Journal of Microbiology (2021) Vol. 59
DOI 10.1007/s12275-021-1326-y
eISSN 1976-3794
pISSN 1225-8873

The transcription factor Cas5 suppresses hyphal morphogenesis during yeast-form growth in *Candida albicans*[§]

Jong-Myeong Kim[1], Hye Yun Moon[2], Dong Wook Lee[2], Hyun Ah Kang[2*], and Jeong-Yoon Kim[1*]

[1]*Department of Microbiology and Molecular Biology, College of Bioscience and Biotechnology, Chungnam National University, Daejeon 34134, Republic of Korea*
[2]*Department of Life Science, College of Natural Science, Chung-Ang University, Seoul 06974, Republic of Korea*

(Received Jun 18, 2021 / Revised Jul 26, 2021 / Accepted Jul 26, 2021)

Candida albicans **is an opportunistic human pathogen that exists as yeast, hyphal or pseudohyphal forms depending on pH, nutrients, and temperature. The morphological transition from yeast to hyphae, which is required for the complete virulence of *C. albicans*, is controlled by many transcription factors that activate or repress hypha-specific genes. The *C. albicans* transcriptional factor Cas5, a key regulator of genes involved in cell wall integrity, affects the susceptibility of *C. albicans* to fluconazole, an inhibitor of ergosterol synthesis. In this study, we found that deletion of *CAS5* in *C. albicans* decreased the expression levels of a set of ergosterol biosynthesis genes, such as *ERG2*, *ERG3*, *ERG5*, *ERG6*, *ERG11*, and *ERG24*, resulting in the accumulation of lanosterol and zymosterol, which are intermediate metabolites in the ergosterol biosynthesis pathway. Interestingly, it was observed that the *cas5*Δ/Δ mutant could not maintain the yeast form under non-hypha-inducing conditions, while the *CAS5*-overexpressing cells could not form hyphae under hypha-inducing conditions. Consistent with these observations, the *cas5*Δ/Δ mutant highly expressed hypha-specific genes, *ALS3*, *ECE1*, and *HWP1*, under non-hypha-inducing conditions. In addition, *CAS5* transcription was significantly downregulated immediately after hyphal initiation in the wild-type strain. Furthermore, the *cas5*Δ/Δ mutant reduced the transcription of *NRG1*, which encodes a major repressor of hyphal morphogenesis, while Cas5 overexpression increased the transcription of *NRG1* under hypha-inducing conditions. Collectively, this study suggests the potential role of Cas5 as a repressor of hypha-specific genes during yeast-form growth of *C. albicans*.**

Keywords: *Candida albicans*, transcription factor Cas5, ergosterol, hypha formation

*For correspondence. (H.A. Kang) E-mail: hyunkang@cau.ac.kr; Tel.: +82-2-820-5863; Fax: +82-2-825-5206 / (J.Y. Kim) E-mail: jykim@cnu.ac.kr; Tel: +82-42-821-6419; Fax: +82-42-822-7367
[§]Supplemental material for this article may be found at http://www.springerlink.com/content/120956.

Introduction

Candida albicans is an important opportunistic fungal pathogen that causes superficial infections as well as life-threatening systemic infections in immunocompromised patients, although it normally resides in yeast form on the mucosal surfaces of healthy people (Calderone and Fonzi, 2001; Gow *et al.*, 2012; Kim, 2016). *Candida albicans* attaches to epithelial or endothelial cells, invades via induced endocytosis or active penetration, and escapes from phagocytes and immune evasion by changing its morphology (Gow *et al.*, 2012; Lee *et al.*, 2015). The commensal-to-pathogen switch of *C. albicans* is closely related to its ability to switch from yeast to hyphal form, rapidly disseminate inside the host, and escape from host defense systems, thus resulting in fatal infection (Calderone and Fonzi, 2001; Gow *et al.*, 2002; Sudbery, 2011).

Candida albicans Cas5, a transcription factor containing two zinc finger domains at the C-terminal region, controls cell wall damage response and cell wall maintenance by regulating the expression of a set of genes encoding glycosylphosphatidylinositol (GPI)-anchored cell wall proteins upon treatment with caspofungin, which inhibits β-1,3-glucan synthesis (Bruno *et al.*, 2006). Cas5 interacts with another transcription factor, Efg1, for binding to some caspofungin-responsive gene promoters to coordinately activate their expression (Xiong *et al.*, 2021). Although *CAS5* does not have an exact ortholog in *Saccharomyces cerevisiae*, *S. cerevisiae* Rlm1 and Mig2 are thought to be functional and structural equivalents of Cas5, respectively, based on the fact that Rlm1-dependent genes in *S. cerevisiae* are homologs of some Cas5 target genes and that *S. cerevisiae* Mig2 and Cas5 share a high degree of similarity (51% identity) at the two C-terminal zinc fingers (Bruno *et al.*, 2006). In addition to cell wall stress responses to caspofungin, Cas5 was found to be required for the virulence of *C. albicans* in worm, toll mutant flies, and murine models (Chamilos *et al.*, 2009; Pukkila-Worley *et al.*, 2009). Cas5 also plays a role in controlling cell cycle dynamics partly in concert with the transcriptional regulators Swi4 and Swi6 under basal and cell wall stress conditions, and it is involved in regulating nuclear division (Xie *et al.*, 2017). Previous studies suggest that Cas5 may play a wider variety of roles than those identified to date.

A previous study reported that deletion of *CAS5* reduced the minimum fungicidal concentration of fluconazole in *C. albicans* and altered the expression of several genes involved in the lipid metabolic process, including *ERG11* and *ERG26* of the ergosterol biosynthesis pathway (Vasicek *et al.*, 2014), implying that Cas5 may participate in a transcriptional network that influences the response of *C. albicans* to fluconazole. Considering that disruption of the cell wall integrity enhances

the susceptibility of *C. albicans* to ergosterol biosynthesis inhibitors (LaFayette *et al.*, 2010), it is plausible that Cas5, a regulator of cell wall integrity, may regulate the expression of genes involved in the ergosterol biosynthesis pathway under basal and cell wall stress conditions. In this study, we demonstrated that Cas5 plays a role in ergosterol biosynthesis. We also showed that Cas5 is involved in the hyphal morphogenesis and maintenance of cell wall integrity in *C. albicans*.

Materials and Methods

Strains, plasmids, media, and growth conditions

The yeast strains constructed and used in this study are listed in Table 1. The plasmids and primers used in this study are listed in Supplementary data Tables S1 and S2. *Candida albicans* cells were routinely grown at 30°C in the yeast extract peptone dextrose (YPD) medium (1% yeast extract, 2% Bacto peptone, 2% glucose). The lithium-acetate method was used to transform *C. albicans*, as described previously (Walther and Wendland, 2003). For the selection of *C. albicans* transformants, synthetic complete (SC) medium (0.67% yeast nitrogen base without amino acids, amino acid dropout mixture, and 2% glucose) was used with appropriate auxotrophic requirements. To induce hyphal formation, 10% (v/v) newborn calf serum (Gibco) was added to each medium and incubated at 37°C. To induce the *MET3* promoter, cells were grown overnight in YPD medium, washed twice in phosphate-buffered saline (PBS), and resuspended in SC-Met-Cys medium (Care *et al.*, 1999).

Staining and microscopic observations

Hyphal morphology was visualized using calcofluor white (CW). Ten μl of each sample was placed on a glass slide and stained with 1 μl of CW (1 mg/ml). For visualization of the nucleus, the cells were stained with Hoechst. Photographs were analyzed using an Olympus BX51 microscope equipped with differential interference contrast optics, appropriate filters, and a charge-coupled device (CCD) camera at 1,000× magnification (Olympus DP71). All images were adjusted using the ImageJ software (NIH).

RNA isolation and quantitative reverse transcription-polymerase chain reaction (RT-PCR)

To isolate total RNA, *C. albicans* cells were grown overnight in 5 ml YPD medium at 30°C. Cultures were inoculated at OD_{600} = 0.3 in 25 ml of fresh YPD medium and grown at 30°C to the exponential phase. The subcultures were harvested by centrifugation and washed twice in PBS. The pellet was stored at -80°C overnight. RNA was isolated using the NucleoSpin RNA kit (MACHEREY-NAGEL), and cDNA was synthesized using the ReverTra Ace™ qPCR RT Kit (TOYOBO). Then, qRT-PCR was carried out using the RealHelix™ qPCR Kit (NanoHelix) in 96 well plates with the following cycle conditions: 95°C for 15 min, 95°C for 20 sec, 50°C for 10 sec, and 60°C for 30 sec for 30 cycles. The melt curve was completed with the following cycle conditions: 95°C for 10 sec and 65°C for 5 sec with an increase of 0.5°C per cycle up to 95°C. All reactions were performed in triplicates. The data were analyzed using the Bio-Rad CFX manager (Bio-Rad).

Statistical analysis

Unpaired t-test was used to determine the significant difference between two independent groups and one-way ANOVA Dunnett's multiple comparisons test was used to determine the significant difference among multiple groups. P-values were calculated by using GraphPad Prism 7 (GraphPad Software).

Table 1. ***Candida albicans*** **strains used in the present study**

Strain	Genotype	Parent	Source
Wild type			
SC5314	Clinical isolate (wild-type strain)		Fonzi and Irwin (1993)
CAI4	*ura3*::imm434/*ura3*::imm434 *iro1*/*iro1*::imm434	SC5314	Fonzi and Irwin (1993)
Gene knockout			
CCS1	*CaCAS5/Cacas5::CaURA3-dpl200*	CAI4	This study
CCS2	*CaCAS5/Cacas5::dpl200*	CCS1	This study
CCS3	*Cacas5::CaURA3-dpl200/Cacas5::CaURA3-dpl200*	CCS2	This study
CCS4	*Cacas5::dpl200/Cacas5::dpl200*	CCS3	This study
Revertant			
CCS5	*Cacas5::dpl200/P_CaCAS5-CaURA3-dpl200*	CCS4	This study
Overexpression			
CAI4[*MET3p-GFP-CaCAS5*]	*CaCAS5/P_CaURA3-MET3p-GFP-CaCAS5*	CAI4	This study
GFP tagging			
HWP1-3xGFPr	*CaHWP1/CaHWP1-3xGFPr*	CAI4	This study
cas5Δ/Δ HWP1-3xGFPr	*Cacas5::dpl200/Cacas5::dpl200 CaHWP1/CaHWP1-3xGFPr*	CCS4	This study
HWP1-GFPr	*CaHWP1/CaHWP1-GFPr*	CAI4	This study
cas5Δ/Δ HWP1-GFPr	*Cacas5::dpl200/Cacas5::dpl200 CaHWP1/CaHWP1-GFPr*	CCS4	This study
Cas5-5myc	*CaCAS5/CaCAS5-5myc-CaURA3-dpl200*	CAI4	This study
Nrg1-13myc	*CaNRG1/CaNRG1-13myc-CaURA3-dpl200*	CAI4	Lee *et al.* (2015)
cas5Δ/Δ NRG1-13myc	*Cacas5::dpl200/Cacas5::dpl200 CaNRG1/CaNRG1-13myc-CaURA3-dpl200*	CCS4	This study

Western blotting analysis

To prepare total protein extraction, *C. albicans* cells were harvested from exponential phase cultures in YPD and re-suspended in 100 µl PBS plus 20 µl of 100% TCA. Cells were lysed using glass beads (0.2 mm) and bead-beating (5 × 2 min) with 2 min ice in between. One ml of 5% TCA was added to the cell lysates. Cell lysates were centrifuged at 13,000 rpm for 30 min at 4°C, washed with cold DW or 100% ethanol, and centrifuged at 13,000 rpm for 30 min at 4°C. The extracted proteins were resuspended in sodium dodecyl sulphate (SDS) sample buffer. After the samples were boiled for 5 min at 98°C, the supernatant was separated on an 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Separated proteins were transferred to polyvinylidene fluoride membranes and blocked with 5% skim milk in TBST for 1 h. Blotted membranes were probed with anti-GFP (1:500; Santa Cruz), anti-myc (1:1,000; Santa Cruz), or anti-PSTAIRE (1:20,000; Santa Cruz) overnight at 4°C. The blots were washed three times for 5 min each with TBST. A secondary antibody, anti-mouse antibody (1:10,000; Santa Cruz), or anti-rabbit antibody (1:5,000; Santa Cruz) diluted in the block solution was incubated for 1 h at room temperature. Blotted membranes were washed three times for 5 min with TBST. Signals were detected using an ECL western blotting kit (Invitrogen), and data were analyzed using the ChemiDoc Imaging System (Bio-Rad).

Analysis of sterols by spectrophotometer and high-performance liquid chromatography (HPLC)

For sterol profile analysis, yeast cell pellets were resuspended in 1 ml of 25% alcoholic potassium hydroxide solution (25 g of KOH and 35 ml of sterile distilled water, brought to 100 ml with 100% ethanol), and then incubated in an 85°C water bath for 1 h. Subsequently, the yeast suspension was added to 500 µl heptane (494526; Sigma-Aldrich) and mixed by vortexing for 1 min. After centrifugation, the supernatants were diluted with 100% ethanol and scanned spectrophotometrically between 240 and 300 nm with a UV-visible spectrophotometer (Thermo Fisher Scientific) (Arthington-Skaggs *et al.*, 1999). For HPLC analysis, the supernatants were dried by evaporation and resuspended in 100% acetone. Using a Cosmosil 5C18-PAQ column (4.6 × 250 mm; Nacalai Tesque), the samples were eluted at 25°C with 90% acetonitrile at a flow rate of 1 ml/min at 203 nm with a Waters 2690 HPLC system and detected by a UV detector.

Results

Candida albicans Cas5 is involved in ergosterol biosynthesis

*Candida albicans cas5*Δ/Δ cells are sensitive to the antifungal drug fluconazole (Vasicek *et al.*, 2014), which inhibits the activity of lanosterol 14 α-demethylase Erg11 (Ghannoum and Rice, 1999), and Cas5 was found to be involved in the expression of *ERG11* and *ERG26* (Vasicek *et al.*, 2014). To investigate the function of the *CAS5* gene in ergosterol biosynthesis, we constructed a *cas5*Δ/Δ mutant by sequentially deleting the two alleles of *CAS5* from the genome of *C. albicans* strain CAI4. The correct deletion of *CAS5* was verified by PCR using the primers listed in Supplementary data Table S1. We

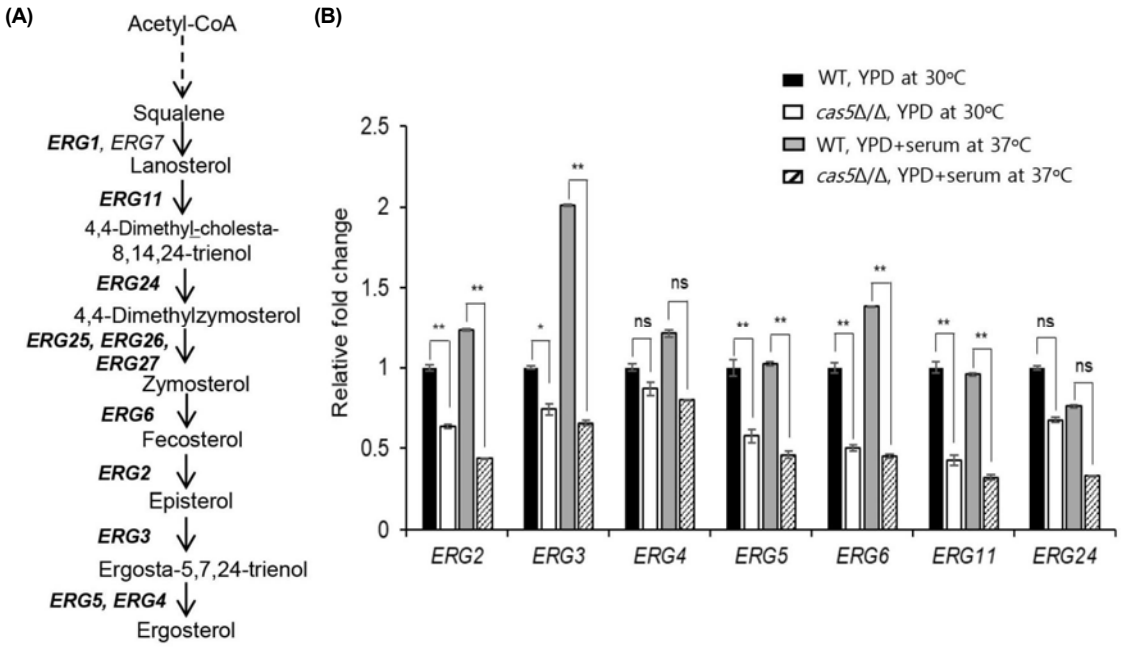

Fig. 1. Cas5 has a profound impact on the expression levels of the ergosterol biosynthesis genes. (A) Ergosterol biosynthesis pathway in *Candida albicans*. (B) Analysis of the expression levels of the ergosterol biosynthesis genes by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in the cells grown in the yeast extract peptone dextrose (YPD) medium at 30°C and in YPD plus 10% serum at 37°C to induce hypha formation. The levels of *ERG* transcripts were normalized by that of *ACT1* transcript and compared to those of *ERG* transcripts in the wild-type (WT) (CAI4) cultivated in YPD at 30°C, which were set to 1. All data are the average of three biologically independent experiments. Unpaired t-test and one-way ANOVA Dunnett's multiple comparisons test were used for statistical analysis. ns, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$; ****$P < 0.001$.

confirmed that the *cas5*Δ/Δ mutant showed increased susceptibility to fluconazole, as previously reported (Vasicek *et al.*, 2014), but was resistant to nystatin (Supplementary data Fig. S1), which causes membrane disruption and ultimately cell death by forming a complex with ergosterol (Mazu *et al.*, 2016). To test the effect of *CAS5* deletion on the expression of genes in the ergosterol biosynthesis pathway, the amount of the transcripts for genes involved in converting lanosterol to ergosterol (Fig. 1A), such as *ERG2*, *ERG3*, *ERG4*, *ERG5*, *ERG6*, *ERG11*, and *ERG24* (Bhattacharya *et al.*, 2020), was measured in wild-type (WT) and *cas5*Δ/Δ cells under yeast-form and hyphal-form growth conditions. The mRNA levels of the tested genes, except *ERG4*, were generally lower in the *cas5*Δ/Δ mutant than in the WT, and the difference in the expression level was much greater when cells were under hyphal-inducing conditions (Fig. 1B). These data suggest that Cas5 is involved in regulating the expression of ergosterol biosynthesis genes.

Next, we investigated whether *CAS5* deletion alters sterol content in *C. albicans* by analyzing total sterol profiles using absorption spectra and HPLC. Samples were prepared from exponentially growing cells under non-hypha-inducing conditions. In the UV spectral analysis (Fig. 2A), the WT strain showed a characteristic four-peak curve between 240 and 300 nm, which results from the presence of ergosterol in the extracted sample (Arthington-Skaggs *et al.*, 1999). In the *cas5*Δ/Δ mutant, the four-peak curve was less evident with lower height compared to the WT strain, indicating that the content of ergosterol is much lower in the *cas5*Δ/Δ mutant than the WT (Fig. 2A). In the sterol profile analysis using HPLC, it was revealed that the peaks corresponding to ergosterol intermediates, such as zymosterol and lanosterol, were increased while the peak for ergosterol was decreased in the *cas5*Δ/Δ mutant (Fig. 2B and C). These data indicate that a lack of Cas5 decreases the amount of ergosterol while increasing intermediate metabolites for ergosterol biosynthesis, which may lead to increased susceptibility to fluconazole in *C. albicans.*

Candida albicans Cas5 is required for maintenance of yeast form

Proper polarization of lipid rafts, which are sterol-and sphingolipid-enriched domains, is related to cell morphogenesis. Blocking ergosterol biosynthesis in *C. albicans* disrupts ergosterol polarization and causes abnormal hyphal morpho-

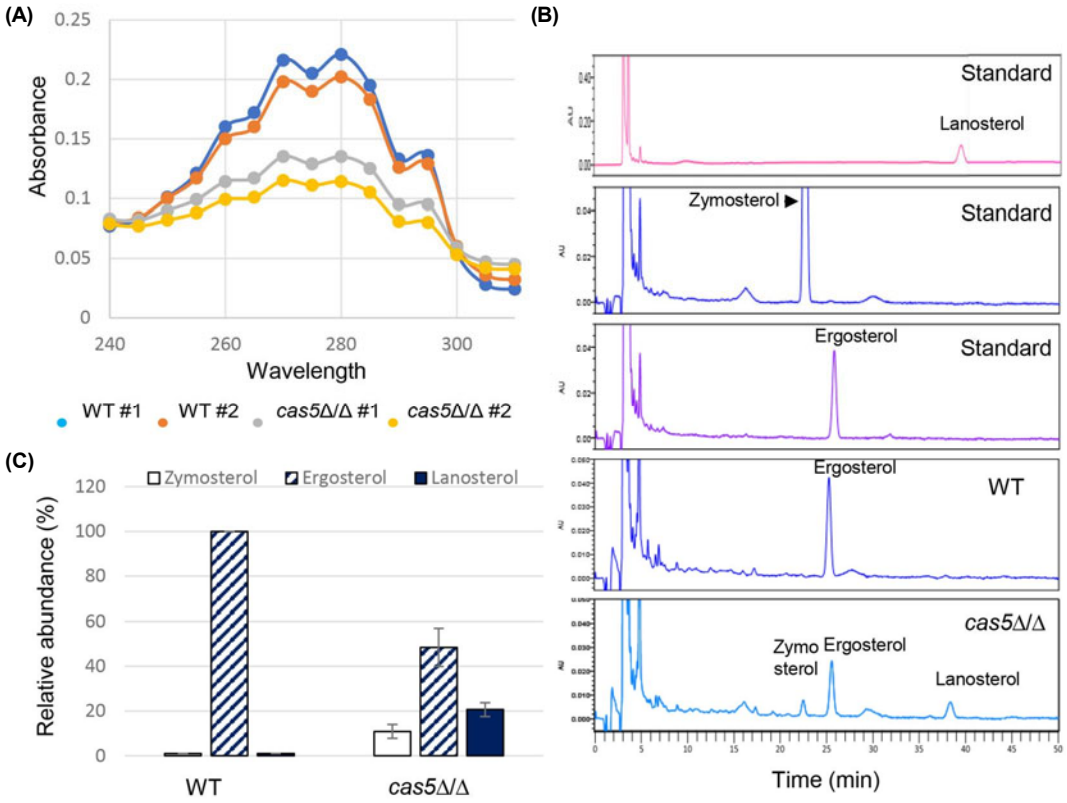

Fig. 2. Cas5 is involved in the biosynthesis of ergosterol. (A) The UV spectral analysis of total sterol profiles in the WT (blue and orange circles) and *cas5*Δ/Δ mutant (gray and yellow circles). (B) High-performance liquid chromatography (HPLC) analysis of sterol compounds in the WT and *cas5*Δ/Δ mutant. Standard sterol compounds; lanosterol, zymosterol, and ergosterol. WT, the cell extract from the WT strain; *cas5*Δ/Δ, the sample from the *cas5*Δ/Δ mutant strain. The representative profiles were shown among three independent experiments. Yeast cells, pre-cultivated in YPD at 30°C overnight, were inoculated and grown in YPD medium for 6 h at 30°C. The yeast cells in the exponential phase were harvested, and the cell pellets were extracted with KOH/EtOH and heptane solution. The obtained cell extracts were scanned spectrophotometrically between 240 and 310 nm or subjected to HPLC analysis. (C) Relative abundance of sterol compounds in the WT and *cas5*Δ/Δ mutant. The amount of individual sterols was calculated based on the area under each peak of the HPLC relative to a known amount of ergosterol, lanosterol, and zymosterol, which were used as standard. For each quantification, three independent culture aliquots were processed and the average ergosterol content of the WT stain was arbitrarily set to 100%.

genesis (Martin and Konopka, 2004). Thus, we were curious about whether the change in ergosterol biosynthesis observed in the *cas5*Δ/Δ mutant might affect hyphal morphogenesis. Interestingly, we found that *cas5*Δ/Δ cells appeared to have elongated and irregular morphologies under non-hypha-inducing conditions (Fig. 3A). The introduction of *CAS5* into the *cas5*Δ/Δ mutant reversed the irregular morphology back to the normal yeast form, indicating that the irregular, filamentous phenotype of *cas5*Δ/Δ was due to *CAS5* deletion (Fig. 3A). They seemed to be able to form hyphae under hypha-inducing conditions, although the width of the hyphae was not uniform (Fig. 3A). To closely observe the morphology, *cas5*Δ/Δ cells under non-hypha-inducing conditions were stained with Calcofluor White, which revealed that they were pseudohyphae, not true hyphae (Fig. 3B). The pseudohyphal form was maintained even when *cas5*Δ/Δ cells were grown to a cell density higher than 10^8 cells/ml (Fig. 3C), at which *C. albicans* cells usually grow in yeast form even in the presence of hypha-inducing serum (Kruppa, 2009). Furthermore, we tested the invasiveness of *cas5*Δ/Δ cells on solid medium because filamentous forms are generally associated with invasive growth. The *cas5*Δ/Δ mutant remained attached to the surface of the solid medium after washing, whereas WT cells were completely washed away (Fig. 3D).

Cas5 overexpression inhibits serum-induced hyphal morphogenesis of *C. albicans*

Because deletion of *CAS5* induced filamentous growth of *C. albicans* under non-hypha-inducing conditions, we reasoned that overexpression of *CAS5* would suppress hyphal morphogenesis under hypha-inducing conditions. To test this hypothesis, we constructed a *CAS5*-overexpressing strain by integrating *MET3p-CAS5-GFP* into the *CAS5* allele of *C. albicans*. The *MET3* promoter was induced in synthetic complete media lacking methionine and cysteine (SC-Met-Cys) (Care *et al.*, 1999), and Cas5 overexpression was confirmed by western blotting (Fig. 4A). The overexpressed Cas5-GFP fusion protein was mostly localized to the nucleus (Fig. 4B). However, since it was reported that Cas5 appeared diffuse throughout the cytoplasm (Xie *et al.*, 2017), we looked closely at the location of Cas5-GFP in more cells and found that the Cas5-GFP protein was also present in the cytoplasm in a subpopulation of cells (Supplementary data Fig. S2), suggesting that Cas5 may be localized in both the nucleus and cytoplasm. The Cas5 overexpressing cells (*MET3p-CAS5-GFP*) were oval in shape during yeast-form growth, but they were not perfectly separated (Supplementary data Fig. S2). Notably, the *MET3p-CAS5-GFP* cells could not form hyphae under serum-induced hyphal growth conditions (Fig. 4C), suggesting

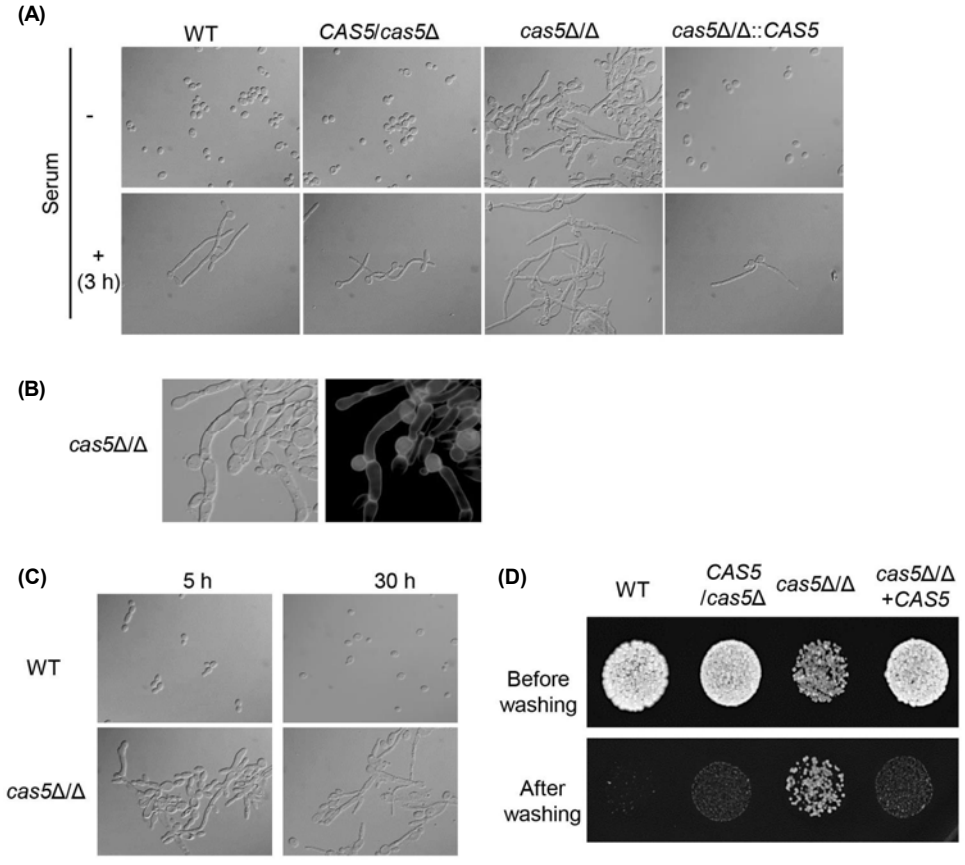

Fig. 3. Cas5 functions to maintain the yeast form under non-hypha-inducing conditions. (A) Cell morphologies of the WT (CAI4), *CAS5*/*cas5*Δ, *cas5*Δ/*cas5*Δ, *cas5*Δ/*cas5*Δ+*CAS5* strains. Cells were grown in YPD at 30°C or YPD plus 10% serum at 37°C for 3 h. (B) *cas5*Δ/Δ cells were stained with Calcofluor White to visualize the cell wall. (C) The pseudohyphal form of *cas5*Δ/Δ cells were maintained at high cell density (10^8 cells/ml). (D) Agar invasion test. The indicated strains were spotted onto YPD plate. Pictures were taken before and after gentle washing with water.

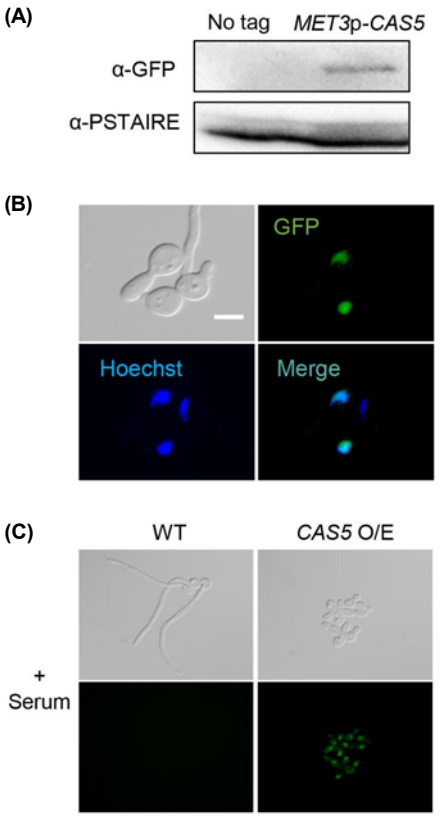

Fig. 4. Cas5 overexpression suppresses the serum-induced hyphal morphogenesis of *C. albicans*. (A) The GFP-fused Cas5 protein was overexpressed in the cells grown in the SC medium without Met and Cys at 30°C. Cdc28 was detected with α-PSTAIRE antibody and used as a loading control. (B) Overexpressed Cas5-GFP was localized in the nucleus. Scale bar, 5 μm. (C) Morphology of Cas5-overexpressing cells that were grown in SC medium plus 10% serum without Met and Cys at 37°C for 3 h.

that Cas5 overexpression blocked hyphal formation by repressing hypha-specific genes.

Cas5 represses hypha-specific genes during yeast-form growth in *C. albicans*

To investigate whether expression of hypha-specific genes is repressed by Cas5, we analyzed the expression of several hypha-specific genes, such as *HWP1* (encoding the hyphal wall protein 1), *ECE1* (coding for endothelin converting enzyme 1), and *ALS3* (encoding amyotrophic lateral sclerosis 3), whose expression levels are known to be very low during yeast-form growth but dramatically rise during hyphal growth (Kadosh and Johnson, 2005; Sudbery, 2011). The mRNA levels of the genes were significantly increased in the *cas5*Δ/Δ mutant compared to the wild-type under non-hypha-inducing conditions (Fig. 5A). To confirm that the hypha-specific genes were controlled by Cas5, GFP-tagged Hwp1 was expressed in the *cas5*Δ/Δ and WT strains. The Hwp1-GFP protein was detected only in *cas5*Δ/Δ, but not in the WT, under yeast-form growth conditions (Fig. 5B and C). Thus, the results lead us to conclude that Cas5 plays an important role in repressing hypha-specific genes during yeast-form growth.

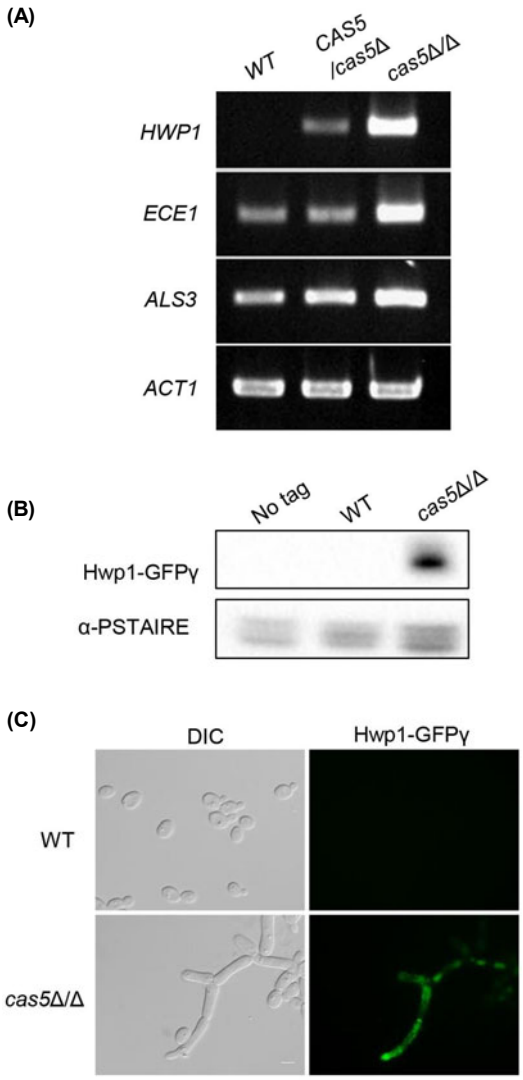

Fig. 5. Cas5 represses the hypha-specific genes during yeast-form growth. (A) qRT-PCR analysis of hypha-specific genes, *HWP1*, *ECE1*, and *ALS3*. mRNAs were extracted from the WT and *cas5*Δ/Δ strains cultivated in YPD at 30°C. *ACT1* was used as an internal control. (B and C) Expression of the Hwp1-GFP protein was detected only in the *cas5*Δ/Δ mutant. Western blotting (B) and fluorescence microscopy (C). Cells were grown in YPD at 30°C overnight and inoculated into fresh YPD at 30°C for 6 h. Scale bar, 5 μm. Cdc28 was detected with α-PSTAIRE antibody and used as a loading control in western blotting analysis.

Expression of Cas5 decreases with the initiation of hyphal growth

If Cas5 functions as a repressor of hyphal morphogenesis, its expression is expected to decrease during hyphal initiation. Indeed, the mRNA level of *CAS5* rapidly decreased immediately after the initiation of hyphal growth induced by serum and then increased gradually with time (Fig. 6A). Furthermore, we confirmed that the amount of Cas5 protein decreased upon hyphal induction by serum, in good agreement with the mRNA levels (Fig. 6B). It has been reported that Nrg1, the major transcriptional repressor of hyphal development, is rapidly downregulated through reduced expression and in-

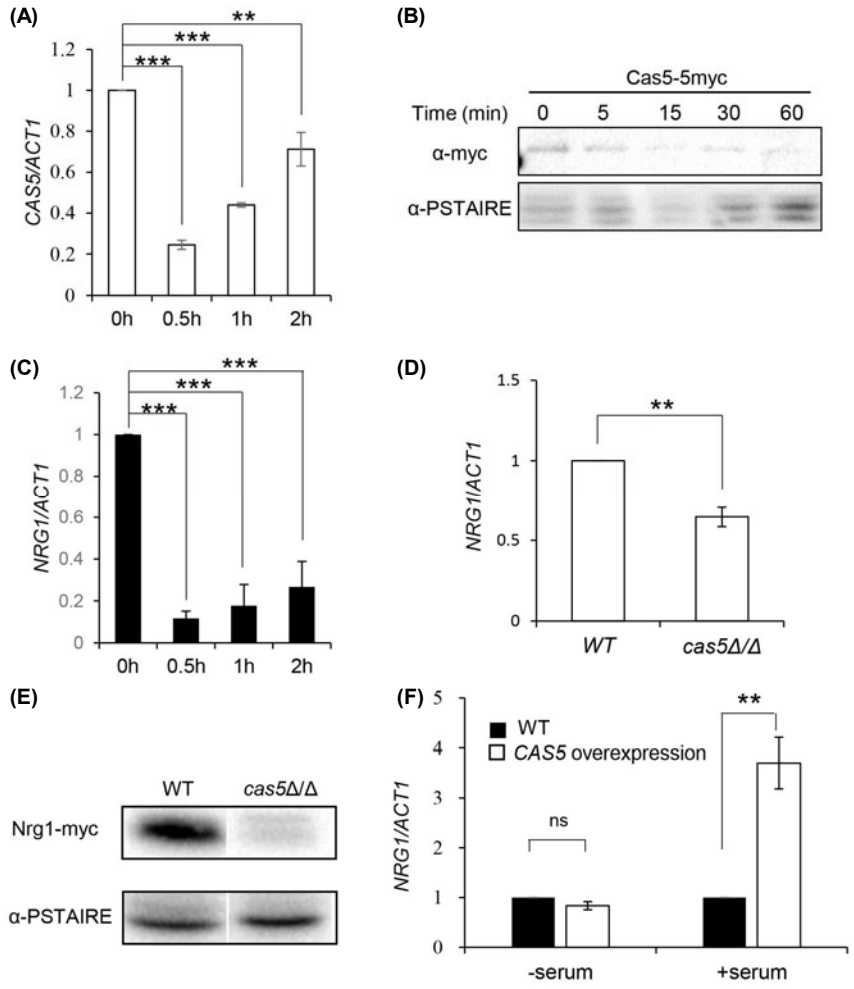

Fig. 6. Expression of Cas5 decreases upon initiation of the hyphal growth. (A and B) *CAS5* mRNA level (A) and myc-tagged Cas5 protein level (B) were measured by qRT-PCR and western blotting, respectively. (C) *NRG1* mRNA level upon hyphal growth initiation in the WT. (D and E) Comparison of *NRG1* mRNA level (D) and myc-tagged Nrg1 protein (E) in the WT and *cas5*Δ/Δ mutant. Cells were grown in YPD plus 10% serum at 37°C for 3 h. Cdc28 was detected with α-PSTAIRE antibody and used as a loading control in western blotting analysis. (F) Cas5 overexpression increased the *NRG1* expression during hyphal growth. All data are the average of three independent experiments. Unpaired t-test and one-way ANOVA Dunnett's multiple comparisons test were used for statistical analysis. ns, not significant; $^{**}P < 0.01$.

creased degradation during hyphal initiation (Braun *et al.*, 2001; Lu *et al.*, 2011, 2014). Because Cas5 and Nrg1 are not only similar in their expression patterns during hyphal initiation (Fig. 6A and C), but also share downstream targets, *HWP1*, *ECE1*, and *ALS3* (Braun *et al.*, 2001; Murad *et al.*, 2001), we speculated that Cas5 and Nrg1 may be genetically related. It was found that *NRG1* transcript levels were lower in the *cas5*Δ/Δ mutant and Nrg1 protein levels were also significantly decreased in the *cas5*Δ/Δ mutant compared to the WT when measured in cells cultured under yeast-form growth conditions (Figs. 5E and 6D). This indicates that the expression of Nrg1, the repressor of hyphal growth in *C. albicans*, was positively correlated with Cas5. We then examined the effect of *CAS5* overexpression on *NRG1* expression. It is noticeable that Cas5 overexpression increased the amount of *NRG1* mRNA by nearly fourfold in cells under hypha-inducing conditions (Fig. 6F). Taken together, these data suggest the possibility of a close relationship between Nrg1 and Cas5 in terms of hyphal morphogenesis of *C. albicans*: Cas5 may be involved in the regulation of *NRG1* transcription, resulting in the repression of hypha-specific genes.

Discussion

Candida albicans Cas5 is a transcription factor that controls the response to environmental stresses, including sensitivity to cell wall stresses induced by caspofungin, hypersensitivity to the antifungal drug fluconazole, and weak virulence in animal infection models (Bruno *et al.*, 2006; Chamilos *et al.*, 2009; Vasicek *et al.*, 2014; Xie *et al.*, 2017; Xiong *et al.*, 2021). In addition, the lack of Cas5 generates altered expression patterns in cell cycle-regulated genes and multinucleated cells (Xie *et al.*, 2017), implying that Cas5 may serve to link environmental stress signals with the cell cycle and division. In this study, we provide a set of data supporting additional functions of Cas5 associated with the regulation of the expression of ergosterol biosynthesis genes and with the repression of hypha-specific genes under non-hypha-inducing conditions.

In yeast, pharmacological or genetic impairment of protein kinase C (Pkc1), a key kinase in the cell wall integrity pathway, renders fungistatic inhibitors of ergosterol biosynthesis, such as fluconazole, fenpropimorph, and terbinafine, fungicidal, and results in attenuated virulence of *C. albicans* in a murine model of systemic infection (LaFayette *et al.*, 2010). The study suggested that the stress response networks con-

tribute to the weakening of drug toxicities without directly blocking the harmful effects of the drug on the cell. Moreover, basal tolerance of *C. albicans* to antifungal drugs can be neutralized by disrupting the capability to defend itself against environmental stresses. Since Cas5 governs the expression of numerous cell wall integrity genes, Cas5 might play a role in the tolerance of *C. albicans* to caspofungin targeting the cell wall (Bruno *et al.*, 2006; Vasicek *et al.*, 2014). In addition, *C. albicans* strains lacking Cas5 are hypersensitive to fluconazole, implying that Cas5 may also help in coping with cell membrane stresses induced by the azole antifungal drug blocking ergosterol biosynthesis (Vasicek *et al.*, 2014). Our data in the present study show that the *cas5*Δ/Δ mutant expresses a set of ergosterol biosynthetic genes at much lower levels than the WT does and accumulates a noticeable amount of lanosterol and zymosterol, intermediate metabolites in the ergosterol biosynthesis pathways (Fig. 2). The mechanism by which Cas5 regulates ergosterol biosynthesis gene expression remains to be answered.

To the best of our knowledge, this is the first study to report that Cas5 maintains yeast-form growth of *C. albicans* by suppressing the expression of hypha-specific genes under non-hypha-inducing conditions. This suggests that Cas5 needs to be mostly in the nucleus during yeast-form growth, even in the absence of environmental stress, as indicated by the Cas5-GFP overexpression in Fig. 4B. A recent study showed that Cas5 governs diverse biological responses under basal conditions, as revealed by chromatin immunoprecipitation of RNA polymerase II coupled with sequencing (Xie *et al.*, 2017). If inferences are made based on these results, Cas5 should be present in the nucleus. However, it was also reported that Cas5 appears to be largely localized to the cytoplasm under basal conditions and translocated to the nucleus if *C. albicans* cells are under physiological and environmental stresses. Cas5 is dephosphorylated by the phosphatase Glc7 in the cytoplasm upon caspofungin treatment before translocation into the nucleus (Xie *et al.*, 2017). Taken together, these results suggest that Cas5 might be present in both the cytoplasm and the nucleus during yeast-form growth under basal conditions. Thus, it would be interesting to investigate how the localization of Cas5 is determined, which kinase phosphorylates Cas5, and how this phosphorylation is involved in the localization and function of Cas5 under basal and stress conditions.

The level of Cas5 decreased upon initiation of hyphal growth (Fig. 6A and B), which is consistent with the role of Cas5 in repressing hypha-specific genes (Fig. 5). Moreover, we found that both the transcription and the protein levels of the transcriptional repressor, Nrg1, were significantly decreased in the *cas5*Δ/Δ mutant (Fig. 6D and E). As Nrg1 suppresses the hyphal growth in *C. albicans* (Murad *et al.*, 2001) and its expression is downregulated under hypha-inducing conditions (Braun *et al.*, 2001), we speculate that Cas5 may be involved in the regulation of the expression of *NRG1* during yeast growth to repress hypha-specific genes. In a previous study, the ChIP-seq analysis of RNA polymerase II (Pol II) indicated a significant reduction of Pol II occupancy in the promoter regions of *ERG3* and *NRG1* in the *cas5*Δ/Δ mutant strain compared to the WT strain (Xie *et al.*, 2017). This strongly implies that CaCas5 can directly control the expression of the *ERG* genes and *NRG1* gene at transcription levels. It will be intriguing future studies to explore the Cas5-medited regulatory network of the *ERG* genes and *NRG1* to elucidate how Cas5 modulates ergosterol biosynthesis positively and hyphal morphogenesis negatively in *C. albicans*.

Acknowledgements

This research was supported by the National Research Foundation of Korea, Grant No. NRF 2020R1A2C101025412 to J.-Y. Kim and Grant No. NRF2018R1A5A1025077 (Advanced Research Center Program) to H.A. Kang.

Conflict of Interest

The authors have no conflict of interest to report.

References

Arthington-Skaggs, B.A., Jradi, H., Desai, T., and Morrison, C.J. 1999. Quantitation of ergosterol content: novel method for determination of fluconazole susceptibility of *Candida albicans*. *J. Clin. Microbiol.* **37**, 3332–3337.

Bhattacharya, S., Sae-Tia, S., and Fries, B.C. 2020. *Candidiasis* and mechanisms of antifungal resistance. *Antibiotics* **9**, 312.

Braun, B.R., Kadosh, D., and Johnson, A.D. 2001. *NRG1*, a repressor of filamentous growth in *C. albicans*, is down-regulated during filament induction. *EMBO J.* **20**, 4753–4761.

Bruno, V.M., Kalachikov, S., Subaran, R., Nobile, C.J., Kyratsous, C., and Mitchell, A.P. 2006. Control of the *C. albicans* cell wall damage response by transcriptional regulator Cas5. *PLoS Pathog.* **2**, e21.

Calderone, R.A. and Fonzi, W.A. 2001. Virulence factors of *Candida albicans*. *Trends Microbiol.* **9**, 327–335.

Care, R.S., Trevethick, J., Binley, K.M., and Sudbery, P.E. 1999. The *MET3* promoter: a new tool for *Candida albicans* molecular genetics. *Mol. Microbiol.* **34**, 792–798.

Chamilos, G., Nobile, C.J., Bruno, V.M., Lewis, R.E., Mitchell, A.P., and Kontoyiannis, D.P. 2009. *Candida albicans* Cas5, a regulator of cell wall integrity, is required for virulence in murine and *Toll* mutant fly models. *J. Infect. Dis.* **200**, 152–157.

Fonzi, W.A. and Irwin, M.Y. 1993. Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics* **134**, 717–728.

Ghannoum, M.A. and Rice, L.B. 1999. Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clin. Microbiol. Rev.* **12**, 501–517.

Gow, N.A.R., Brown, A.J.P., and Odds, F.C. 2002. Fungal morphogenesis and host invasion. *Curr. Opin. Microbiol.* **5**, 366–371.

Gow, N.A., van de Veerdonk, F.L., Brown, A.J., and Netea, M.G. 2012. *Candida albicans* morphogenesis and host defence: discriminating invasion from colonization. *Nat. Rev. Microbiol.* **10**, 112–122.

Kadosh, D. and Johnson, A.D. 2005. Induction of the *Candida albicans* filamentous growth program by relief of transcriptional repression: a genome-wide analysis. *Mol. Biol. Cell.* **16**, 2903–2912.

Kim, J.Y. 2016. Human fungal pathogens: why should we learn? *J. Microbiol.* **54**, 145–148.

Kruppa, M. 2009. Quorum sensing and *Candida albicans*. *Mycoses* **52**, 1–10.

LaFayette, S.L., Collins, C., Zaas, A.K., Schell, W.A., Betancourt-Quiroz, M., Gunatilaka, A.A.L., Perfect, J.R., and Cowen, L.E. 2010. PKC signaling regulates drug resistance of the fungal patho-

gen *Candida albicans* via circuitry comprised of Mkc1, calcineurin, and Hsp90. *PLoS Pathog.* **6**, e1001069.

Lee, H.J., Kim, J.M., Kang, W.K., Yang, H., and Kim, J.Y. 2015. The NDR kinase Cbk1 downregulates the transcriptional repressor Nrg1 through the mRNA-binding protein Ssd1 in *Candida albicans*. *Eukaryot. Cell* **14**, 671–683.

Lu, Y., Su, C., Unoje, O., and Liu, H. 2014. Quorum sensing controls hyphal initiation in *Candida albicans* through Ubr1-mediated protein degradation. *Proc. Natl. Acad. Sci. USA* **111**, 1975–1980.

Lu, Y., Su, C., Wang, A., and Liu, H. 2011. Hyphal development in *Candida albicans* requires two temporally linked changes in promoter chromatin for initiation and maintenance. *PLoS Biol.* **9**, e1001105.

Martin, S.W. and Konopka, J.B. 2004. Lipid raft polarization contributes to hyphal growth in *Candida albicans*. *Eukaryot. Cell* **3**, 675–684.

Mazu, T.K., Bricker, B.A., Flores-Rozas, H., and Ablordeppey, S.Y. 2016. The mechanistic targets of antifungal agents: an overview. *Mini Rev. Med. Chem.* **16**, 555–578.

Murad, A.M., Leng, P., Straffon, M., Wishart, J., Macaskill, S., MacCallum, D., Schnell, N., Talibi, D., Marechal, D., Tekaia, F., *et al.* 2001. *NRG1* represses yeast-hypha morphogenesis and hypha-specific gene expression in *Candida albicans*. *EMBO J.* **20**, 4742–4752.

Pukkila-Worley, R., Peleg, A.Y., Tampakakis, E., and Mylonakis, E. 2009. *Candida albicans* hyphal formation and virulence assessed using a *Caenorhabditis elegans* infection model. *Eukaryot. Cell* **8**, 1750–1758.

Sudbery, P.E. 2011. Growth of *Candida albicans* hyphae. *Nat. Rev. Microbiol.* **9**, 737–748.

Vasicek, E.M., Berkow, E.L., Bruno, V.M., Mitchell, A.P., Wiederhold, N.P., Barker, K.S., and Rogers, P.D. 2014. Disruption of the transcriptional regulator Cas5 results in enhanced killing of *Candida albicans* by Fluconazole. *Antimicrob. Agents Chemother.* **58**, 6807–6818.

Walther, A. and Wendland, J. 2003. An improved transformation protocol for the human fungal pathogen *Candida albicans*. *Curr. Genet.* **42**, 339–343.

Xie, J.L., Qin, L., Miao, Z., Grys, B.T., Diaz, J.C., Ting, K., Krieger, J.R., Tong, J., Tan, K., Leach, M.D., *et al.* 2017. The *Candida albicans* transcription factor Cas5 couples stress responses, drug resistance and cell cycle regulation. *Nat. Commun.* **8**, 499.

Xiong, K., Su, C., Sun, Q., and Lu, Y. 2021. Efg1 and Cas5 orchestrate cell wall damage response to caspofungin in *Candida albicans*. *Antimicrob. Agents Chemother.* **65**, e01584-20.