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

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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 glycosylphosphatidylinositol (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 complementary 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. *sun41*Δ/Δ 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 *sun41*Δ/Δ 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 *sun41*Δ/Δ 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 *efg1*Δ/Δ mutant grows as elongated yeast cells under most conditions and is highly defective for biofilm formation *in vitro* (28).

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TABLE 1 Strains used in this study

Strain[a]	Genotype	Reference
PCY87 (WT)	*ade2::hisG/ade2::hisG ura3::imm^{434}/ura3::imm^{434} ENO1/eno1::ENO1-tetR-ScHAP4AD-3×HA-ADE2 rps1::URA3/RPS1*	37
MBY38 (*tetO-UME6*)	*ade2::hisG/ade2::hisG ura3::imm^{434}/ura3::imm^{434} ENO1/eno1::ENO1-tetR-ScHAP4AD-3×HA-ADE2 tetO-UME6::URA3/UME6*	34
PCY50 (*tetO-UME6 hgc1*Δ/Δ)	*ade2::hisG/ade2::hisG ura3::imm^{434}/ura3::imm^{434} ENO1/eno1::ENO1-tetR-ScHAP4AD-3×HA-ADE2 hgc1*Δ*::frt/hgc1*Δ*::SAT1 tetO-UME6::URA3/UME6*	37
PCY21 (*tetO-UME6 efg1*Δ/Δ)	*ade2::hisG/ade2::hisG ura3::imm^{434}/ura3::imm^{434} ENO1/eno1::ENO1-tetR-ScHAP4AD-3×HA-ADE2 efg1*Δ*::frt/efg1*Δ*::SAT1 tetO-UME6::URA3/UME6*	This study
MBY179 (*tetO-UME6 sun41*Δ/Δ)	*ade2::hisG/ade2::hisG ura3::imm^{434}/ura3::imm^{434} ENO1/eno1::ENO1-tetR-ScHAP4AD-3×HA-ADE2 sun41*Δ*::frt/sun41*Δ*::SAT1 tetO-UME6::URA3/UME6*	This study
JKC915 (WT)	*HIS1/his1::FRT tetR*	45
MBY208 (*tetO-UME6*)	*HIS1/his1::FRT tetR UME6/FLP-CaNAT1 tetO-UME6*	This study

[a] WT, wild type.

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. *ume6*Δ/Δ 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*Δ/Δ 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 *tetO-UME6* (MBY38) and *tetO-UME6 hgc1*Δ/Δ (PCY50) strains were described previously (34, 37). In order to construct the *tetO-UME6 efg1*Δ/Δ strain (PCY21), primer pairs 1/2 and 3/4 (see Table S1 in the supplemental material for a list of all primers used in this study) were used to generate PCR products corresponding to the 5′ and 3′ flanking regions (just outside the open reading frame), respectively, of *EFG1*. The 5′ flank was digested with KpnI and XhoI, and the 3′ flank was digested with NotI 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*Δ*::SAT1* fragment, which was used to transform the *tetO-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 *tetO-UME6 sun41*Δ/Δ strain (MBY179), using primer pairs 5/6 and 7/8 (see Table S1 in the supplemental material) to generate 5′ and 3′ flanking regions, respectively, by PCR. A different version of the *tetO-UME6* strain (MBY208), along with a wild-type *tetR* control strain (JKC915), was used exclusively in the experiment involving the reconstituted three-dimensional model of the human oral mucosa. In order to generate MBY208, PCR fragments 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 ApaI, and the *UME6* fragment at positions −45 to +443 was digested with SacII and NcoI. These fragments were then cloned stepwise into plasmid pJK1000 (45). The resulting construct was digested with KpnI and NcoI to release an *FLP-CaNAT1-tetO-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.

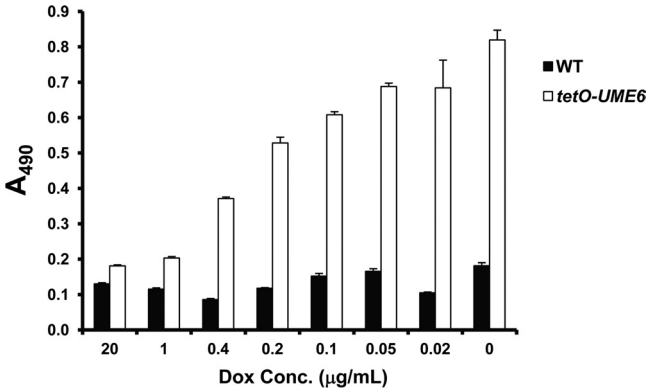

FIG 1 *UME6* expression level is correlated with increased biofilm formation. Suspensions of 1×10^6 cells/ml of the indicated strains were allowed to form biofilms on 96-well polystyrene plates in Lee's pH 6.8 medium at 30°C either in the absence of Dox or in the presence of the indicated Dox concentrations.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

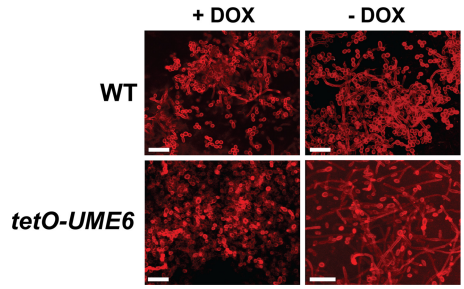

FIG 2 *UME6* expression promotes hyphal growth in *C. albicans* biofilms. CSLM was used to visualize cells in the bottommost layer of biofilms formed on 6-well polystyrene plates by the indicated strains in the presence or absence of 20 μg/ml Dox. *C. albicans* cells were stained with concanavalin A for 1 h in the dark at 37°C. Bar = 25 μm.

Media and growth conditions. Standard growth conditions for all strains included solid or liquid yeast extract-peptone-dextrose (YEPD) 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 at 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 1×10^6 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-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay, as reported previously (18, 48). Biofilms for CSLM were developed under static conditions at 37°C for 24 h in 6-well tissue culture-treated polystyrene plates.

RNA preparation and Northern analysis. RNA extractions were performed by using a hot acid phenol protocol (50). Northern analysis was carried out and blot images were visualized as described previously (33). *ACT1* and rRNA were used as loading controls. Primers used to generate probes for the Northern analysis are shown in Table S1 in the supplemental material.

Confocal scanning laser microscopy. CSLM was performed by using biofilms stained for 1 h in the dark at 37°C with 25 μg/ml concanavalin A-Alexa Fluor 594 conjugate (catalog number C-11253; Molecular Probes, Eugene, OR), as described previously (29). Biofilms were visualized by CSLM using a Zeiss LSM 510 upright confocal microscope with a Zeiss Achroplan 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 (51–54). 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 1×10^5 *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 bottommost 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

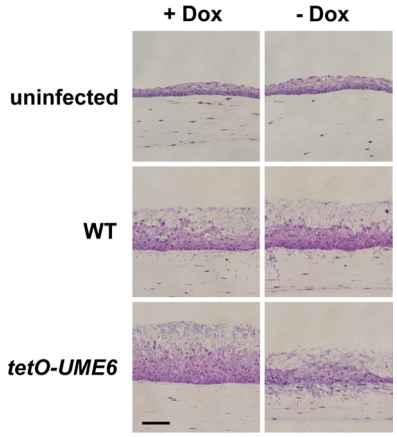

FIG 3 *UME6* expression drives *C. albicans* tissue invasion in a reconstituted three-dimensional model of oropharyngeal candidiasis. A three-dimensional organotypic model of the human oral mucosa was infected with 1×10^5 cells of the indicated *C. albicans* strains in the presence or absence of 20 μg/ml Dox. Following a 24-h infection period, cultures were fixed in formaldehyde, embedded in paraffin, stained with H&E, and visualized by light microscopy. Bar = 100 μm.

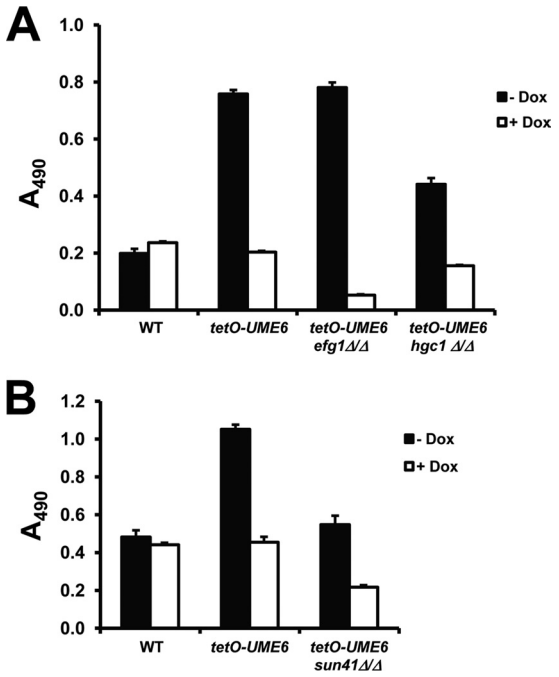

FIG 4 Hgc1 and Sun41 are important for the ability of *UME6* expression to cause enhanced biofilm formation. Suspensions of 1×10^6 cells/ml of the indicated strains were allowed to form biofilms for 24 h on 96-well polystyrene plates in Lee's medium, pH 6.8, (A) or in minimal medium (B) at 30°C in the presence or absence of 20 μg/ml Dox. Biofilm formation was assessed by using a standard colorimetric XTT reduction assay (18, 48). Error bars represent standard deviations ($n = 8$).

indicate a direct correlation between *UME6* expression levels and *C. albicans* biofilm formation and suggest that increasing the number of hyphal cells in a biofilm can lead to significantly enhanced overall biofilm growth.

***UME6* expression promotes tissue invasion in a reconstituted human model of oropharyngeal candidiasis.** In order to determine the effect of *C. albicans UME6* expression on biofilm formation and tissue invasion of a host mucosal surface, we used a 3-dimensional model of the human oral mucosa (51–54). A total of 1×10^5 cells of both the *tetO-UME6* and wild-type control strains were 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-hgc1*Δ/Δ mutant (37), biofilm formation ability was assessed in the presence and absence of Dox. The *tetO-UME6 efg1*Δ/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 efg1*Δ/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 *efg1*Δ/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 hgc1*Δ/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 sun41*Δ/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 *sun41*Δ/Δ strain (26, 27), the *tetO-UME6 sun41*Δ/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.

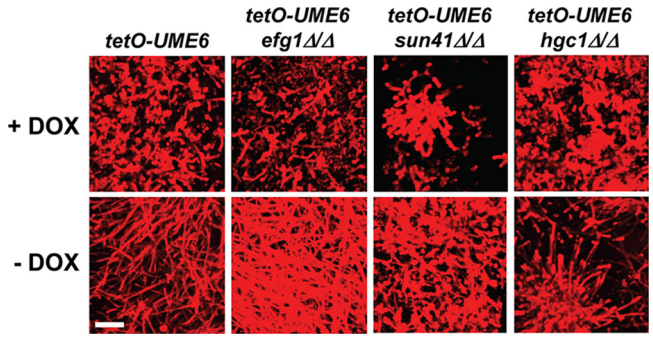

FIG 5 Hgc1 and Sun41 are important for *UME6*-driven hyphal growth in *C. albicans* biofilms. CSLM was used to visualize cells in the top layer of biofilms formed on 6-well polystyrene plates by the indicated strains in the presence or absence of 20 μg/ml Dox. *C. albicans* cells were stained with concanavalin A for 1 h in the dark at 37°C. Bar = 25 μm.

Hgc1 and Sun41 are important for *UME6*-driven hyphal growth in biofilms. In order to specifically examine the effect of *efg1*Δ/Δ, *sun41*Δ/Δ, and *hgc1*Δ/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 efg1*Δ/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 sun41*Δ/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 *sun41*Δ/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 hgc1*Δ/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 *efg1*Δ/Δ and *sun41*Δ/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 *efg1*Δ/Δ filamentation defect in either solid or liquid medium. Consistent with our biofilm results (Fig. 5), the *tetO-UME6 sun41*Δ/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 sun41*Δ/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 *sun41*Δ/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.

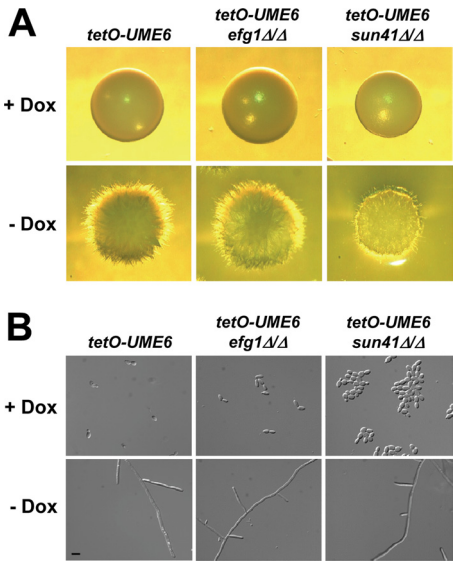

FIG 6 Sun41 is important for *UME6*-driven filamentous growth under solid, non-filament-inducing conditions. (A) Colonies of the indicated strains were grown on solid YEPD medium at 30°C for 2 days in the presence or absence of 20 μg/ml Dox and visualized by light microscopy. (B) The indicated strains were grown in liquid YEPD medium at 30°C overnight to an OD_{600} of ~1.0 in the presence or absence of 20 μg/ml Dox and visualized by using DIC microscopy. Bar = 10 μm.

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 *hgc1*Δ/Δ mutation does not affect this process [37]). *tetO-UME6 efg1*Δ/Δ and *tetO-UME6 sun41*Δ/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*, *ECE1*, and *ALS3*) showed significantly reduced expression levels in the *tetO-UME6 efg1*Δ/Δ mutant but significantly increased expression levels in the *tetO-UME6 sun41*Δ/Δ mutant. Levels of the *RBT1* transcript showed a minor increase in the *tetO-UME6 efg1*Δ/Δ strain and a small decrease in the *tetO-UME6 sun41*Δ/Δ mutant. In contrast, induction of *PHR1* and *HGC1* by Ume6 did not appear to be significantly affected in the *tetO-UME6 efg1*Δ/Δ and *tetO-UME6 sun41*Δ/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-

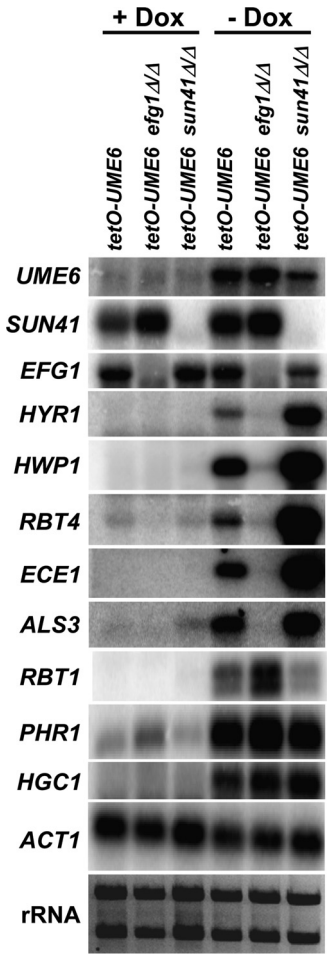

FIG 7 Efg1 and Sun41 differentially affect the ability of *UME6* to induce certain filament-specific transcripts. The indicated strains were grown as described in the legend of Fig. 6B. Cells were harvested, and total RNA was prepared for Northern analysis. Blots were probed for the indicated transcripts. Each lane was loaded with 3 μg of total RNA. *ACT1* and rRNA are shown as loading controls.

mote enhanced formation of biofilms. Here, we identify one such mechanism, which is mediated by the transcriptional regulator Ume6. As *UME6* levels rise and *C. albicans* transitions from yeast to a nearly complete hyphal population, we demonstrate that there is a corresponding increase in biofilm formation above levels typically observed for a wild-type strain. This result suggests a direct correlation between increased hyphal growth (and/or increased expression levels of genes associated with hyphal growth) and enhanced *C. albicans* biofilm formation. Our findings are consistent with previous observations that while the initiation stage of *C. albicans* biofilms involves attachment of yeast cells to a surface, subsequent biofilm growth and development involve the generation of pseudohyphal and hyphal filaments as well as the formation of a dense exopolymeric matrix (14–18). Interestingly, while most wild-type *C. albicans* biofilms maintain a bottom layer of yeast cells, our *tetO-UME6* biofilm appears to be nearly completely hyphal, even in the bottom layer, when *UME6* is expressed at high levels. Because the *tetO-UME6* strain is typically grown in the yeast form (in the presence of Dox) prior to the start of biofilm assays, our results suggest that following the initiation step, the yeast form appears to be dispensable for enhanced *C. albicans* biofilm formation. It is important to note, however, that the ability of *C. albicans* cells within a biofilm to transition from filaments to yeast appears to be critical for biofilm dispersion, since we have previously demonstrated that constitutive high-level *UME6* expression significantly inhibits this process (36).

In the clinical setting, biofilm formation can occur on both abiotic surfaces (e.g., catheters, denture materials, and implanted medical devices) as well as mucosal surfaces (11–14). While *UME6* expression and increased hypha formation appear to clearly enhance biofilm formation on solid abiotic surfaces (equivalent to catheters and implanted medical devices), we have also shown that in the context of a biotic surface, the reconstituted three-dimensional model of the human oral mucosa, a *C. albicans* strain expressing constitutive high levels of *UME6* shows significantly increased tissue invasion. These findings are consistent with our previous observation that *UME6* expression promotes hypha formation, tissue invasion, and virulence in a mouse model of systemic candidiasis (34) and also suggest that shifting the morphology of cells in a *C. albicans* biofilm to hyphae is an important step in the pathogenesis of mucosal candidal infections.

Hypha-dependent and cell wall-dependent mechanisms important for *UME6*-driven enhanced biofilm formation. There are a number of mechanisms that may account for our observation that *UME6*-driven hyphal growth promotes *C. albicans* biofilm formation. *UME6* encodes a key transcriptional regulator of *C. albicans* hyphal development and is known to induce a variety of filament-specific transcripts (33, 35). Several of these target transcripts encode key adhesins, such as the Als3 agglutinin-like protein and Hwp1, a mammalian transglutaminase substrate mimic, which are known to play critical roles in biofilm formation, most likely by functioning as mediators of cell-cell adherence (16, 22–24, 60, 61). Overexpression of either *ALS3* or *HWP1* was previously 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 (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 *sun41*Δ/Δ 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).

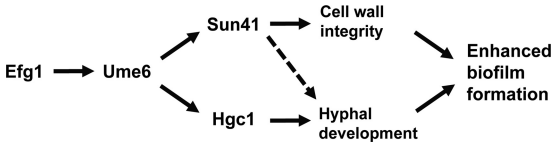

FIG 8 Model for roles of Efg1, Hgc1, and Sun41 in *UME6*-driven enhanced *C. albicans* biofilm formation. Ume6 functions downstream of Efg1 and upstream of both Hgc1 and Sun41 to promote biofilm development. *UME6* expression is known to cause transcriptional induction of the Hgc1 cyclin-related protein, 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.

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 sun41*Δ/Δ mutant, like the *sun41*Δ/Δ 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 sun41*Δ/Δ 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 sun41*Δ/Δ 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 *sun41*Δ/Δ 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 *efg1*Δ/Δ 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 *efg1*Δ/Δ 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 efg1*Δ/Δ 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

least partially explain the differential effects of *efg1*Δ/Δ and *sun41*Δ/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.

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