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Convergent Regulation of *Candida albicans* Aft2 and Czf1 in Invasive and Opaque Filamentation

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ABSTRACT

Candida albicans is the most common fungal pathogen of mucosal infections and invasive diseases in immuno-compromised humans. The abilities of yeast–hyphal growth and white–opaque switching affect *C. albicans* physiology and virulence. Here, we showed that *C. albicans* Aft2 regulator was required for embedded filamentous growth and opaque cell-type formation. Under low-temperature matrix embedded conditions, Aft2 functioned downstream of Czf1-mediated pathway and was required for invasive filamentation. Moreover, deletion of *AFT2* significantly reduced opaque cell-type formation under *N*-acetylglucosamine (GlcNAc) inducing conditions. Ectopic expression of *CZF1* slightly increased the white-opaque switching frequency in the *aft2Δ/Δ* mutant, but did not completely restore to wild-type levels, suggesting that Czf1 at least partially bypassed the essential requirement for Aft2 in response to opaque-inducing cues. In addition, multiple environmental cues altered *AFT2* mRNA and protein levels, such as low temperature, physical environment and GlcNAc. Although the absence of Czf1 or Efg1 also increased the expression level of *AFT2* gene, deletion of *CZF1* remarkably reduced the stability of Aft2 protein. Furthermore, *C. albicans* Aft2 physically interacted with Czf1 under all tested conditions, whereas the interaction between Aft2 and Efg1 was barely detectable under embedded conditions, supporting the hypothesis that Aft2, together with Czf1, contributed to activate filamentous growth by antagonizing Efg1-mediated repression under matrix-embedded conditions. J. Cell. Biochem. 116: 1908–1918, 2015.

KEY WORDS: Candida albicans; Aft2; FILAMENTOUS GROWTH; WHITE–OPAQUE SWITCHING; INVASIVE FILAMENTATION

Candida albicans is a well-known fungal commensal pathogen that remains perpetually associated with its mammalian hosts. It serves as a part of the normal microbial flora in most healthy people, but causes superficial mucosal infections as well as life-threatening systemic diseases in immuno-compromised individuals. The molecular mechanisms and virulence determinants that contribute to the transition from commensalism to pathogenicity have been extensively studied during the last few years [Mayer et al., 2013; Noble, 2013; Pande et al., 2013; Perez et al., 2013]. Among them, one of the most important properties of *C. albicans* is the capacity to undergo a reversible morphological transition, which is closely associated with pathogenesis during pathogen–host interactions [Mayer et al., 2013]. Numerous studies have suggested that *C. albicans* exhibits a wide range of morphological plasticity, including the yeast-to-hyphal transition and the white-to-opaque transition [Brown and Gow, 1999; Whiteway and Bachewich, 2007; Morschhauser, 2010]. The diversity of morphogenetic processes allows *C. albicans* to adapt to a wide variety of environmental stimuli, which provides more opportunities for this fungus to survive in different host niches.

Yeast–hypha morphogenesis in *C. albicans* can be triggered by various environmental cues in vitro, including high temperature, nutrient limitation, ambient pH, serum, hypoxia, and physical embedding [Biswas et al., 2007; Sudbery, 2011; Huang, 2012]. Multiple environmental sensing and signal transduction pathways are involved in the regulation of yeast–hypha morphogenesis in *C. albicans*, including Cph1-mediated MAPK pathway, Efg1-mediated cAMP pathway, and Czf1-mediated matrix embedding pathway

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[Biswas et al., 2007]. The transcription factor Cph1, which is required for the function of MAP kinase module, confers a defective hyphal formation on many solid hypha-inducing media, but exhibits a much less role in response to serum induction [Liu et al., 1994]. The APSES protein Efg1 plays a central role in several signaling networks that regulate *C. albicans* morphogenesis and virulence [Doedt et al., 2004]. The *efg1Δ/Δ* mutant strain exhibits a severe defect in hyphal development in response to most hypha-inducing conditions, but shows remarkably enhanced hyphal growth under low-temperature matrix embedded conditions. The identification and characterization of the *CZF1* gene reveals an alternative morphogenetic pathway in *C. albicans* [Brown et al., 1999; Kumamoto and Vinces, 2005]. Homozygous *czf1* null mutants can undergo filamentation under standard induction conditions. However, cells lacking Czf1 protein are defective in filamentous development under matrix-embedded conditions. The molecular mechanisms of Czf1-mediated matrix embedding morphogenesis have being investigated during the past several years [Giusani et al., 2002; Vinces et al., 2006]. Chromatin immuno-precipitation suggests that *CZF1* expression is highly regulated through the coordinate binding of Efg1 and Czf1 to its promoter. The yeast two-hybrid interaction also confirms that Efg1 and Czf1 interact with each other physically. These findings demonstrate that *CZF1* might promote filamentation by antagonizing Efg1-mediated repression. In addition, recent studies also showed that deletion of *CDC35* or *FLO8* results in hyper-filamentation under matrix-embedded conditions [Cao et al., 2006], while deletion of *RAC1*, a highly conserved G protein, fails to form filaments when cells are embedded in an agar matrix [Bassilana and Arkowitz, 2006]. However, the exact roles of these proteins in Czf1-mediated morphogenesis are not yet clear. Other components of this alternative signaling pathway remain to be identified.

C. albicans also has the capacity to switch frequently and reversibly, from the normal round-to-oval yeast form (white) to elongated bean-shaped cell morphology (opaque) [Morschhauser, 2010]. The white-opaque transition has profound effects on the abilities of *C. albicans* colonization, survival and pathogenesis in the hostile environment, which contributes to a better adaptation to specific host niches. The molecular basis of genetic control of white-opaque switching has recently been elucidated [Huang et al., 2006; Zordan et al., 2007; Hernday et al., 2013]. White–opaque switching is mainly controlled by mating type-like (*MTL*) locus and the master white-opaque regulator Wor1. The majority of natural isolates of *C. albicans* have two heterozygous *MTL* alleles, *MTLa* and *MTLα*, and fail to switch between white and opaque cells. This block is attributed to the repression of *WOR1* expression by the heterodimeric a1–α2 repressor, which is encoded by *MTLa* and *MTLα*, respectively. Loss of *MTL* heterozygosity relieves this repression, and allows the formation of opaque cells. Only *C. albicans MTL*-homozygous strains can switch to the mating-competent opaque form in a high frequency, and undergo efficient mating with opposite mating types [Miller and Johnson, 2002]. Although there are significant differences in expression patterns and colony morphologies, some major regulators involved in hyphal development are also required for white–opaque switching, including Efg1 and Czf1 [Biswas et al., 2007; Sonneborn et al., 1999; Vinces and Kumamoto, 2007]. A recent study revealed that the stability of the white and opaque phases is maintained by the interlocking transcriptional positive feedback loops consisting of Efg1, Czf1, Wor1, and Wor2 [Zordan et al., 2007]. The transcription factors Czf1, Wor1, and Wor2 are positive regulators that contribute to the formation of opaque cells, whereas Efg1 acts as a repressor of white–opaque switching to maintain the white state. Notably, Efg1 exerts its regulatory activity by repressing the positive regulator Wor2 in white cells, whereas Czf1 inhibits Efg1 activity in opaque cells. Furthermore, some other transcription factors involved in the white-opaque transition have also been found, such as Zcf37, Cph1, and Wor3 [Wang et al., 2011; Hernday et al., 2013; Ramirez-Zavala et al., 2013]. Interestingly, the switching between white and opaque cells is not only governed by the transcriptional network, but also induced by numerous environmental signals [Ramirez-Zavala et al., 2008]. *N*-acetylglucosamine (GlcNAc), low temperature and high CO_2 are considered as potent inducers of the switching and essential for the stabilization of opaque cells [Huang et al., 2009; Huang et al., 2010]. A recent study demonstrated that *C. albicans* white and opaque cells undergo distinct programs of filamentous growth [Si et al., 2013]. Standard filament-inducing conditions inducing white cell filamentation fail to induce opaque cell filamentation. Significantly, opaque cells undergo efficient filamentation in response to distinct environmental cues, including sorbitol, GlcNAc, low nitrogen, and low phosphate. Moreover, several studies showed that some transcription factors are implicated in the regulation of opaque cell filamentation, including Cph1, Ume6, and Rfg1 [Ramirez-Zavala et al., 2013; Si et al., 2013]. In addition, Bcr1 and its downstream regulators Cup9, Nrg1, and Czf1 are shown to control opaque cell filamentation by the cAMP-signalling pathway in *C. albicans* [Guan et al., 2013]. However, the molecular mechanisms of filamentous growth in opaque cells are not yet fully understood.

Our previous studies have demonstrated that *C. albicans* Aft2, as a transcriptional regulator, plays an important role in filamentous growth under hypha-inducing conditions [Liang et al., 2010; Xu et al., 2013]. In this work, we found that Aft2 regulator was required for filamentous growth under matrix-embedded conditions, which functioned downstream of Czf1-mediated pathway. The *aft2Δ/Δ* mutant also exhibited an obviously low frequency of white–opaque switching and higher expression levels of opaque-phase specific genes in comparison with the wild-type and complemented cells. However, ectopic expression of *CZF1* under the control of the *ADH1* promoter only partially rescued the defect of the *aft2Δ/Δ* mutant in white-opaque switching. Interestingly, we also found that the lack of Efg1 or Czf1 regulator resulted in an increase in the *AFT2* mRNA and protein expression. Further results suggested that Aft2 protein was unstable in the *czf1Δ/Δ* mutant, and had a physical interaction with Czf1. Moreover, Efg1 regulator failed to interact with Aft2 protein when cells were embedded in agar matrix, supporting the hypothesis that Aft2 might exert its positive regulatory effect when Efg1-mediated repression was relieved. In conclusion, our data characterized Aft2 as a new regulator involved in embedded growth and opaque filamentation, and suggested its potential relationship with other major transcription factors. These findings provided a new understanding of Efg1–Czf1 mediated signal transduction pathways.

MATERIALS AND METHODS

STRAINS AND GROWTH CONDITIONS

C. albicans strains used in this study are listed in Table I. BWP17 or CAI-4 was used as the wild-type control strain in the functional analysis. Cells were routinely cultivated in YPD (1% yeast extract, 2% peptone, 2% glucose) supplemented with 80 μg/ml uridine or synthetic drop-out medium (0.67% yeast nitrogen base without amino acids, 2% glucose, 0.2% complete mixture lacking specific amino acid) for selection of specific transformants. YPS medium (1% yeast extract, 2% peptone, 2% surcose) was also used when necessary. Synthetic complete medium supplemented with 1 mg/ml 5-fluoroorotic acid (5-FOA) was used to select Ura-derivatives. For embedding experiments, 2% agar was added into YPD medium. Modified Lee′s medium containing 2.5% *N*-acetylglucosamine (GlcNAc) and 10 μM KH2PO4 was adjusted to pH 7.0 and used for white-to-opaque switching assays [Guan et al., 2013].

PLASMID AND STRAIN CONSTRUCTIONS

For the construction of the *AFT2* over-expression plasmid, a 3.0 kb fragment (containing the entire ORF and its transcription terminator) was amplified from the BWP17 genome, and subcloned into the *Pst*I–*Cla*I sites of pBES116-P_{ADH1}-URA3 plasmid. The resulting plasmid was linearized with *Asc*I, and transformed into the *C. albicans* BWP17, *cph1Δ/Δ*,*efg1Δ/Δ* and *czf1Δ/Δ* mutants to generate strains that over-expressed *AFT2* gene under the control of the strong *ADH1* promoter, respectively. Similar strategies were used for the construction of the *CPH1, EFG1 and CZF1* over-expression strains.

For the quantitative analysis of gene expression, the pGFP–P_{AFT2}–URA3 reporter system, which contained the *AFT2* promoter and the codon-optimized GFP reporter, was generated as described previously [Barelle et al., 2004]. Briefly, the *AFT2* promoter region was amplified, and subcloned into the *Xho*I–*Hin*dIII sites of pGFP plasmid. Then, the resulting plasmid was linearized with *Stu*I, and transformed into the corresponding *C. albicans* strain to generate promoter–reporter system. The *MTL* homozygous strains of *C. albicans* were obtained by targeted disruption with the MTLa–HIS1 cassette as described previously [Noble and Johnson, 2005]. The upstream and downstream flanking sequences of *MTLa* locus were amplified from the BWP17 genome, and the selectable *HIS1* marker was amplified from the pSN52 template. Then, the fusion PCR

TABLE I. Strains and plasmids used in this study

Strains or plasmids	Genotype	Source
Strains		
CAI4	*ura3Δ::λimm434/ura3Δ::λimm434*	Jiangye Chen
cph1Δ/Δ	CAI4 *cph1::hisG/cph1::hisG*	Julia Koehler
efg1Δ/Δ	CAI4 *efg1::hisG/efg1::hisG*	Julia Koehler
czf1Δ/Δ	CAI4 *czf1::hisG/czf1::hisG*	Carol Kumamoto
BWP17	*ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*	Dana Davis
aft2Δ/Δ	BWP17 *aft2::ARG4/aft2::dpl200*	Liang et al., 2010
aft2Δ/Δ+AFT2	BWP17 *aft2::ARG4/aft2::dpl200, pCR4-AFT2*	Liang et al., 2010
BWP17+AFT2$_{ADH1p}$	BWP17 *ADE2:: pBES116-P_{ADH1}-AFT2-URA3*	This study
aft2Δ/Δ+CZF1$_{ADH1p}$	BWP17 *aft2::ARG4/aft2::dpl200 ADE2:: pBES116-P_{ADH1}-CZF1-URA3*	This study
aft2Δ/Δ+CPH1$_{ADH1p}$	BWP17 *aft2::ARG4/aft2::dpl200 ADE2:: pBES116-P_{ADH1}-CPH1-URA3*	This study
aft2Δ/Δ+EFG1$_{ADH1p}$	BWP17 *aft2::ARG4/aft2::dpl200 ADE2:: pBES116-P_{ADH1}-EFG1-URA3*	This study
czf1Δ/Δ+AFT2$_{ADH1p}$	CAI4 *czf1::hisG/czf1::hisG ADE2:: pBES116-P_{ADH1}-AFT2-URA3*	This study
cph1Δ/Δ+AFT2$_{ADH1p}$	CAI4 *cph1::hisG/cph1::hisG ADE2:: pBES116-P_{ADH1}-AFT2-URA3*	This study
efg1Δ/Δ+AFT2$_{ADH1p}$	CAI4 efg1::hisG/efg1::hisG ADE2:: pBES116-P_{ADH1}-AFT2-URA3	This study
BWP17 *(Δ/α)*	*MTLa::HIS1/MTLα BWP17*	This study
aft2Δ/Δ (Δ/α)	*MTLa::HIS1/MTLα* BWP17 *aft2::ARG4/aft2::dpl200*	This study
aft2Δ/Δ+AFT2 (Δ/α)	*MTLa::HIS1/MTLα* BWP17 *aft2::ARG4/aft2::dpl200, pCR4-AFT2*	This study
BWP17+*AFT2$_{ADH1p}$ (Δ/α)*	*MTLa::HIS1/MTLα* BWP17 *ADE2:: pBES116-P_{ADH1}-AFT2-URA3*	This study
aft2Δ/Δ+CZF1$_{ADH1p}$(Δ/α)	*MTLa::HIS1/MTLα* BWP17 *aft2::ARG4/aft2::dpl200 ADE2:: pBES116-P_{ADH1}-CZF1-URA3*	This study
CAI4+ P_{AFT2}-GFP	*ura3Δ::λimm434/ura3Δ::λimm434 RPS10::pGFP- P_{AFT2}-URA3*	This study
czf1Δ/Δ+ P_{AFT2}-GFP	*CAI4 czf1::hisG/czf1::hisG RPS10::pGFP- P_{AFT2}-URA3*	This study
efg1Δ/Δ+ P_{AFT2}-GFP	*CAI4 efg1::hisG/efg1::hisG RPS10::pGFP- P_{AFT2}-URA3*	This study
CAI4-AFT2-HA	*ura3Δ::λimm434/ura3Δ::λimm434 AFT2/AFT2-3×HA-URA3*	This study
cph1Δ/Δ- AFT2-HA	CAI4 *cph1::hisG/cph1::hisG AFT2/AFT2-3×HA-URA3>*	This study
efg1Δ/Δ- AFT2-HA	CAI4 *efg1::hisG/efg1::hisG AFT2/AFT2-3×HA-URA3*	This study
czf1Δ/Δ- AFT2-HA	CAI4 *czf1::hisG/czf1::hisG AFT2/AFT2-3×HA-URA3*	This study
BWP*17-AFT2-FLAG*	BWP17 *AFT2/AFT2-6×His-FLAG-HIS1*	This study
BWP*17- CZF1-HA*	BWP17 *CZF1/CZF1-3×HA-URA3*	This study
BWP*17- EFG1-HA*	BWP17 *EFG1/EFG1-3×HA-URA3*	This study
BWP*17-AFT2-FLAG-CZF1-HA*	BWP17 *AFT2/AFT2-6×His-FLAG-HIS1 CZF1/CZF1-3×HA-URA3*	This study
BWP*17-AFT2-FLAG-EFG1-HA*	BWP17 *AFT2/AFT2-6×His-FLAG-HIS1 EFG1/EFG1-3×HA-URA3*	This study
Plasmids		
pBES116	ADE2-URA3-ADE2, AscI fragment in pBluescript II KS (+)	Gerald Fink
pBES116-P_{ADH1}-URA3	∼1.8 kb *ADH1* promoter in *pBES116*	Xu et al., 2013
pBES116-P_{ADH1}-AFT2-URA3	∼3.0 kb *AFT2* ORF-terminator in *pBES116-P_{ADH1}-URA3*	Xu et al., 2013
pBES116-P_{ADH1}-CZF1 -URA3	∼2.0 kb *CZF1* ORF-terminator in *pBES116-P_{ADH1}-URA3*	This study
pBES116-P_{ADH1}-CPH1-URA3	∼2.5 kb CPH1 ORF-terminator in *pBES116-P_{ADH1}-URA3*	This study
pBES116-P_{ADH1}-EFG1-URA3	∼2.4 kb *EFG1* ORF-terminator in *pBES116-P_{ADH1}-URA3*	This study
pGFP	containing ∼0.7kb GFP reporter gene	Alistair Brown
pGFP-P_{AFT2}-URA3	∼1.0 kb *AFT2* promoter fragment in *pGFP*	This study
pSN52	containing ∼2.4 kb C. *dubliniensis* HIS1 marker	Suzanne Noble
pFA-HA-URA3	*containing a 3×HA-URA3* cassette	Xu et al., 2013
p6HF-Act1-HIS1	*containing a 6×his*-FLAG-HIS1 cassette	Takashi Umeyama

reaction was performed to generate the MTLa–HIS1 disruption cassette.

The strains producing epitope-tagged proteins were achieved by PCR-mediated homologous recombination as previously described [Cheng et al., 2013]. The Aft2::3×HA–URA3, Efg1::3×HA–URA3 or Czf1::3×HA–URA3 cassette containing the flanking homology regions was amplified from the pFA–HA–URA3 template, respectively. The Aft2::6×His–FLAG–HIS1 cassette containing the flanking homology regions was amplified from the p6HF–Act1–HIS1 template. The Aft2::3×HA–URA3 cassette was transformed into the CAI-4 or mutant strains to generate correct recombinants that expressed Aft2–HA fusion protein under its own promoter. For the construction of *C. albicans* double epitope-tagged strains, both Aft2::6×His–FLAG–HIS1 and Efg1::3×HA-URA3 (or Czf1::3×HA–URA3) cassettes were sequentially transformed into the BWP17 strain by homologous recombination.

MATRIX-EMBEDDING ASSAY

Growth and embedding of *C. albicans* cells in agar medium were performed as previously described with minor modifications [Brown et al., 1999; Vinces et al., 2006]. Cells from overnight cultures were inoculated into fresh YPD medium, and re-cultivated to mid-exponential phase at room temperature. The harvested cells were washed and serially diluted to concentrations of 10^3 cells/ml with sterilized water. About one hundred of *C. albicans* cells were mixed with 50 ml of molten YPD 2% agar, and decanted into a 100-by-15-mm petri dish. The plates were allowed to solidify at room temperature, overlaid with a thin layer of YPD agar culture, and incubated at 25°C for the indicated time period. For RNA isolation, cells grown in embedded agar medium at 25°C for 6 days were harvested by mechanical disruption in cold phosphate-buffered saline buffer. The slurry was filtered through 400 micron nylon mesh, and the cells were collected and used for RNA extraction.

WHITE-OPAQUE SWITCHING ASSAYS

C. albicans white-opaque assays were performed as previously described with minor modifications [Huang et al., 2010]. Modified Lee′s 2.5% glucose or 2.5% GlcNAc was chosen as the sole carbon source, respectively. For solid switching assay, 20 μg/ml phloxine B dyes were added into the agar medium to exclusively stain opaque colonies. Strains were routinely grown on appropriate solid medium for 4-5 days at 25°C, and white cell colonies were inoculated into tubes containing fresh liquid Lee′s glucose medium. The overnight cultures were collected, diluted and plated into Lee′s GlcNAc agar plates. The plates were then incubated at 25°C for seven days before imaged. For liquid switching assay, white cell samples collected from the agar plates were also inoculated into Lee′s glucose medium. Then, the overnight cultures were collected, washed with sterilized water, and re-grown in 50 ml of fresh Lee′s medium with 2.5% GlcNAc as the sole carbon source at 25°C for 24 h. After incubation, the cells exhibiting the normal round-to-oval yeast cell type (white cell) or elongate bean-shaped cell type (opaque cell) were recorded, respectively. The white-to-opaque switching frequency was presented as the percentage of white cells that had switched to opaque phase.

For farnesol-mediated *C. albicans* cells killing assay, 60 μM *E, E*-farnesol (Sigma-Aldrich) was added to the medium, which relies on the fact that opaque cells are more sensitive to farnesol killing than white cells under aerobic conditions [Langford et al., 2009; Langford et al., 2013]. Briefly, white cells from the agar plates were pre-grown in Lee′s Glucose medium at 25°C for 24 h. The strains were then collected, re-suspended in 20 ml fresh Lee′s GlcNAc medium to an OD_{600} of 0.1, and incubated to exponential phase at 25°C with shaking. Subsequently, cell suspensions were divided into two equal parts: one was treated with 60 μM farnesol, and the other in the absence of farnesol was used as a control. After incubation at 25°C for 20 min, the number of colonies recovered from farnesol treated and untreated cultures were determined by serial dilution followed by plate counts. Cell survival was calculated according to the percentage of viable *C. albicans* cells. These experiments were performed at least three times independently. Data are presented as means ± SD of three independent experiments.

RNA ISOLATION AND QUANTITATIVE REAL-TIME PCR

Total RNA was extracted by the phenol-chloroform method as previously described [Cao et al., 2006]. The overall quality of RNA was determined by A260/A280 and analyzed by agarose gel electrophoresis. Quantitative real-time PCR was performed in triplicate and repeated in three independent experiments with the Mastercycler ep realplex system. Independent reaction mixtures were carried out by the same DNA template for both the genes of interest and the *ACT1* reference gene using the SYBR Green qPCR SuperMix (TransGen Biotech) according to the instructions. The relative fold changes in gene expression were determined by the $2^{-\Delta\Delta CT}$ method. Data are presented as means ± SD of three independent experiments.

WESTERN BLOTTING AND IMMUNOPRECIPITATION

Overnight cultures of the strains producing Aft2-HA protein were re-cultivated to mid-exponential phase in YPD medium at 25°C for 6 h. For protein stability assay, cells were incubated in YPD medium for 4 h before exposure to protein synthesis inhibitor cycloheximide (CHX) for another 2 h [Chen and Noble, 2012]. Protein extracts were prepared by breakage with glass beads as previously described [Chen and Noble, 2012]. Protein concentration of the lysates was measured by the Bradford assay. Lysates were analyzed by SDS–PAGE, and immunoblotted with high-affinity mouse anti-HA monoclonal antibody (Sigma–Aldrich) for HA-tagged proteins. Immunoblots were probed with anti-α tubulin antibody (Novus Biologicals) as a loading control.

For co-immunoprecipitation assay, cell lysates were obtained by breakage with glass beads under nondenaturing conditions [Chen and Noble, 2012]. About 2 μg rabbit anti-HA polyclonal antibody (Santa cruz) was added into lysates containing 10 mg of proteins, and incubated overnight at 4°C. Then, the immuno-complex was precipitated by 60 μl of agarose beads (PureProteome Protein A, Millipore). After incubation for 4 h at 4°C, beads were washed six times with yeast lysis buffer gently. Bound proteins were eluted, and subjected to Western blot with mouse anti-FLAG monoclonal antibody (Sigma–Aldrich) for FLAG-tagged proteins.

RESULTS

C. ALBICANS Aft2 REGULATOR IS ESSENTIAL FOR INVASIVE FILAMENTOUS GROWTH UNDER LOW-TEMPERATURE EMBEDDED CONDITIONS

Previous studies have found that many regulators play an important role in response to the physical environmental cues, including Cph1, Efg1, and Czf1 [Giusani et al., 2002; Kumamoto and Vinces, 2005]. Here, we identified Aft2 as a new positive regulator of morphogenesis during embedded growth. The *aft2Δ/Δ* mutant showed a severe growth defect in filamentation when cells were embedded within YPD agar medium at 25°C. Ectopic expression of *AFT2* under its own promoter fully rescued the filamentous defect of the *aft2Δ/Δ* mutant to the wild-type level (Fig. 1A). Moreover, compared with the wild-type and *AFT2*-complemented strains, the *aft2Δ/Δ* mutant was also defective in filamentous growth when cells were embedded in YPS 2% agarose medium at 25°C, which was consistent the previous report [Brown et al., 1999] and suggested that chemical components were not contributors of invasive filamentation under matrix-embedded conditions (data not shown).

To explore genetically the relationship between *C. albicans* Aft2 and other major regulators of invasive filamentation, we performed epistasis analysis by over-expression strategy. The Cph1, Efg1, and Czf1 regulators were chose as key components of three well-characterized morphogenetic signaling pathways. Gene overexpression was validated by real-time quantitative PCR (Fig. S1). Over-expression of *AFT2* in the *czf1Δ/Δ* mutant restored the production of filamentous colonies, while *CZF1* over-expression failed to suppress the morphogenetic defects of the *aft2Δ/Δ* mutant (Fig. 1A). In addition, the *aft2Δ/Δ* mutant over-expressing *CPH1* or *EFG1* under the control of the strong *ADH1* promoter was still defective in filamentation during matrix-embedded growth. Furthermore, *AFT2* over-expression in the *cph1* or *efg1* null mutant background had no obvious effect on morphogenesis (Fig. 1B). These findings suggested that Aft2 was a critical downstream effector of the Czf1-mediated matrix embedding pathway, but was independent of Cph1-mediated MAPK pathway and Efg1-mediated cAMP pathway. Further study revealed that deletion of *AFT2* significantly reduced the expression levels of four well-characterized hyphae-specific genes in response to physical environmental cues (Fig. 1C). These include

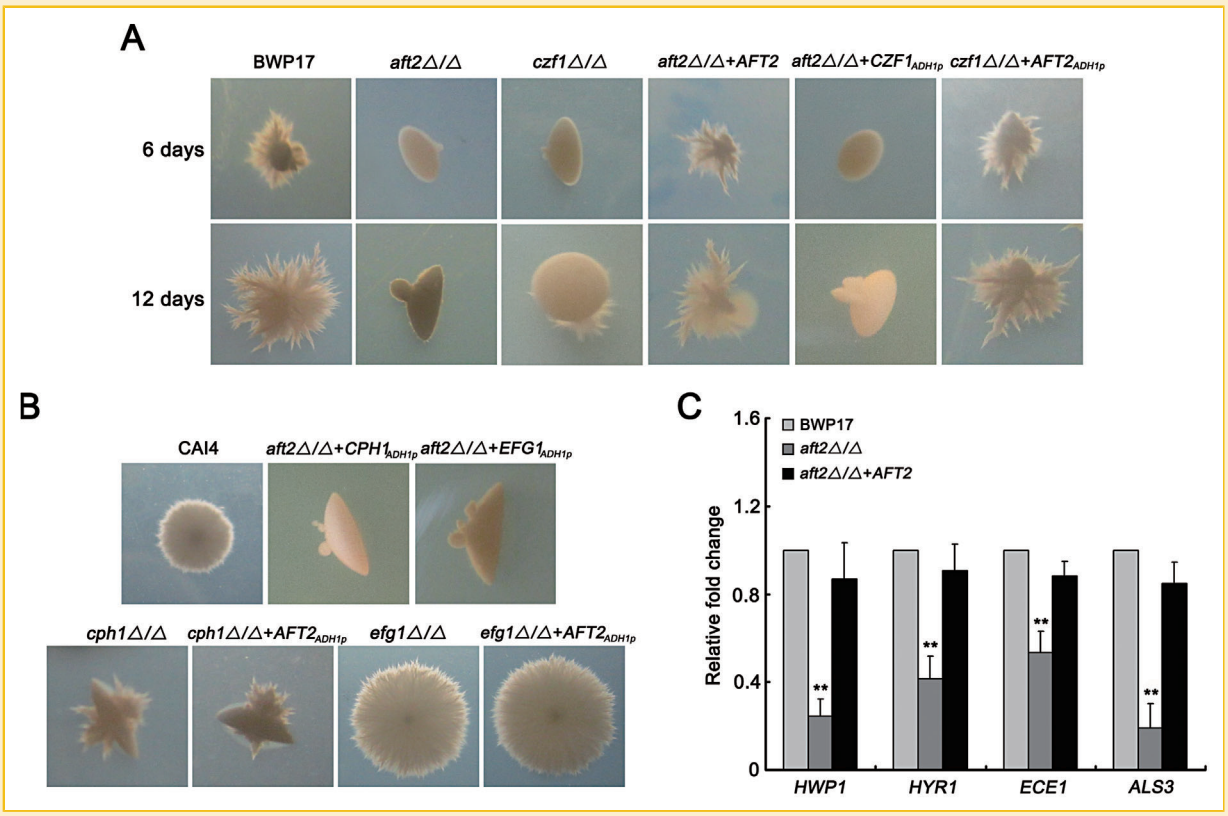

Fig. 1. Role of *C. albicans* Aft2 regulator in embedding growth. (A, B) The indicated *C. albicans* cells were mixed with molten YPD 2% agar, and incubated at 25°C. Each strain was tested in duplicate and in two independent experiments. Representative colonies were photographed at the times indicated. (C) Cells embedded in YPD agar medium for 6 days were harvested by mechanical disruption in cold PBS buffer and used for RNA extraction. Expression analyses of selected hyphae-specific genes were assessed by quantitative real-time PCR, and data are presented as means ± SD of three independent experiments. **, *P*-value < 0.01 compared with the wild-type strain as measured by a two-tailed unpaired Student's *t* test.

genes encoding hyphae-associated cell wall proteins Hwp1 and Ece1, GPI-anchored cell wall protein Hyr1 and cell surface glycoprotein Als3, which are required for filamentous growth in *C. albicans*. Taken together, our results indicate that *C. albicans* Aft2 acts downstream of Czf1 regulator to activate the expression of hyphal-related genes under matrix-embedded conditions.

THE *aft2Δ/Δ* MUTANT IS DEFECTIVE IN WHITE–OPAQUE SWITCHING

Based on the fact that the regulators Efg1 and Czf1 have been identified as parts of white–opaque switching transcriptional circuit [Zordan et al., 2007], we further investigate the importance of Aft2 regulator in opaque cell-type formation. To relieve *MTL* heterozygosity-mediated repression, we constructed the *MTLΔ/α* mutant by targeted disruption of one allele of the *MTL* locus [Miller and Johnson, 2002; Guan et al., 2013]. In this study, GlcNAc was used as a potent inducer of white-opaque switching in *C. albicans* [Huang et al., 2010]. Our results found that Aft2 regulator was also required for GlcNAc-induced white–opaque switching (Fig. 2A). Compared with the wild-type and complemented strains, the *aft2Δ/Δ* mutant exhibited an obviously reduction in opaque cell-type formation when grown on agar plates. Moreover, deletion of *AFT2* resulted in a severe growth defect in opaque cell filamentation. The opaque colonies of the *aft2Δ/Δ* mutant remained smooth, whereas colony morphology of the complemented strain was wrinkled and fuzzy after 7 days of incubation at 25°C. As expected, over-expression of *AFT2* under the control of the strong *ADH1* promoter stimulated

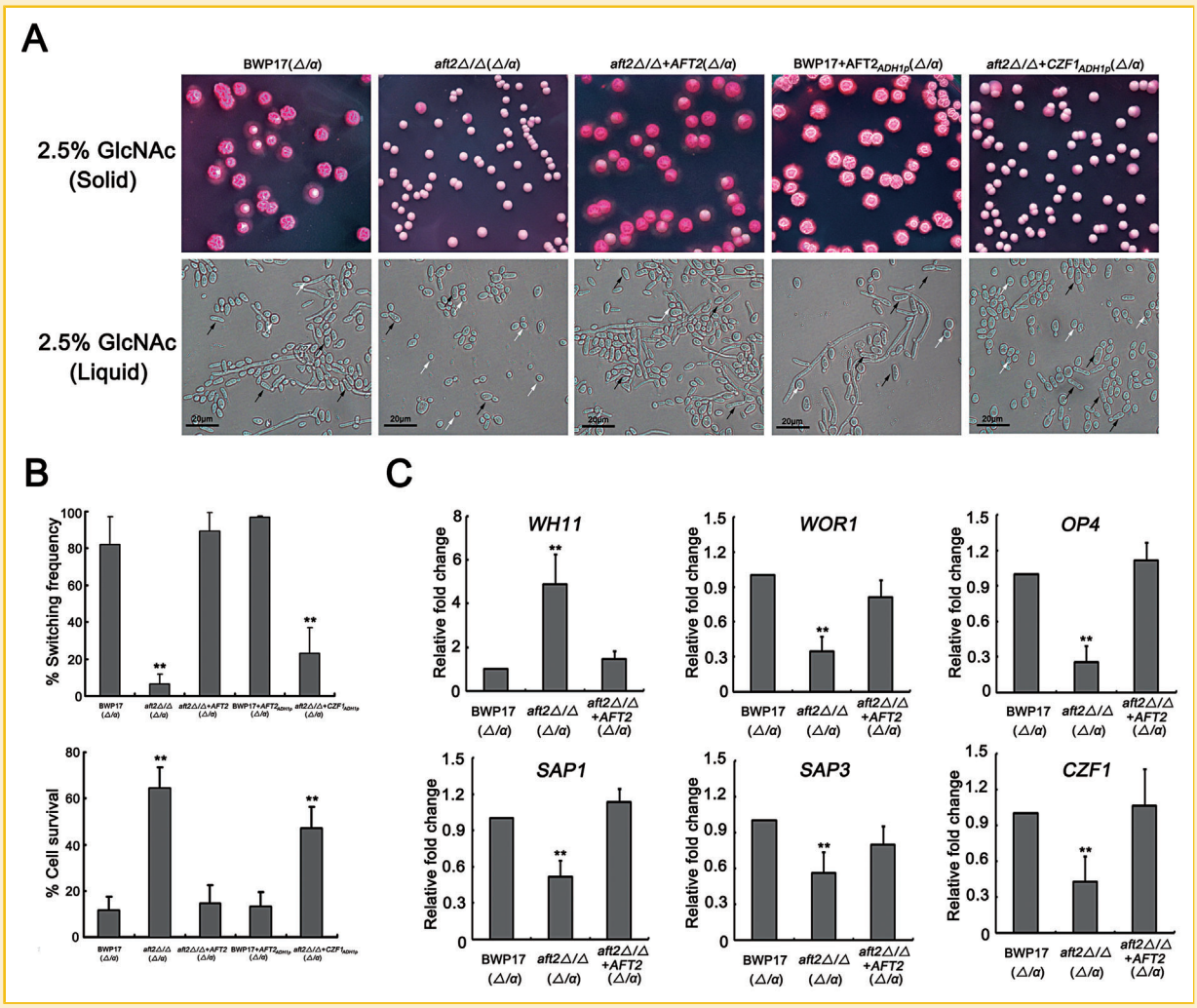

Fig. 2. *C. albicans* Aft2 regulator has a profound effect on opaque cell filamentation. (A) White-phase cells were pre-grown in YPD medium overnight, plated onto Lee's GlcNAc plates with 20 μg/ml phloxine B, and then incubated at 25°C in air for 7 days. For liquid switching assay, overnight cultures were inoculated into fresh liquid GlcNAc medium, and re-cultivated to mid-exponential phase for 6 h before imaged. Representative opaque cells are identified with solid arrows, and white arrows point to representative white cells. (B) Quantification opaque cell-type formation by switching frequency and farnesol killing assays. Switching frequency was calculated as the percentage of opaque cells (larger, elongate or bean-shaped cell form), and the viability of *C. albicans* cells was quantified by serial dilution followed by plate counts. (C) Expression analyses of the white-phase specific *WH11* gene and the opaque-phase specific *WOR1*, *OP4*, *SAP1* and *SAP3* genes. Mid-exponential cells were pre-grown in liquid Lee's GlcNAc medium at 25°C for 24 h, and used for RNA isolation. Transcript levels of target genes were quantified by real-time PCR and normalized to the expression of the *ACT1* reference gene. The results are presented as means ± SD of three independent experiments. **, *P*-value < 0.01 compared with the wild-type strain as measured by a two-tailed unpaired Student's *t* test

white cells to switch to the opaque phase, and nearly all of these mutants produced filamentous opaque cells. To determine the frequency of opaque cell-type formation, quantitative switching assays were performed [Ramirez-Zavala et al., 2013] (Fig. 2B, top panel). The switching frequency of the *aft2Δ/Δ* mutant was 4.36 ± 3.04%, which was much lower than those of the wild-type (81.94 ± 14.57%) and complemented (88.02 ± 9.68%) strains, while almost all of colonies of the *AFT2* over-expression mutant (99.16 ± 0.88%) were homogeneously opaque phase. To quantitatively determine the white-opaque switching capacity of *C. albicans* strains under liquid inducing conditions, we performed farnesol killing assays (Fig. 2B, bottom panel). Previous studies have observed that *C. albicans* opaque cells are more sensitive to franesol killing under aerobic conditions [Langford et al., 2013]. After 20 min of treatment with 60 μM farnesol, approximately 12.16 ± 5.14% of wild-type cells were able to evade the killing by the farnesol, whereas 63.92 ± 7.37% cells were survived in the *aft2Δ/Δ* mutant. As expected, cell survival of the *aft2Δ/Δ* mutant was significantly reduced to 16.49 ± 6.13% by integrating a wild-type *AFT2* fragment under the control of its native promoter, and 13.43 ± 5.76% for the *AFT2* over-expression strains. These observations indicated that the *aft2Δ/Δ* mutant had more white-phase cells in response to liquid GlcNAc induction, which was consistent with the above finding that the presence of Aft2 regulator was required for the efficient opaque cell-type formation.

It has been well-known that Czf1 serves as a positive regulator of white-opaque switching [Vinces and Kumamoto, 2007]. Therefore, we further explore the potential correlation between *AFT2* and *CZF1* in this morphogenetic process (Fig. 2B). Over-expression of *CZF1* in the *aft2Δ/Δ* mutant increased the frequency of white-opaque switching to 26.81 ± 12.77%, but failed to completely reach wild-type levels. Farnesol killing assays showed that cell survival of the *aft2Δ/Δ* mutant over-expressing *CZF1* was about 48.46 ± 7.18%, which was lower than that of the single *aft2Δ/Δ* mutant. Taken together, these results suggested that ectopic *CZF1* expression could at least partially bypass the requirement for Aft2 in opaque cell-type formation.

To further explore the possible mechanism by which Aft2 affected white-opaque switching, the expression levels of white-phase specific genes *WH11* and opaque-phase specific genes (*WOR1*, *OP4*, *SAP1*, and *SAP3*) were analyzed by quantitative real-time PCR assay (Fig. 2C). The results showed that the *aft2Δ/Δ*

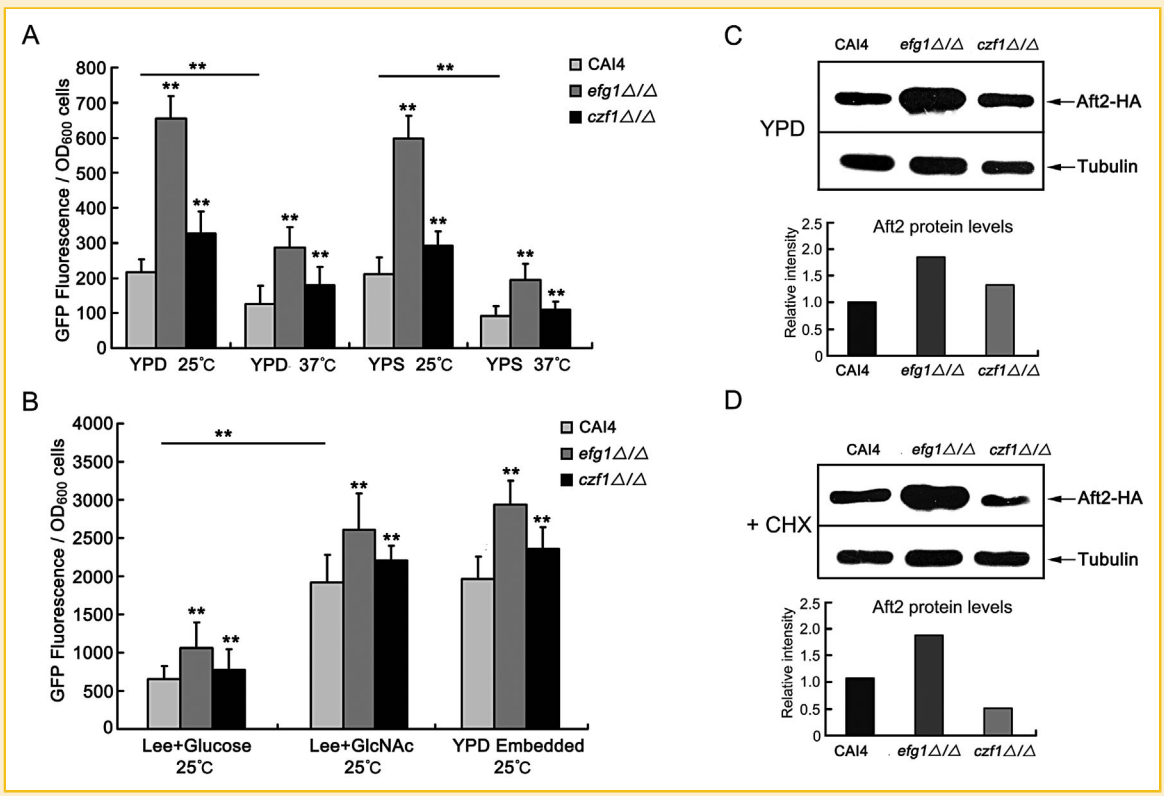

Fig. 3. Quantitative analysis of *AFT2* mRNA and protein expression levels in *C. albicans*. (A, B) Derivatives of *C. albicans* CAI-4 strain expressing the GFP reporter gene under control of the *AFT2* promoter were incubated under the indicated conditions. The intensities of GFP fluorescence were measured by quantitative fluorescence microscopy. At least three independent isolates of each strain were used for the assay, and data are presented as means ± SD of three independent experiments. ** indicates *P*-value < 0.01 compared with CAI-4 levels. (C) Protein levels of Aft2 in the CAI-4 derivatives were analyzed by Western blotting using the anti-HA antibody. For the cycloheximide (CHX) chase assay, cells were further treated with 50 μg/ml CHX for 2 h, and harvested for Western blotting analysis. The results shown are representative of at least three independent experiments. Protein band intensities were quantified by measuring band pixel density using ImageJ software, and normalized to the respective α-tubulin control of CAI-4 strains without CHX treatment.

mutant exhibited higher white-phase specific gene expression in comparison to wild-type cells under GlcNAc inducing conditions. Moreover, deletion of *AFT2* obviously reduced the transcription of *WOR1*, a master regulator of white–opaque switching, and the transcription of opaque-phase specific genes. In addition, the expression of *CZF1* in the *aft2Δ/Δ* mutant was also decreased. Taken together, these data revealed that *C. albicans* Aft2 was required for opaque cell-type formation by altering the expression of phase-specific genes.

MULTIPLE STIMULI TRIGGER THE EXPRESSION OF *AFT2* IN *C. ALBICANS*

To further investigate the molecular mechanisms of Aft2 in embedding growth and white-opaque switching, we analyzed gene expression patterns of *AFT2* in these cellular processes by the GFP-based reporter system. The analysis revealed that *AFT2* expression was highly induced in response to low temperature, whereas carbon source had little effect on *AFT2* mRNA levels (Fig. 3A). Furthermore, in accordance with the above observations, the presence of GlcNAc and physical environment also obviously increased *AFT2* expression (Fig. 3B). In addition, we found that Efg1 and Czf1 were involved in the regulation of *AFT2* expression under all tested conditions (Fig. 3A, B). Loss of Efg1 or Czf1 in *C. albicans* led to much higher expression levels of *AFT2* in response to environmental cues, which might be attributed to the alleviation of repression, functional redundancy and/or compensatory upregulation mechanisms.

To better understand the possible regulatory mechanism, we examined Aft2 protein levels in both wild-type and mutant strains (Fig. 3C). Western blot assays showed that Aft2 protein levels were up to 1.8-fold higher in the *efg1Δ/Δ* mutant when compared to those of wild-type cells. Likewise, the levels of Aft2 protein were increased by approximately 32.1% in the *czf1Δ/Δ* mutant (Fig. 3C, top panel). Subsequently, we examined Aft2 protein stability in the mutant cells by the cycloheximide (CHX) chase assay. The assay suggested that the concentration of Aft2 protein in the *efg1Δ/Δ* mutant remained unchanged upon CHX treatment. However, Aft2 protein levels in the *czf1Δ/Δ* mutant decreased by approximately 48.4% following 2 h of CHX treatment, indicating that Aft2 protein was destabilized and prone to degradation when Czf1 regulator was absent. Taken together, our current data suggest that *AFT2* expression is transcriptionally regulated by multiple environmental cues. Loss of Efg1 and Czf1 elevated *AFT2* mRNA and protein expression levels, but the stability of Aft2 is significantly reduced in the *czf1Δ/Δ* mutant, revealing an important role of these two regulators in the regulation of *AFT2* expression.

Aft2 INTERACTS WITH Czf1 IN VIVO

In this study, we found that Aft2 functioned downstream of Czf1 to activate filamentation under matrix-embedded conditions, and loss of Czf1 reduced the stability of Aft2 protein in *C. albicans*. In addition, Efg1 has been previously shown to be a repressor of invasive filamentation [Lachke et al., 2003; Zordan et al., 2007]. To further define the relationship among these regulators, we examined whether Aft2 interacts with Efg1 and/or Czf1 by immunoprecipitation analysis (Fig. 4). HA epitope was fused to the C-terminal end of

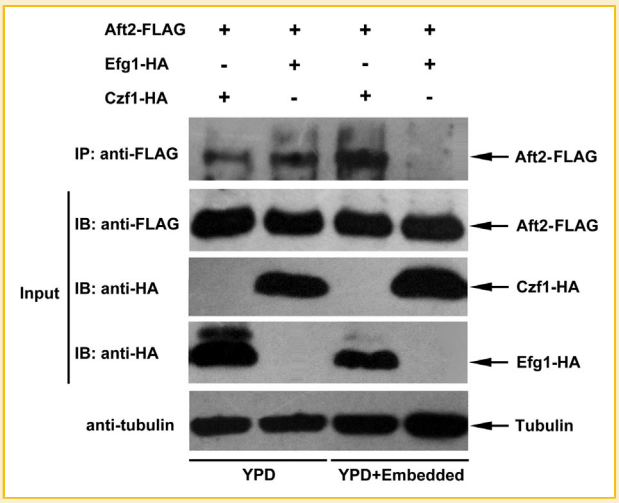

Fig. 4. Efg1 and Czf1 physically interact with Aft2 in vivo. Overnight cultures of *C. albicans* double epitope-tagged strains (Aft2-FLAG/Efg1-HA or Aft2-FLAG/Czf1-HA) were collected, and re-cultivated to mid-exponential phase in 100 ml liquid YPD medium at 25°C for 6 h. Cells growing in embedded agar medium were incubated at 25°C for 6 days before harvested. Whole cell extracts were prepared by breakage with glass beads under non-denaturing conditions, and subjected to immuno-precipitation with anti-HA polyclonal antibody (Santa cruz) and Pureproteome protein A agarose beads (Millopore). Then, the precipitated proteins were separated by SDS-PAGE, and probed with anti-FLAG monoclonal antibody (Sigma–Aldrich) to identify Aft2-FLAG. The input controls were analyzed by Western blotting with anti-FLAG monoclonal antibody (Sigma–Aldrich) or anti-HA monoclonal antibody (Sigma–Aldrich), respectively. Immunoblots were also probed with anti-α tubulin antibody (Novus Biologicals) as a loading control.

Czf1 or Efg1 protein, whereas Aft2 was tagged at its C terminus with FLAG epitope. Specificity of the antibody in this assay was confirmed with whole cell extracts containing only the FLAG-tagged or HA-tagged fusion proteins (Fig. S2). The assay showed that Aft2–FLAG could be co-immunoprecipitated with Czf1-HA when cells grown under both normal YPD and agar-embedded conditions, indicating that Aft2 physically interacted with Czf1 directely or in the presence of other unknown co-factors. Additionally, co-immunoprecipitation experiments revealed that Aft2 had a physical interaction with Efg1 under normal YPD conditions, but the interaction was barely detectable when cells were subjected to physical environmental cues. Taken together, these findings support a hypothesis that, under matrix-embedded conditions, the dissociation of the Aft2–Efg1 protein complex confers to the activation of Aft2, and subsequently coordinates Czf1 to regulate invasive filamentation.

DISCUSSION

The fungus *C. albicans* is often a benign member of the human mucosal flora, and becomes pathogenic in the immuno-compromised host. One distinguishing feature of *C. albicans* biology is its ability to grow in different morphological forms, which is important for fungal virulence [Mayer et al., 2013]. Although numerous environmental sensing and signal transduction pathways were

found to govern these phenotypic transitions in liquid cultures, relatively little is known about the strategies that *C. albicans* growth on host surfaces undergoes reversible morphological transitions [Kumamoto and Vinces, 2005; Biswas et al., 2007; Mayer et al., 2013]. In the host environment, *C. albicans* yeast forms are generally found to reside on the skin, mucosal and intestinal surfaces, including buccal mucosa, vagina mucosa and gastrointestinal tract. The interaction of this opportunistic pathogen with a surface can alter its physiology and lead to the formation of hyphal and/or opaque cells [Kumamoto and Vinces, 2005]. Hyphal cells allow *C. albicans* to disrupt the epithelial surfaces, invade deeper tissues and penetrate the blood vessel systems to cause systemic infection. Opaque cells, the mating-competent form of this fungus, are better colonizers of the skin and mammalian gastrointestinal tract, facilitating the colonization and survival in various host niches [Morschhauser, 2010]. Therefore, a further understanding of regulatory mechanisms involved in the control of dimorphism on host surfaces will provide new insights into *C. albicans* pathogenicity.

Previous studies demonstrated that, during growth on host surfaces, *C. albicans* was subjected to multiple environmental cues including both contact and low temperature [Brown et al., 1999; Petrovska and Kumamoto, 2012]. Contact-dependent cues mimicking the in vivo conditions encountered in host tissues contribute to both filamentation and mating. Both Efg1 and Czf1 have been identified as regulators of contact-dependent filamentous development and white–opaque switching, establishing a link between filamentation and mating [Giusani et al., 2002; Zordan et al., 2007]. In this study, we characterized Aft2 as another regulator in these two processes. Deletion of *AFT2* caused a profound defect in the yeast–hyphal transition during low-temperature matrix-embedded conditions. Epistasis analysis showed that *AFT2* over-expression efficiently masked morphogenetic defects of the *czf1Δ/Δ* mutant, revealing that Aft2 and Czf1 functioned in the same pathway. However, ectopic *CZF1* expression had little effect on the *aft2Δ/Δ* mutant. These observations suggested that Aft2 acted downstream of Czf1 regulator to control invasive filamentation under matrix-embedded conditions. Interestingly, over-expression of Cph1 or Efg1 was unable to complement the function of Aft2, indicating that Cph1-mediated MAPK pathway and Efg1-mediated cAMP pathway was independent of Aft2-mediated embedded filamentous growth, which were also confirmed by the finding that ectopic expression of *AFT2* in a single *cph1Δ/Δ* or *efg1Δ/Δ* mutant background did not affect morphological transitions in response to physical environmental cues. When cultured in the medium with GlcNAc as the sole carbon source, a potent inducer of white to opaque switching, the *aft2Δ/Δ* mutant exhibited an extremely low frequency of white-opaque switching by downregulating the expression of

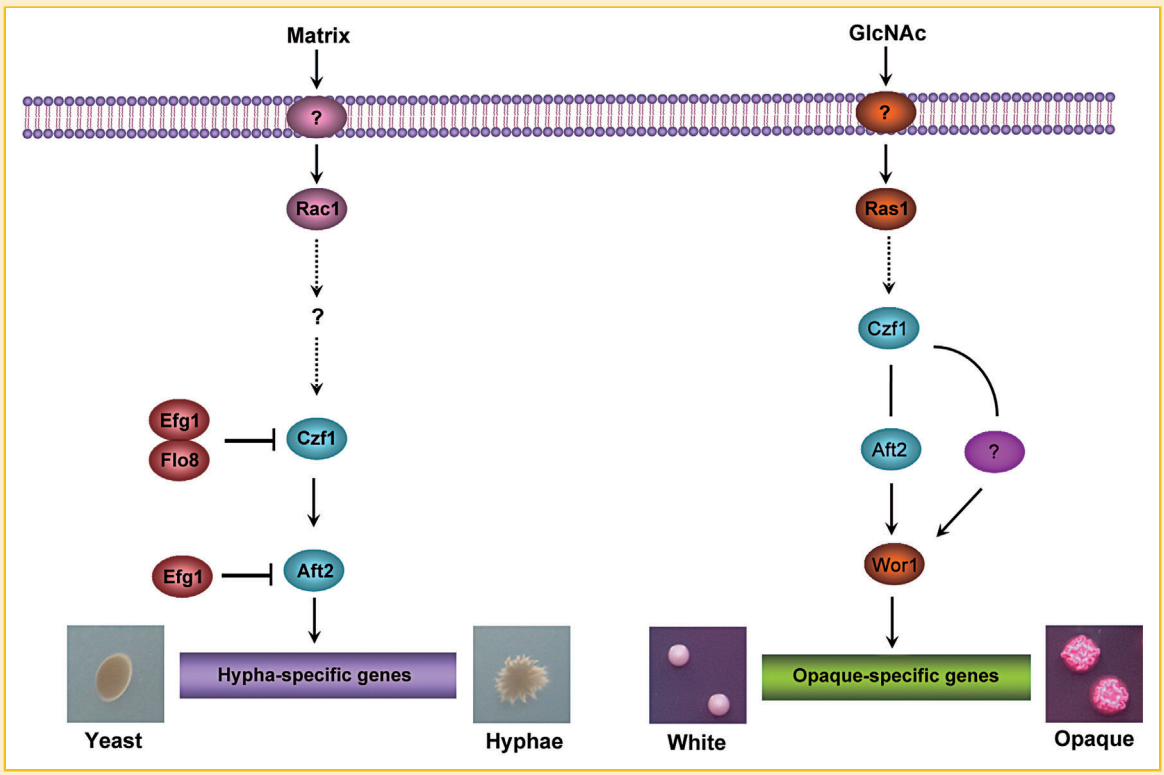

Fig. 5. A model of Aft2 in invasive filamentous growth and white–opaque switching in *C. albicans*. Under matrix-embedded conditions, the expression of *AFT2* is released from the Efg1-mediated repression. Subsequently, Aft2 physically interacts with Czf1, and functions downstream of Czf1 to activate hyphae-specific genes. However, in GlcNAc-containing medium, Aft2 and Czf1 operate through two distinct signaling pathways and ectopic expression of Czf1 can partially bypass the requirement for Aft2 in white–opaque switching. Dashed lines indicate unclear relationships, and blunt arrows indicate negative effects.

opaque-specific genes. Over-expression of *CZF1* slightly increased the switching frequency of the *aft2Δ/Δ* mutant, but did not completely normalize to wild-type levels. These results indicated that Aft2 was essential for opaque cell-type formation, and Czf1 partially bypass the requirement for Aft2 in white-opaque switching. Further studies also confirmed that deletion of *AFT2* remarkably reduced the expression levels of both hyphae-specific genes and opaque-specific genes under these tested conditions. In conclusion, our data found that Aft2 is a new regulator to govern morphogenesis and mating.

n addition, co-immunoprecipitation experiments revealed that Aft2 interacted with Efg1 and Czf1 under normal YPD conditions, respectively. During growth under matrix-embedded conditions, Efg1 was dissociated from the Efg1-Aft2 protein complex, whereas Aft2 was still functionally associated with Czf1. Previous studies have showed that Efg1 represses filamentous growth and inhibits white-opaque switching, whereas Czf1 had an opposite positive effect [Giusani et al., 2002]. Therefore, we proposed a simple model about the relative importance of Aft2-mediated pathway (Fig. 5). Under matrix-embedded conditions, the genetic interaction between Aft2 and Efg1 confirmed the hypothesis that Aft2 promoted filamentous growth by antagonizing Efg1-mediated repression. The dissociation of Aft2-Efg1 protein complexes conferred to the derepression of Aft2 regulator, and subsequently the direct cooperative interaction between Aft2 and Czf1 effectively promoted morphogenetic transition. However, in the presence of GlcNAc inducer, ectopic expression of Czf1 can partially bypass the requirement for Aft2 in white-opaque switching, suggesting that Aft2 and Czf1 might operate through two distinct signaling pathways. Taken together, our study provides a new insight into the molecular mechanisms that control invasive filamentation and mating during *C. albicans*–host interactions.

AUTHOR CONTRIBUTIONS

N.X. and Y.J.D. planned and performed experiments; N.X., Y.J.D., Q.L.Y., Bin.Z., M.Z., C.J., Y.L.C. analyzed data; N.X., Y.J.D., Bia. Z., L.J.X. and M.C.L. contributed reagents/materials/analysis tools; N.X., Y.J.D. and M.C.L. wrote the paper. All authors discussed the results and commented on the manuscript.

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