Roles of Candida albicans Sfl1 in Hyphal Development

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The ability to switch between different morphological forms is an important feature of Candida albicans and is relevant to its pathogenesis. Many conserved positive and negative transcription factors are involved in morphogenetic regulation of the two dimorphic fungi Candida albicans and Saccharomyces cerevisiae. In S. cerevisiae, the transcriptional repressor Sfl1 and the activator Flo8 function antagonistically in invasive and filamentous growth. We have previously reported that Candida albicans Flo8 is a transcription factor essential for hyphal development and virulence in C. albicans. To determine whether a similar negative factor exists in C. albicans, we identified Candida albicans SFL1 as a functional homolog of the S. cerevisiae sfl1 mutant. SFL1 is a negative regulator of hyphal development in C. albicans. Deletion of C. albicans SFL1 enhanced filamentous growth and hypha-specific gene expression in several media and at several growth temperatures. Overexpression of the SFL1 led to a significant reduction of filament formation. Both deletion and overexpression of the SFL1 attenuated virulence of C. albicans in a mouse model. Deleting FLO8 in an sfl1/sfl1 mutant completely blocked hyphal development in various growth conditions examined, suggesting that C. albicans SFL1 may act as a negative regulator of filamentous growth by antagonizing Flo8 functions. We suggest that, similar to the case for S. cerevisiae, a combination of dual control by activation and repression of Flo8 and Sfl1 may contribute to the fine regulatory network in C. albicans morphogenesis responding to different environmental cues.

Candida albicans is a serious opportunistic fungal pathogen of humans. It can cause various forms of candidiasis ranging from superficial mucosal infections to life-threatening systemic diseases, especially in immunocompromised patients (34). One of the important properties of C. albicans that is known to contribute to its pathogenicity and virulence is its ability to reverse the morphological transition from the yeast form to the pseudohyphal or hyphal form. Accordingly, some mutants blocked in yeast or pseudohyphal forms under all laboratory conditions examined are avirulent in mouse models (4, 29, 31).

Morphogenesis in C. albicans is subject to both positive and negative controls. Multiple positive signaling pathways have been characterized, including the Cph1-mediated mitogen-activated protein kinase cascade (27), the Efg1- and Flo8-mediated cyclic AMP (cAMP)-dependent protein kinase A (PKA) signaling pathway (8, 42), and the Rim101-mediated pH response signaling pathway (11). The negative control is mediated mainly by Tup1 through Rfg1 and Nrg1. A lack of any one of these regulators leads to constitutive filamentous growth and derepression of hypha-specific genes under non-filament-inducing conditions (5, 6, 24, 33). In Saccharomyces cerevisiae, the Tup1-Ssn6 transcriptional repression complex is recruited to regulatory promoters via pathway-specific DNA-binding proteins in response to various environmental signals (23), including Nrg1, Rnx1, Sfl1, Mig1, and alpha2 (10, 13, 36, 43, 46). In C. albicans, the homologs of Nrg1, Rnx1 (Rfg1), and Mig1, but not Sfl1, have been identified and characterized. SFL1 of S. cerevisiae was originally identified in a genetic screen for suppressors of flocculation. Its N-terminal sequence has a high degree of similarity with the DNA-binding domain (heat shock factor [HSF] domain) of heat shock transcription factors (17). Dependent upon the HSF domain, Sfl1 can bind specifically to heat shock elements with an inverted DNA repeat 5’AGAA-n-TTCT3’ (10). Genes with heat shock elements, such as FLO11, STA1, and SUC2, are repressed by Sfl1 and derepressed in sfl1 mutants (25, 37, 41). Sfl1 can form a multimer via its coiled-coil domain, and this multimerization is believed to be important for its DNA binding activity. Tpk2, a catalytic subunit of the cAMP-dependent PKA, inactivates Sfl1 by phosphorylation and releases Sfl1 from DNA (10, 35). Sfl1 can interact with the TPR motifs of Ssn6 and represses transcription by recruiting an Ssn6-Tup1 complex. Sin4 and Srb10, components of a specific RNA polymerase II subcomplex that are required for Ssn6-Tup1 repression, are also required for Sfl1 repression (10).

Flo8 is a transcription factor that is critical for invasive growth and flocculation in haploids and pseudohyphal growth in diploids of S. cerevisiae (28). It functions downstream of the cAMP-dependent PKA pathway (38). Interestingly, Flo8 has been shown to bind to the same region of the FLO11 promoter as Sfl1, and phosphorylation of Flo8 by Tpk2 is required for its interaction with the FLO11 promoter both in vivo and in vitro (35). Candida albicans has a Flo8 homolog. (We use the prefixes Sc and Ca in this report to distinguish S. cerevisiae and C. albicans genes and proteins, respectively, where there may be confusion.) The Candida albicans Flo8 (CaFlo8) is essential for hyphal development and hypha-specific gene expression and is also important for Candida pathogenicity, since a flo8/flo8 mu-

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Sfl1 and Flo8 act antagonistically in the regulation of hyphal development. Our genetic analysis of S. cerevisiae wanted to determine whether a similar regulator exists in C. albicans regulating the invasive/filamentous growth in S. cerevisiae pathway in ScFlo8, CaFlo8 may function downstream of the cAMP/PKA taut is avirulent in a mouse model of systemic infection. Like ScFlo8, CaFlo8 may function downstream of the cAMP/PKA pathway in C. albicans SFL1. We cloned a C. albicans SFL1 homolog by sequence comparison and functional complementation of an S. cerevisiae SFL1 promoter region in C. albicans with primers 5′-CTGGGAATCCGATACATGAGTCTTATTTGTACAATTCATC-3′ and 5′-CCCTGGATTCTTATTTGTACAATTCATC-3′. The first copy of SFL1 was disrupted by the transformation of C. albicans HIS1 into BWP17. C. albicans ARG4 was used to replace the second copy of SFL1. The homozygous sfl1::sfl1 mutants were identified by PCR and Southern blot analysis.

### Materials and Methods

**Strains and culture conditions.** The Candida albicans and Saccharomyces cerevisiae strains used in this study are listed in Table 1. Yeast strains were routinely grown on YPD (1% yeast extract, 2% peptone, 2% glucose) medium or on synthetic complete medium with 2% glucose (SCD) for selection of prototrophic strains. YPS with 1% agar was used for colony morphology assay under embedded conditions (7). Media were used for yeast and hyphal growth as described previously (9, 16, 19, 27). YP refers to YPD without glucose. SCGL refers to SC supplemented with 0.1% glucose. SLAD refers to synthetic low-ammonia medium. Spider medium contained 1% nutrient broth and 0.4% potassium phosphate (pH 7.2) (27). Lee's medium or media containing 10% serum (GIBCO) were used for hyphal induction.

**C. albicans SFL1 disruption.** CaSFL1 was deleted based on PCR recombination by the method of Wilson et al. (48). C. albicans BWP17 was used as the parent strain for SFL1 deletion. Primers 5′-ATGAGTCAAATTCTTGACTGTTCTCATTGCTACGACAACTGCTACTCCTCAACTCAAAGAGTTTTCCCAAGTCACGAGCCTTTCTGAGGTTGCAAACTCACCAGACTCGTCCCTTATAGTGTGGTTGCTCAACTACAGTGGATGGTGAATGTGAGCGGATA and 3′-TGGTGGCAAACTCACCAGACTCGTCCCTTATAGTGTGGTTGCTCAACTACAGTGGATGGTGAATGTGAGCGGATA) were used to amplify C. albicans HIS1, URA3, and ARG4 from plasmids pGEM-HIS1, pGEM-URA3, and pRS-ARG4, SpeI, respectively. The first copy of SFL1 was disrupted by the transformation of C. albicans HIS1 into BWP17. C. albicans ARG4 was used to replace the second copy of SFL1. The homozygous sfl1::sfl1 mutants were identified by PCR and Southern blot analysis.

### Table 1. Yeast strains and plasmids used in this study

| Strain or plasmid | Genotype or description | Reference |
|------------------|------------------------|-----------|
| **Strains** | | |
| C. albicans | | |
| SC5314 | Wild type | 16 |
| CAF2-1 | URA3::ura3::Imm434 | 16 |
| CAH4 | ura3::Imm434::ura3::Imm434 | 16 |
| BWP17 | ura3::Imm434::ura3::Imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG SFL1::sfl1::HIS1 | 48 |
| CAL1 | ura3::Imm434::ura3::Imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG SFL1::sfl1::HIS1 | This study |
| CAL2 | ura3::Imm434::ura3::Imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG sfl1::ARG4::sfl1::HIS1 | This study |
| CAL3 | ura3::Imm434::ura3::Imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG sfl1::ARG4::sfl1::HIS1 flo8::hisG/fo8::hisG-Ura3::hisG | This study |
| CAL4 | ura3::Imm434::ura3::Imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG sfl1::ARG4::sfl1::HIS1 flo8::hisG/fo8::hisG | This study |
| CCF3 | ura3::Imm434::ura3::Imm434 flo8::hisG/fo8::hisG-Ura3::hisG | 8 |
| CCF4 | ura3::Imm434::ura3::Imm434 flo8::hisG/fo8::hisG-Ura3::hisG | 8 |
| **S. cerevisiae** | | |
| CG680 | MATα/α, wild type | 19 |
| HLY334 | MATα/a-3-52 | 27 |
| XPY108α | MATα sfl1::HpyB ura3-52 | 35 |
| XPY108α/α | MATα/α sfl1::HpyB/sfl1::HpyB ura3-52 | 35 |
| **Plasmids** | | |
| pVTU102 | S. cerevisiae URA3 2 μm vector | 45 |
| pVTU-CaSFL1 | 2.42-kb full-length CaSFL1 in pVTU | This study |
| pBES116 | C. albicans URA3 vector, integration at ADE2 | 15 |
| pBES116-SFL1 | 3.7-kb CaSFL1 fragment including 1.3-kb CaSFL1 promoter region in pBES116 | This study |
| pBES116 | | |
| pBA1-GFP | 0.7-kb GFP ORF in pBA1 | This study |
| pBAS1-GFP | 3.1-kb CaSFL1-GFP fusion fragment in pBA1 | This study |
| pBA1-SFL1 | 2.42-kb full-length CaSFL1 in pBA1 | This study |
| pBA1-FLO8 | 1.7-kb full-length CaFLO8 in pBA1 | 8 |

TCTTCACTTGAGTGTCGCAAACAGCGCTGCTACTCCTCAACTAGAGGTTTTCCCAAGTACGCAGCTTTCTGAGGTTGCAAACTCACCAGACTCGTCCCTTATAGTGTGGTTGCTCAACTACAGTGGATGGTGAATGTGAGCGGATA and 3′-TGGTGGCAAACTCACCAGACTCGTCCCTTATAGTGTGGTTGCTCAACTACAGTGGATGGTGAATGTGAGCGGATA were used to amplify C. albicans HIS1, URA3, and ARG4 from plasmids pGEM-HIS1, pGEM-URA3, and pRS-ARG4, SpeI, respectively. The first copy of SFL1 was disrupted by the transformation of C. albicans HIS1 into BWP17. C. albicans ARG4 was used to replace the second copy of SFL1. The homozygous sfl1::sfl1 mutants were identified by PCR and Southern blot analysis.
CATAC, and then an SFL1-GFP fragment amplified by overlap PCR was digested with BamHI and ligated with BglII-digested pBA1, generating pBA1-SFL1-GFP. The pBA1-GFP or pBA1-SFL1-GFP was digested with Ascl and introduced into CAI4 for GFP or SFL1-GFP expression.

**Northern blotting.** RNA was prepared and subjected to Northern analysis with the probes indicated. PCR products were used for probing C. albicans SFL1, ECE1, HWP1, and ACT1. The primers used were 5′ CAATCGTGCCGCTGGGAGTTC and 5′ TATATCTATTTCTTCTTTATG for SFL1, 5′ GGCATCC ACCATGCTCC and 5′ GTGCTACTGAGCGCGGCACTTC for ECE1, 5′ TGCT CCAGGTACTGAATCCGC and 5′ GGCAGATGGTGTGAGTTGCG for HWP1, and 5′ CGGTTATTGATATTTTAGTCG and 5′ ACCATGCCCCAGATCAATC for ACT1. The sizes of mRNAs on Northern blots correlated with the expected lengths based on information from the *Candida Genome Database.*

**Virulence assay.** The newly plated *C. albicans* strains were grown in liquid YPD at 30°C overnight, suspended in physiological saline solution, counted in a hemacytometer, and adjusted to a concentration of 5 × 10⁶ cells/ml. ICR male mice from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences, were used for the virulence assay. Eight ICR male mice weighing from 18 to 21 g for each strain were injected in the lateral tail veins with 0.1 ml cells. The survival of mice was observed and recorded continuously for at least 25 days after injection (9).

**RESULTS**

**Identification of *Candida albicans* SFL1.** A search of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) indicates that a *Candida albicans* protein named Sfl1 (orf19.454) shares the highest similarity with *Saccharomyces cerevisiae* Sfl1 (25% identical). The GenBank accession number for the *C. albicans* SFL1 (CaSFL1) nucleotide sequence is XM_710795 (22). Like *S. cerevisiae* Sfl1 (ScSfl1), *C. albicans* Sfl1 (CaSfl1) contains an HSF-like domain that could potentially bind to inverted repeats of nGAAn, called heat shock elements, which are present in the target promoters, a hydrophobic coiled-coil region proposed to be essential for the regulation of homotrimer formation. In addition, CaSfl1 possesses four glutamine-rich regions that are absent in ScSfl1 (Fig. 1A), which are believed to be involved in protein-protein interactions (39).

To determine whether CaSfl1 is a functional homolog of *S. cerevisiae* Sfl1, we examined the ability of CaSfl1 to suppress the flocculation and hyperfilamentous growth of *sfl1* mutants. An expression plasmid, pVTU-CaSFL1, containing the entire ORF of CaSFL1 under control of the ADH1 promoter, was introduced into an *C. albicans* Sfl1 (CaSfl1) mutant. Ectopically expressed CaSfl1 suppressed the flocculent phenotype of a haploid *sfl1* mutant. *S. cerevisiae* strains HLY334 (wild type [WT]) and XPY108α (sfl1) carrying pVTU102 or pVTU-CaSFL1 were grown in YPD to saturation, allowed to settle for 15 min, and then photographed. (C) Ectopically expressed CaSFL1 suppresses the pseudohyphal development phenotype of a diploid *sfl1* mutant. *S. cerevisiae* strains CG08 (wild type) and XPY108α (sfl1) carrying pVTU102 or pVTU-CaSFL1 were grown on SLAD plates at 30°C for 2 days.

**Deletion of SFL1 promotes filament formation in *C. albicans.* To elucidate the role of *SFL1* in the hyphal development of *C. albicans*, we deleted two copies of *SFL1* by PCR-based homologous recombination, as described previously (48) (Table 1). Deletion of both alleles of *SFL1* enhanced filamentous growth in nutrient-poor media. On solid YPD medium, the *C. albicans* *sfl1/sfl1* mutant generated smooth colonies and was indistinguishable from the wild-type strain CAF2-1, even after 7 days of incubation at 30°C. When incubated on YPD plates for 6 days at 30°C, the *sfl1/sfl1* mutant produced rough wrinkled colonies, in contrast to the wild-type, heterozygous, and revertant strains, which all formed smooth colonies (Fig. 2A, upper panels). Increased germination of the *sfl1/sfl1* mutant was also observed in liquid media. After growth in SCD medium at 30°C for 6 h, the *sfl1/sfl1* mutant was a mixture of yeast and filaments, with about 30% of the cells developing into hyphae (Fig. 2A, lower panel). The increase in filamentous growth was caused by the *SFL1* deletion, as the phenotype was reversed by reintroducing wild-type *SFL1* back into its own locus under control of the *SFL1* endogenous promoter. In *C. albicans*, the expression of hypha-specific genes, such as *ECE1* and *HWP1*, correlates with hyphal morphogenesis. Consistent with the phenotype, the transcription levels of *ECE1* and *HWP1* increased in the *sfl1/sfl1* mutant in liquid SCD medium at 30°C but were undetectable in wild-type, heterozygous, or revertant strains, indicating that deletion of *SFL1* enhanced the expression of hypha-specific genes under a non-hypha-inducing condition (Fig. 2B). Interestingly, the *sfl1/sfl1* mutant did not form hyphae in YPD liquid medium at 30°C, but removal of glucose from the YPD medium promoted filament formation (Table 2). The *sfl1/sfl1* mutant also enhanced filamentous growth in other liquid media, including the glucose-depleted medium SCLD, the nitrogen-limited medium SLAD, and the nutrient-deprived medium Spider, suggesting that Sfl1 acts as a repressor of hyphal development in *C. albicans.*

**Overexpression of SFL1 inhibits hyphal development.** To further confirm the repressive effect of Sfl1 on the hyphal development of *C. albicans*, we overexpressed *SFL1* under the control of the *ADH1* promoter. On solid serum-containing media, the *SFL1* overexpression strain produced mostly stunted hyphal cells in the initial hours but formed round and fuzzy colonies after 3 days of incubation at 37°C, whereas the wild-type strain produced long hyphae and generated florid filamentous colonies (Fig. 3). More obviously, on solid Lee’s medium, wild-type strains formed wrinkled colonies surrounded by long invasive filaments, while the *SFL1* overexpres-
Sfl1 is localized in the nucleus in yeast and hyphal cells. To verify its presumptive function as a transcriptional regulator, we examined the subcellular localization of Sfl1 in C. albicans. A strain expressing the Sfl1-GFP fusion protein was constructed under the control of ADH1 promoter as described in Materials and Methods. By direct fluorescence observation, we found Sfl1-GFP constitutively localized in the nucleus both in yeast and hyphal form (Fig. 5). Additionally, we observed that the localization of the Sfl1-GFP was not altered in a tpk2 null mutant of C. albicans or in response to exogenous cAMP (data not shown), indicating that the cAMP-dependent PKA pathway had no effect on Sfl1 localization, at least when the protein is overexpressed, which is consistent with the localization pattern of ScSfl1 (35). The nuclear localization of overexpressed Sfl1-GFP is consistent with a predicted function of CaSfl1 in transcriptional regulation.

The lack of Sfl1 reduces the threshold of hyphal induction. In the laboratory, environmental conditions influence the morphological state of C. albicans. Serum is a strong extracellular stimulus for germination. High temperature (37°C), a high ratio of CO2 to O2, neutral pH, and nutrient-poor media also stimulate hyphal growth. Conversely, low temperature, air,
acidic pH, and rich media promote blastospore growth. To determine whether CaSfl1 is regulated in response to a hyphal induction signal, we compared levels of hyphal growth of the sfl1\!/sfl1 mutant and a wild-type control under various growth conditions. At high temperature (37°C), the sfl1\!/sfl1 mutant developed into true hyphae in all liquid media examined, whereas the wild-type strain grew in yeast form in medium at low pH (Lee’s, pH 4) and required serum for true hyphal growth.

### TABLE 2. Filamentous growth in liquid media

| Temp (°C) | Medium | Growth |
|-----------|--------|--------|
|           |        | Wild type | sfl1/sfl1 mutant | sfl1/sfl1 flo8/flo8 mutant | sfl1/sfl1+FLO8 mutant |
| 37        | Lee’s, pH 4 | – | ++ | – | ++++ |
|           | Lee’s, pH 8 | ++ | ++++ | – | ++++ |
|           | YPD     | +  | ++++ | – | ++++ |
|           | YPD + serum | ++ | ++++ | – | ++++ |
|           | SCLD    | +++| ++++ | – | ++++ |
|           | SLAD    | +++| ++++ | – | ++++ |
| 30        | YPD     | –  | –   | –   | +++ (30) |
|           | YP      | –  | ++ (20) | –  | ++++ (100) |
|           | Spider  | –  | ++++ (100) | – | ++++ (100) |
|           | SCD     | –  | ++++ (30) | – | ++++ (80) |
|           | SCLD    | –  | ++++ (70) | – | ++++ (90) |
|           | SLAD    | –  | ++++ (60) | – | ++++ (90) |
|           | Lee’s, pH 4 | – | ++ (10) | – | +++ (30) |
|           | Lee’s, pH 8 | – | ++++ (60) | – | ++++ (80) |
|           | YPD + serum | – | ++++ (100) | – | ++++ (100) |
|           | SCD + serum | – | ++++ (100) | – | ++++ (100) |
|           | SLAD + serum | – | ++++ (100) | – | ++++ (100) |
| 25        | YPD     | –  | –   | –   | –   |
|           | YP      | –  | –   | –   | –   |
|           | SCD     | –  | –   | –   | –   |
|           | SCLD    | –  | + (2) | – | + (10) |
|           | SLAD    | –  | + (5) | – | + (30) |
|           | Spider  | –  | + (5) | – | + (20) |
|           | Lee’s   | –  | –   | –   | –   |
|           | YPD + serum | – | –   | –   | –   |
|           | YP + serum | – | –   | –   | –   |
|           | SCD + serum | – | –   | –   | –   |
|           | SCLD + serum | – | –   | –   | –   |
|           | SLAD + serum | – | ++ (50) | – | ++++ (80) |
|           | Spider + serum | – | ++ (30) | – | ++++ (80) |
|           | Lee’s + serum | – | ++ (30) | – | ++++ (70) |

a Wild-type (CAI4+pBES116), sfl1/sfl1 (CAL2+pBES116), sfl1/sfl1 flo8/flo8 (CAL4+pBES116), and sfl1/sfl1+FLO8 (CAL2+pBA1-FLO8) strains were grown in liquid media. The media used for yeast and hyphal growth were as described in Materials and Methods.

b –, yeast growth; +, filamentous growth. The level of cell filamentous growth is indicated by the number of + symbols. The percentage of hyphal cells in a given cell population counted is in parentheses.

![FIG. 3. Sfl1 functions as a repressor in hyphal development. The colony morphologies of the wild-type (WT) strain with vector (CAI4+pBES116) or overexpressed SFL1 (CAI4+pBA1-SFL1) and the sfl1/sfl1 mutant with vector (CAL2+pBES116) or overexpressed SFL1 (CAL2+pBA1-SFL1), plated on solid serum-containing medium and Lee’s medium at 37°C for 3 and 5 days, respectively, are shown.](image-url)
induction in liquid YPD (Table 2). In the other liquid media, combinations of high temperature with neutral pH or nutrient limitation were sufficient for hyphal induction. The absence of Sfl1 reduced the threshold of pH in hyphal induction, and the mutant was no longer dependent on serum for hyphal growth at 37°C.

At an intermediate temperature (30°C), the wild-type strain SC5314 or CAF2-1 could not induce true hyphae in all liquid media examined. In contrast, the sfl1/sfl1 mutant could form true hyphae in all media except YPD. The ratio of filament formation varied from 10% in low pH medium (Lee’s, pH 4) to 100% in nutrient-limited medium (Spider without a carbon source) (Table 2). Addition of serum was not sufficient to release the inhibition of hyphal induction in wild-type cells but was sufficient to promote the sfl1/sfl1 mutant cells to develop true hyphae. The absence of Sfl1 reduced the threshold of sensing nutrient starvation and serum at 30°C.

At a lower temperature (25°C), wild-type strains favored growth in the yeast form, but about 2 to 5% of the sfl1/sfl1 mutant cells could develop into short hyphae in nutrient-poor media, including SCLD, SLAD, and Spider (Table 2). The hyphal induction was inhibited in Spider supplemented with 2% glucose or 2% mannitol. Addition of 10 to 30% serum to the nutrient-poor media increased the hyphal induction by 6- to 25-fold. The combinative effect was only observed in SCLD, SLAD, and Spider and not in other liquid media, indicating that serum is an additional input that activates hyphal development at low temperature. The lack of Sfl1 reduces the temperature threshold for nutrients and serum sensing. Our results showed that serum, high temperature, neutral pH, and nutrient starvation each have an additive effect on hyphal induction in the sfl1/sfl1 mutant. Sfl1 functions as a new negative regulator of hyphal development.

**Flo8 is required for germination and hyphal development in sfl1/sfl1 mutants.** In *S. cerevisiae*, the Sfl1 repressor and Flo8 activator play antagonistic roles in controlling the expression of FLO11 via a common promoter element (35). To address the relationship between Sfl1 and Flo8 in the regulation of *C. albicans* hyphal development, we deleted CaFLO8 in a *C. albicans* sfl1/sfl1 mutant. In the constructed sfl1/sfl1 flo8/flo8 double mutant the repressive effect of Sfl1 on hyphal induction was abolished, and the mutant failed to form filaments in all liquid media examined (Table 2), resulting in a phenotype similar to that of the flo8/flo8 mutant (Fig. 6). Thus, flo8/flo8 loss of function blocks the hyperfilamentous phenotype of sfl1/sfl1 loss of function. On the other hand, overexpression of FLO8 in *C. albicans* wild-type strain CAI4 did not activate germination (data not shown), but overexpression of FLO8 in the sfl1/sfl1 mutant enhanced filamentous growth in all liquid media and even in YPD medium at 30°C (Table 2). These data suggest that Sfl1 may inhibit hyphal development by antagonizing Flo8 functions.

**Both Sfl1 and Flo8 have dual effects on hyphal growth under embedded conditions at low temperature.** In embedded conditions such as in YPS, *C. albicans* can be induced to filament
because of contact with agar (7). At 37°C, wild-type colonies generated long heterogeneous filaments in 2 days, and the sfl1/sfl1 mutant generated more and longer filaments than the wild type, whereas the flo8/flo8 mutant produced smooth colonies surrounded with very short filaments in which most cells were yeast form and a few cells were elongated. The sfl1/sfl1 double mutant showed a phenotype similar to that of flo8/flo8 mutants. At 37°C, wild-type colonies were yeast form and a few cells were elongated. The sfl1/sfl1 double mutant generated more and longer filaments than the flo8/flo8 double mutant. Overexpression of FLO8 increased the hyphal formation in all four strains examined (Fig. 7A, fourth row). The difference is that the colonies formed homogeneous filaments in the presence of Sfl1 but formed heterogeneous filaments in the absence of the Sfl1. These data show that Flo8 functions as an activator and Sfl1 functions as a repressor of filamentous growth at 37°C under embedded conditions. This is similar to their roles in hyphal development exhibited in aerobic conditions.

At 24°C, colonies of the wild-type strain generated some filaments after 3 days of incubation, and the colonies contained both yeast and hyphal cells. The sfl1/sfl1 mutant formed colonies with longer heterogeneous filaments (Fig. 7B, upper two rows). The flo8/flo8 mutant produced fluffy colonies with more filaments that were mostly hyphal cells (8). Surprisingly, the sfl1/sfl1 flo8/flo8 double mutant formed smooth colonies, in which almost all cells were yeast and a few cells were elongated (Fig. 7B, upper two rows). Overexpression of SFL1 in the wild-type or the sfl1/sfl1 mutant inhibited hyphal formation, but it had no significant effects on flo8/flo8 or sfl1/sfl1 flo8/flo8 mutants (Fig. 7A, third row). Overexpression of FLO8 increased the hyphal formation in all four strains examined (Fig. 7A, fourth row). The difference is that the colonies formed homogeneous filaments in the presence of Sfl1 but formed heterogeneous filaments in the absence of the Sfl1. These data show that Flo8 functions as an activator and Sfl1 functions as a repressor of filamentous growth at 37°C under embedded conditions. This is similar to their roles in hyphal development exhibited in aerobic conditions.

As a repressor of hyphal development, Sfl1 is required for germination in sfl1 mutants. Cell and colony morphologies of wild-type (WT) (CAI4+pBES116), sfl1/sfl1 (CAL2+pBES116), flo8/flo8 (CCF4+pBES116), and sfl1/sfl1 flo8/flo8 (CAL4+pBES116) strains are shown. Cells were grown in liquid medium (YPD plus 10% serum) at 37°C for 3.5 h or plated on solid serum-containing medium and incubated at 37°C for 5 days.

**FIG. 6.** Flo8 is required for germination in sfl1 mutants. Cell and colony morphologies of wild-type (WT) (CAI4+pBES116), sfl1/sfl1 (CAL2+pBES116), flo8/flo8 (CCF4+pBES116), and sfl1/sfl1 flo8/flo8 (CAL4+pBES116) strains are shown. Cells were grown in liquid medium (YPD plus 10% serum) at 37°C for 3.5 h or plated on solid serum-containing medium and incubated at 37°C for 5 days.

**DISCUSSION**

**Sfl1 functions as a repressor of hyphal development.** In *C. albicans*, the yeast-to-hypha transition is triggered by various environmental cues, such as serum, neutral pH, high temperature, starvation, CO₂, and matrix (3, 47). Many transcriptional regulatory factors that may mediate environmental responses have been identified and characterized. For example, Rim101 is required for pH-regulated morphogenesis (12), Czf1 functions as a positive regulator in a matrix-sensing pathway (7), GCN4 is involved in nitrogen-regulated morphogenesis (44), and Stp1 and Stp2 play important roles in amino acid sensing (32). On the other hand, a large number of positive and negative transcriptional regulators, including Efg1, Flo8, Cph2, Tec1, Tup1, Ssn6, Rgt1, and Nrg1, are indicated to play critical roles in the general control of morphogenesis. These factors may integrate signals from different signaling pathways and generate a final transcriptional response for hyphal development (3, 14, 26, 47).

In this study, we identified a *C. albicans* homolog of *S. cerevisiae* Sfl1. Like ScSfl1, CaSfl1 functions as a negative regulator of filamentous growth in *C. albicans*. Deletion of SFL1 enhances filamentous growth in several media, and overexpression of SFL1 inhibits hyphal development. Growth temperatures, serum, high temperature, neutral pH, and nutrient starvation each had an additive effect on hyphal induction in the sfl1/sfl1 mutant. Lack of Sfl1 reduces the threshold of hyphal induction responding to multiple extracellular stimuli. Unlike the case for the other heat shock factors, SFL1 expression is not responsive to heat shock, which is similar to that of SFL1 in *S. cerevisiae*. In *C. albicans*, the level of SFL1 expression remains constant in various growth conditions (data not shown). Its protein level and cellular localization are also the same in all growth conditions examined. Therefore, Sfl1 might be regulated at the level of transcriptional activity or DNA binding.

In *S. cerevisiae*, Sfl1 has been shown to interact with the TPR motifs of Ssn6 as well as Sin4 and Srb10 in response to envi-
Environmental signals (10). Sn6-Tup1 and specific subunits of the RNA polymerase II holoenzyme are required for Sfl1 repression function. Biochemical evidence demonstrates that Sfl1 is present at the promoters of three Sn6-Tup1-repressible genes: *FLO11*, *HSP26*, and *SUC2* (10). Sfl1 inhibits transcription by recruiting Sn6-Tup1, which is responsible for the repression of over 180 genes in *S. cerevisiae* (20). The Sn6-Tup1 complex represses multiple subsets of genes when recruited to promoters by sequence-specific DNA binding repressors (30). In *C. albicans*, the negative regulator Sn6 is postulated to form a complex with Tup1 (18, 21). Sfl1 may act as a repressor by interacting with Sn6, which in turn represses the transcription of target genes. Binding sites for the HSF domain were found at the promoters of several of hypha-specific genes, including *HWPI* and *ECE1* (2, 40). The *HWPI* mRNA level was increased 100-fold in an *sfl1/sfl1* mutant compared with a wild-type strain in YPD at 37°C but was reduced dramatically in an *SFL1*-overexpressing strain (data not shown). Consistent with its putative binding to specific DNA via the HSF domain, Sfl1 is localized in the nucleus independent of growth forms. We suggest that Sfl1 may bind to specific promoter sequences of hypha-specific genes and repress their expression by recruiting Sn6-Tup1 complex and Srb/mediator proteins.

**Antagonistic effects of Sfl1 and Flo8 on hyphal development.** In *S. cerevisiae*, the transcriptional activator Flo8 and the repressor Sfl1 function downstream of the PKA pathway, antag-

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**FIG. 7. Effects of Sfl1 and Flo8 on hyphal growth under embedded conditions.** The wild-type (WT) strain (CAI4), *sfl1/sfl1* mutant (CAL2), *flo8/flo8* mutant (CCF4), and *sfl1/sfl1 flo8/flo8* mutant (CAL4) carrying vector (pBES116), overexpressed *SFL1* (pBA1-SFL1), or overexpressed *FLO8* (pBA1-FLO8) were plated with molten YPS agar and grown at 37°C for 2 days (A) or at 24°C for 3 days (B). Cells and colonies were photographed with a microscope using ×40 and ×10 lenses.
onistically controlling expression of FLO11 and the dimorphic filamentous transition in response to nutrient cues (35). Both Flo8 and Sfl1 can bind to the same region of the FLO11 promoter. Phosphorylation by Tpk2 promotes Flo8 binding and activation of the FLO11 promoter and relieves repression by prohibiting dimerization and DNA binding by Sfl1. A double-barreled mechanism was proposed to illustrate a finer network of checks and balances to modulate gene expression in S. cerevisiae, by controlling the ratios of phosphorylated and unphosphorylated forms of both Sfl1 and Flo8 (35). The combination of dual control by the activation of Flo8 and the relief of repression of Sfl1 may contribute to control morphogenesis of C. albicans responding to different environmental cues in aerobic conditions. Similarly, in C. albicans, Sfl1 functions as a negative regulator and Flo8 acts as a positive regulator of filamentous growth in liquid and solid media (Table 2; Fig. 6). The hyperfilamentous phenotype of the sfl1/sfl1 mutant was abolished by deleting FLO8 but enhanced by overexpression of FLO8 (Table 2). These data suggest that Sfl1 may inhibit filamentous development by antagonizing Flo8 functions. The mechanism for the antagonizing roles of Sfl1 and Flo8 in hyphal development is likely similar to that in S. cerevisiae.

In microaerophilic conditions, C. albicans Sfl1 may act as an activator as well as a repressor. In presence of Flo8, Sfl1 functions as a repressor of hyphal development; deletion of Sfl1 caused the formation of stronger heterogeneous filaments at both 24°C and 37°C (Fig. 7A and B). In absence of Flo8, Sfl1 acts as a repressor at high temperature but an activator at low temperature. When embedded in agar at 24°C, overexpression of SFL1 enhances filamentous growth, whereas deletion of SFL1 blocks the filament formation in an flo8/flo8 mutant (Fig. 7B). Consistent with our data, S. cerevisiae SFL1 was recently reported to be an activator involved in transcriptional control of the stress-responsive gene HSP30 (1). Interestingly, the strain that Ansanay Galeote et al. used is derived from S288C, which contains a mutated FLO8 gene (1), but Pan and Heitman used a sigma background strain containing a wild-type FLO8 gene for study of Sfl1 (35). The existence of Flo8 may prevent Sfl1 from binding to the promoter and then inhibit its activating effect as well as its repressing effect on target genes in response to physical environmental cues. Our observations suggest that Sfl1 has a dual function in filamentous growth of C. albicans: it acts as a repressor of hyphal development antagonizing activation of the Flo8 but functions as an activator releasing from inhibition of the Flo8 in matrix at low temperature. Further investigation should help in elucidating the mechanisms of Sfl1 and Flo8 in regulation of hyphal development.

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