.1371/journal.pone.0029707.
15. Banerjee M, Thompson DS, Lazzell A, Carlisle PL, Pierce C, Monteagudo G, Lopez-Ribot JL, Kadosh D. 2008. UME6, a novel filamentation- specific regulator of Candida albicans hyphal extension and virulence. Mol. Biol. Cell 19:1354–1365.
16. Carlisle PL, Banerjee M, Lazzell A, Monteagudo C, Lopez-Ribot JL, Kadosh D. 2009. Expression levels of a filament-specific transcriptional regulator are sufficient to determine Candida albicans morphology and virulence. Proc. Natl. Acad. Sci. U. S. A. 106:599–604.
17. Zeidler U, Lettner T, Lassign C, Muller M, Lajko R, Hintner H, Breitenbach M, Bito A. 2009. UM6 is a crucial downstream target of other transcriptional regulators of true hyphal development in Candida albicans. FEMS Yeast Res. 9:126–142.
18. Uppuluri P, Chaturvedi AK, Srinivasan A, Banerjee M, Ramasubrama- niam AK, Kohler JR, Kadosh D, Lopez-Ribot JL. 2010. Dispersion as an important step in the Candida albicans biofilm developmental cycle. PLoS Pathog. 6:e1000828. doi:10.1371/journal.ppat.1000828.
19. Carlisle PL, Kadosh D. 2010. Candida albicans Ume6, a filament-specific
transcriptional regulator, directs hyphal growth via a pathway involving Hgc1 cyclin-related protein. Eukaryot. Cell 9:1320–1328.
38. Gonzalez-Novo A, Correa-Bordes J, Labrador L, Sanchez M, Vazquez de Aldana CR, Jimenez J. 2008. Sep7 is essential to modify septin ring dynamics and inhibit cell separation during Candida albicans hyphal growth. Mol. Biol. Cell 19:1509–1518.
39. Park HO, Bi E. 2007. Central roles of small GTPases in the development of cell polarity in yeast and beyond. Microbiol. Mol. Biol. Rev. 71:48–96.
40. Sinha I, Wang YM, Philp R, Li CR, Yap WH, Wang Y. 2007. Cyclin-dependent kinases control septin phosphorylation in Candida albicans hyphal development. Dev. Cell 13:421–432.
41. Wang A, Raniga PP, Lane S, Liu H. 2009. Hyphal chain formation in Candida albicans: Cdc28-Hgc1 phosphorylation of Efg1 represses cell separation genes. Mol. Cell. Biol. 29:4406–4416.
42. Wang Y. 2009. CDKs and the yeast-hyphal decision. Curr. Opin. Microbiol. 12:644–649.
43. Zheng X, Wang Y, Wang Y. 2004. Hgc1, a novel hypha-specific G1 cyclin-related protein regulates Candida albicans hyphal morphogenesis. EMBO J. 23:1845–1856.
44. Zheng XD, Lee RT, Wang YM, Lin QS, Wang Y. 2007. Phosphorylation of Rga2, a Cdc28 GAP, by CDK/Hgc1 is crucial for Candida albicans hyphal growth. EMBO J. 26:3760–3769.
45. Shen J, Cowen LE, Griffin AM, Chan L, Kohler JR. 2008. The Candida albicans pescadillo homolog is required for normal hypha-to-yeast morphogenesis and yeast proliferation. Proc. Natl. Acad. Sci. U. S. A. 105:20918–20923.
46. Reuss O, Vik A, Kolter R, Morschhauser J. 2004. The SAT1 flipper, an optimized tool for gene disruption in Candida albicans. Gene 341:119–127.
47. Guthrie C, Fink GR. 1991. Guide to yeast genetics and molecular biology. Academic Press, San Diego, CA.
48. Pierce CG, Uppuluri P, Tristan AR, Wormley FL, Jr, Mowat E, Ramage G, Lopez-Ribot JL. 2008. A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to anti-fungal susceptibility testing. Nat. Protoc. 3:1494–1500.
49. Ramage G, Vande Walle K, Wickey BI, Lopez-Ribot JL. 2001. Standardized method for in vitro antifungal susceptibility testing of Candida albicans biofilms. Antimicrob. Agents Chemother. 45:2473–2479.
50. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (ed). 1992. Current protocols in molecular biology. Greene Publishing Associates and Wiley-Interscience, New York, NY.
51. Dongari-Bagtzoglou A, Kashleva H. 2006. Development of a highly reproducible three-dimensional organotypic model of the oral mucosa. Nat. Protoc. 1:2012–2018.
52. Dongari-Bagtzoglou A, Kashleva H. 2006. Development of a novel three-dimensional in vitro model of oral Candida infection. Microbiol. Pathog. 40:271–278.
53. Villar CC, Kashleva H, Mitchell AP, Dongari-Bagtzoglou A. 2005. Invasive phenotype of Candida albicans affects the host proinflammatory response to infection. Infect. Immun. 73:4588–4595.
54. Villar CC, Kashleva H, Nobile CJ, Mitchell AP, Dongari-Bagtzoglou A. 2007. Mucosal tissue invasion by Candida albicans is associated with E-cadherin degradation, mediated by transcription factor Rim101p and protease Sap5p. Infect. Immun. 75:2126–2135.
55. Nakayama H, Mio T, Nagahashi S, Kokado M, Arisawa M, Aoki Y. 2000. Tetracycline-regulatable system to tightly control gene expression in the pathogenic fungus Candida albicans. Infect. Immun. 68:6712–6719.
56. Blankenship JR, Fanning S, Hamaker JJ, Mitchell AP. 2010. An extensive circuitry for cell wall regulation in Candida albicans. PLoS Pathog. 6:e1000752. doi:10.1371/journal.ppat.1000752.
57. Goyard S, Knechtle P, Chauvel M, Mallet A, Prevost MC, Proux C, Coppee JY, Schwartz P, Dromer F, Park H, Filler SG, Janbon G, d’Enfert C. 2008. The Yak1 kinase is involved in the initiation and maintenance of hyphal growth in Candida albicans. Mol. Biol. Cell 19:2251–2266.
58. Kelly MT, MacCallum DM, Clancy SD, Odds FC, Brown AJ, Butler G. 2004. The Candida albicans CaACE2 gene affects morphogenesis, adherence and virulence. Microbiol. 53:969–983.
59. Kumamoto CA. 2005. A contact-activated kinase signals Candida albicans invasive growth and biofilm development. Proc. Natl. Acad. Sci. U. S. A. 102:5576–5581.
60. Ene IV, Bennett RJ. 2009. Hwp1 and related adhesins contribute to both mating and biofilm formation in Candida albicans. Eukaryot. Cell 8:1913–1913.
61. Nobile CJ, Nett JE, Andes DR, Mitchell AP. 2006. Function of Candida albicans adhesion Hwp1 in biofilm formation. Eukaryot. Cell 5:1604–1610.
62. Nobile CJ, Andes DR, Nett JE, Smith FJ, Yue F, Phan QT, Edwards JE, Filler SG, Mitchell AP. 2006. Critical role of Bcr1-dependent adhesins in C. albicans biofilm formation in vitro and in vivo. PLoS Pathog. 2:e63. doi:10.1371/journal.ppat.0020063.
63. Braun BR, Kadosh D, Johnson AD. 2001. NRG1, a repressor of filamentous growth in C. albicans, is down-regulated during filament induction. EMBO J. 20:4753–4761.
64. Guisani AD, Vincen M, Kumamoto CA. 2002. Invasive filamentous growth of Candida albicans is promoted by CzGlp-dependent relief of Efg1p-mediated repression. Genetics 160:1749–1753.
65. Sohn K, Urban C, Brunner H, Rupp S. 2003. EFG1 is a major regulator of cell wall dynamics in Candida albicans as revealed by DNA microarrays. Mol. Microbiol. 47:89–102.
66. Carlisle PL, Kadosh D. 14 December 2012, posting date. A genome-wide transcriptional analysis of morphology determination in Candida albicans. Mol. Biol. Cell [Epub ahead of print.] doi:10.1091/mcb.E12-01-0065.