Genetic Analysis of Sirtuin Deacetylases in Hyphal Growth of Candida albicans

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ABSTRACT Candida albicans is a major human fungal pathogen that encounters varied host environments during infection. In response to environmental cues, C. albicans switches between ovoid yeast and elongated hyphal growth forms, and this morphological plasticity contributes to virulence. Environmental changes that alter the cell’s metabolic state could be sensed by sirtuins, which are NAD⁺-dependent deacetylases. Here, we studied the roles of three sirtuin deacetylases—Sir2, Hst1, and Hst2—in the hyphal growth of C. albicans. We made single, double, and triple sirtuin knockout strains and tested their ability to switch from yeast to hyphae. We found that true hypha formation was significantly reduced by the deletion of SIR2 but not HST1 or HST2. Moreover, the expression of hypha-specific genes HWP1, ALS3, and ECE1 decreased in the sir2Δ/Δ mutant compared to the wild type. This regulation of hypha formation was likely dependent on the deacetylase activity of Sir2, as a similar defect in hypha formation was observed when an asparagine known to be required for deacetylation was mutated. Finally, we found that Sir2 and Hst1 were localized to the nucleus, with Sir2 specifically focused in the nucleolus. This nuclear localization suggests a role for Sir2 and Hst1 in regulating gene expression. In contrast, Hst2 was localized to the cytoplasm. In conclusion, our results suggest that Sir2 plays a critical and nonredundant role in hyphal growth of C. albicans.

IMPORTANCE Candida albicans is one of the most common causes of hospital-acquired systemic fungal infections in the United States. It can switch between ovoid yeast and elongated hyphal growth forms in response to environmental cues. This morphological transition is essential for its survival in the host. Thus, identifying regulators involved in this process can lead to new therapies. In this study, we examined the contribution of three regulators called sirtuins (Sir2, Hst1, and Hst2) to the yeast-to-hypha transition of C. albicans. We found that loss of Sir2 but not Hst1 or Hst2 hampered hypha formation. Moreover, the defect was caused by the loss of the catalytic activity of Sir2. Our study may lay the groundwork for discovering novel targets for antifungal therapies.

KEYWORDS Candida albicans, sirtuin, filamentous growth, nucleolar localization
important for pathogenicity, since the hyphal form enables penetration into host organs, whereas the yeast form is adapted to dissemination through the bloodstream. Mutants that are defective in this morphological transition are attenuated in virulence in animal models of systemic infections (6–8). The morphological transition from yeast to hyphae is accompanied by the expression of many virulence factors. For example, adhesins, such as Als3 and Hwp1, mediate adherence of C. albicans to host cell surfaces (9, 10). In addition, secreted hydrolases, such as secreted aspartic proteases, facilitate active penetration into host cells (11). Moreover, the first identified cytolytic peptide toxin in C. albicans, Ece1, is highly expressed by hyphae and damages epithelial membranes (12, 13).

Other factors that assist in the transition from yeast to hyphae are transcription regulators, including deacetylases. For example, binding of the deacetylase Hda1 to the promoters of hypha-specific genes is required for hyphal maintenance by blocking the repressor, Nrg1 (14). In addition, the Set3/Hos2 deacetylase complex regulates morphogenesis of C. albicans by adjusting transcriptional kinetics of key morphogenic regulators (15). Finally, the deacetylase Rpd31 promotes hyphal extension, in part by activating transcription of the positive regulator UME6 (16). These deacetylases are all classical deacetylases that deacetylate lysines using water to hydrolyze the acetyl group (17).

Sirtuins belong to a different family of deacetylases that couple lysine deacetylation to cleavage of NAD⁺, a coenzyme that is central to metabolism in all living cells. Sirtuins are found in organisms ranging from bacteria to humans, regulating numerous cellular processes, including transcription, metabolism, and cell aging (18–20). In pathogenic fungi, sirtuin deacetylases impact virulence. For example, in another human fungal pathogen Candida glabrata, the sirtuin Hst1 regulates genes required for antifungal resistance and oxidative stress response (21). In addition, the sirtuin Sir2 represses genes encoding adhesins, whose expression in low NAD⁺ promotes urinary tract infections (22). In C. albicans, there are five sirtuin-coding genes—SIR2, HST1, HST2, HST3, and orf19.2963—with similarity to Homo sapiens SIRT5. C. albicans Hst3, which deacetylates H3K56, is essential for normal hyphal growth, chromatin structure maintenance, and cell viability (23). Sir2 maintains the hypoacetylated state of the transcriptionally silent heterochromatin assembled at ribosomal DNA (rDNA) and telomeres (24–26). Sir2 may also affect phenotypic switching (27) and regulate the replicative life span of C. albicans in a dose-dependent manner, such that cells with more SIR2 have extended lifespans (28). The role of C. albicans sirtuins has also been examined in the switch between two types of yeast cells called white and opaque. White cells are unable to mate, whereas opaque cells are mating competent (29). C. albicans lacking HST1 had a reduced frequency of switching from the opaque to the white state, whereas cells lacking HST2 had decreased switching from white to opaque (30). However, the functions of Sir2, Hst1, and Hst2 have not been well studied in the yeast-to-hypha morphological transition, an important virulence trait of C. albicans.

To study the roles of Sir2, Hst1, and Hst2 in the formation of hyphae, we made single-, double-, and triple-knockout strains using CRISPR (31) and compared the abilities of wild-type and knockout strains to form hyphae in two types of liquid media. In both yeast extract-peptone-dextrose (YPD) and spider media, hypha formation was significantly reduced in a sir2 single mutant strain, but it was not affected by the deletion of HST1, HST2, or both. Double- and triple-deletion strains that lacked SIR2 behaved similarly to the sir2 single deletion, suggesting no redundant roles among the three sirtuins in hypha formation under the conditions tested. Moreover, the expression of the hypha-specific genes HWP1, ALS3, and ECE1 decreased in the sir2 mutant compared to the wild type. Furthermore, we found that the defect in hypha formation was likely due to the loss of Sir2 deacetylation activity, since it was disrupted by a point mutation of an amino acid required for catalysis. Finally, we found that Sir2 was localized to the nucleolus and Hst1 was localized to the whole nucleus, suggesting a role in regulating gene expression. In contrast, Hst2 was localized to the cytoplasm. In conclusion, our
results suggest that Sir2 is important for hypha formation and thus for the virulence of *C. albicans*.

**RESULTS**

**Sir2 is required for maximal true hypha formation.** To investigate the function of the three sirtuins, Sir2, Hst1, and Hst2, in the morphological transition of *C. albicans*, we used a recyclable CRISPR-Cas9 system (31) to construct mutant strains that included homozygous deletions of each sirtuin and all possible double and triple deletions. In addition, addback strains were made by incorporating the wild-type or epitope-tagged gene sequences into the genome of mutant strains at the endogenous loci. First, we tested whether the three sirtuins play roles in yeast-to-hypha transition triggered by elevating the temperature to 37°C (32). For this liquid filamentation assay, we inoculated *C. albicans* yeast-form cells grown overnight at 30°C into fresh YPD at 37°C and checked hypha formation microscopically after 3 h. The cell morphologies were divided into three categories as previously described (33): yeast, pseudohyphae, and true hyphae (Fig. 1A and B). We found that cells lacking Sir2 showed 1.5 to 2 times more pseudohyphae and half as many true hyphae compared to the wild type (Fig. 1C). This trend was also observed for the double and triple mutants in which *SIR2* was deleted (Fig. 1F). The percentages of yeast cells in all mutant strains were similar to that of the wild type, which is 20 to 30%. The percentages of true hyphae and pseudohyphae
were restored to the wild-type levels by adding back SIR2 at its original locus with either a Myc or green fluorescent protein (GFP) tag (Fig. 1C), indicating that the defect was due to loss of SIR2. In contrast to SIR2, deletion of HST1 or HST2 did not affect yeast-to-hypha transition under these conditions (Fig. 1D and E). Our results suggest that Sir2 is required for maximal true hypha formation when C. albicans cells are transferred from an overnight culture at 30°C into fresh YPD at 37°C.

Although hyphae can be induced solely by elevating the temperature in media with the preferred carbon source, glucose, many widely used hypha-inducing media have poor carbon sources, such as mannitol in spider medium. To determine whether Sir2, Hst1, and Hst2 function in the morphological transition of C. albicans in media with a poor carbon source, we performed the liquid filamentation assay in spider medium at 37°C with the same mutant strains (Fig. 2). Similar to the results observed in YPD at 37°C, single, double, and triple mutants in which SIR2 was deleted had half as many true hyphae compared to the wild type (Fig. 2A and D). In contrast to YPD, we found that in spider medium the strains lacking Sir2 had 1.5 to 2 times more yeast cells compared to the wild type (Fig. 2A and D), whereas the percentages of pseudohyphae in all strains were similar, which is around 20% (Fig. 2A and D). Adding back SIR2 restored the percentages of true hyphae and yeast cells to the wild-type level (Fig. 2A). Comparable to the results observed in YPD at 37°C, the deletion of HST1 and HST2 did not affect yeast-to-hypha transition in spider medium at 37°C (Fig. 2B and C). Our results suggest that Sir2 is required for C. albicans yeast-to-hypha transition at 37°C in spider medium, which contains a nonfavorable carbon source.

Expression of hypha-specific genes was affected by Sir2 deletion. C. albicans hypha formation is associated with the expression of hypha-specific genes (34, 35). HWP1, ECE1, and ALS3 encode virulence factors and are highly expressed specifically in hyphal cells. As sir2Δ/Δ mutant had reduced true hypha formation, we tested whether the expression of hyphal-specific genes was affected. We analyzed the mRNA levels of HWP1, ECE1, and ALS3 in the wild-type, sir2Δ/Δ, and the SIR2 addback strains in spider medium at 37°C using reverse transcriptase followed by quantitative PCR (Fig. 3). In
the absence of SIR2, cells expressed less HWP1, with a 2- to 3-fold difference between the wild-type and sir2Δ/Δ strains, consistent with a defective hyphal program (Fig. 3A). In addition, adding back SIR2 with either a Myc or GFP tag restored HWP1 expression to wild-type levels (Fig. 3A). Moreover, qualitatively identical results were obtained with ECE1 (Fig. 3B) and ALS3 (Fig. 3C). We performed a similar experiment on cells grown in YPD at 37°C, and we also detected decreased expression of HWP1, ECE1, and ALS3 (see Fig. S1A to C in the supplemental material).

As expression of hypha-specific genes is repressed by Nrg1, Tup1, and Rfg1 (34, 36–39), it was possible that increased expression of these factors in sir2Δ/Δ strains inhibits filamentation. We therefore tested the expression of NRG1, TUP1, and RFG1 in the same samples analyzed for hypha-specific gene expression. However, their expression did not change in the sir2Δ/Δ strain compared to the wild-type upon hyphal induction in spider medium (Fig. 3D to F) or YPD (see Fig. S1D to F). Taken together, our results suggest that Sir2 is required for yeast-to-hypha transition of C. albicans at both the cellular and the expression level.

The defect in true hypha formation of sir2Δ/Δ cells is due to the loss of deacetylase activity. Sir2 and its homologous proteins are deacetylases that act on both histone and nonhistone substrates (40–43). To determine whether the deacetylase activity is required for hypha formation in C. albicans, we first identified a conserved asparagine residue that is required for deacetylase activity in Saccharomyces cerevisiae (44, 45). Based on an alignment of C. albicans Sir2 (CaSir2) with its homologs from S. cerevisiae (ScSir2) and Kluyveromyces lactis (KISir2) (Fig. 4A) (46), we replaced the conserved asparagine residue (N329) in C. albicans Sir2 with alanine. Mutation of

**FIG 3** Expression of hypha-specific genes was reduced in Sir2 deletion strain. mRNA levels of the hypha-specific genes HWP1 (A), ECE1 (B), and ALS3 (C) and the negative regulators of hyphal growth NRG1 (D), TUP1 (E), and RFG1 (F) were measured by quantitative RT-PCR before (0 h-YPD, 30°C) and after (3 h-spider medium, 37°C) hypha induction. The fold induction was normalized to the wild type at time zero. The data represent the average of four independent replicates with error bars representing SEM. Asterisks show statistically significant differences (***, P < 0.001; ****, P < 0.0001) based on two-way analysis of variance (ANOVA) with multiple comparisons.
the equivalent asparagine in ScSir2 disrupts its deacetylase activity (44). To generate the catalytic mutant, the wild-type Sir2 with a Myc tag was first reintroduced into the endogenous locus in the sir2Δ/Δ strain. Then, the N329A point mutation was introduced into the SIR2-MYC gene through CRISPR. The expression of Myc-tagged Sir2 and Sir2-N329A was similar as detected by immunoblotting (Fig. 4B). Compared to the reintroduced wild-type Sir2-Myc, which fully recapitulated the yeast-to-hypha transition in YPD and spider medium at 37°C (Fig. 1C and 2A), the SIR2-N329A point mutation resulted in fewer true hyphae in both media (Fig. 4C and D). The effect of this mutation is similar to that of the sir2Δ/Δ mutation. Further analysis of the expression of hypha-specific genes revealed that while the addback strain with wild-type Sir2 fully restored their expression (Fig. 3), strains expressing the Sir2-N329A point mutation displayed the same defective induction of hypha-specific genes as that in the sir2Δ/Δ strain.
Altogether, these data suggest that the defect in true hypha formation of \textit{sir2}\textsubscript{D}/\textit{D} is due to the loss of its deacetylase activity.

**Subcellular localization of Sir2, Hst1, and Hst2.** To narrow down the likely deacetylation targets of Sir2, Hst1, and Hst2, we investigated their subcellular distribution. To this end, we integrated \textit{SIR2-GFP}, \textit{HST1-GFP}, and \textit{HST2-GFP} into the genomes of \textit{sir2}\textsubscript{D}/\textit{D}, \textit{hst1}\textsubscript{D}/\textit{D}, and \textit{hst2}\textsubscript{D}/\textit{D} strains at their endogenous loci. First, we confirmed the expression of the GFP-tagged sirtuins by immunoblotting (Fig. 5A). All three sirtuins were successfully expressed under their native promoters. Furthermore, the GFP fusion did not interfere with the function of Sir2, since the \textit{SIR2-GFP} addback strain restored true hypha formation and hyphal gene expression to the wild-type level (Fig. 1C, 2A, and 3).

Next, we examined the subcellular localization of the three sirtuins in both yeast cells and hyphae using fluorescence microscopy (Fig. 5B). The wild-type strain with no GFP integration was used as a negative control for background fluorescence. For Sir2-GFP, the fluorescence was concentrated in a discrete part of the nucleus both in yeast cells and hyphae. In contrast to Sir2-GFP, Hst1-GFP was distributed throughout the nucleus in both yeast and hyphal cells. Finally, Hst2-GFP was found throughout the cell, in some cases appearing to be excluded from the nucleus. Thus, the three sirtuins have distinct subcellular localizations.

In \textit{S. cerevisiae}, Sir2 is concentrated in the nucleolus, where it regulates recombination between the rDNA repeats (47). A similar role is expected for CaSir2, which deacetylates histones within the rDNA array (25, 26). To determine whether the subnuclear focus we observed for Sir2-GFP coincides with the nucleolus, we tagged the nucleolar protein Nop1 with red fluorescent protein (RFP) (Fig. 6). The wild-type strain with no GFP integration was used as a negative control for background fluorescence. For Sir2-GFP, the fluorescence was concentrated in a discrete part of the nucleus both in yeast cells and hyphae. In contrast to Sir2-GFP, Hst1-GFP was distributed throughout the nucleus in both yeast and hyphal cells. Finally, Hst2-GFP was found throughout the cell, in some cases appearing to be excluded from the nucleus. Thus, the three sirtuins have distinct subcellular localizations.
nuclear proteins, whereas Hst2 is a cytoplasmic protein. These localization patterns are consistent in yeast and hyphal cells. These different localization patterns indicate distinct functions for Sir2, Hst1, and Hst2 in *C. albicans*, which may explain their different roles in filamentation.

**DISCUSSION**

In this study, we explored the roles of three sirtuins, Sir2, Hst1, and Hst2, in the hyphal induction of *C. albicans*. We made single, double, and triple mutants of the sirtuins and tested their ability to form hyphae. Our liquid filamentation assay indicated the importance of Sir2, but not Hst1 or Hst2, in hypha formation at 37°C. Consistent with the defect in hyphal formation in the *sir2* mutant, the expression of hypha-specific genes decreased in the absence of *SIR2*. Moreover, the defect in hypha formation in the *sir2* mutant resulted from the loss of the deacetylase activity of Sir2. We found that the contribution of Sir2 to hyphal growth was not achieved through regulating expression of the negative regulators of hyphal growth. Our study of morphological transitions is relevant to the virulence of *C. albicans*, since the yeast-to-hypha transition in liquid media would mimic filamentation of *C. albicans* in bodily fluids or within phagocytic cells. Finally, we recorded the subcellular localization of the three sirtuins. Sir2 is mainly localized to the nucleolus, as was previously observed in *S. cerevisiae* (47, 48). This finding is consistent with its role in regulating the rDNA tandem repeats (25, 26). However, given the silencing function of *C. albicans* Sir2 at subtelomeres (24–26), Sir2 may also be present at lower, less detectable levels in the rest of the nucleus and regulate factors involved in filamentation. We also observed the nuclear localization of Hst1 and the cytoplasmic localization of Hst2. The presence of Sir2 and Hst1 in the nucleus suggests a role in regulating gene expression.

Our results differ from those presented previously (27), which showed that the deletion of *SIR2* resulted in spontaneous filamentation and wrinkled colony morphologies, with a high frequency of switching among colony morphologies. In contrast, we report reduced filamentation and did not observe wrinkled colonies when we streaked our *sir2Δ/Δ* strains. These differences might arise from different growth conditions or strains. We note that we observed the same phenotype for two independent deletions, and the phenotype reverted to wild-type when we added back the *SIR2* gene.

Our study revealed that expression of hypha-specific genes decreased in the
absence of Sir2. This finding likely reflects the reduced number of hyphae because these genes are only expressed in hyphal cells. Moreover, these genes are unlikely to be the direct targets of Sir2 because direct targets are expected to increase in expression in the absence of histone deacetylation by Sir2. It was theoretically possible that Sir2 directly regulates genes that repress filamentation and that increased expression of these genes causes reduced filamentation. However, the expression of three negative regulators, NRG1, TUP1, and RFG1, did not change in the absence of Sir2. Nevertheless, our results indicated that the contribution of Sir2 to hyphal induction depends on its deacetylase activity. Thus, the hypha-specific genes are likely indirectly influenced by Sir2 through its regulation of other genes or proteins, and it will be necessary to identify these direct genomic and protein targets of Sir2 to understand the mechanism by which Sir2 promotes hyphal induction. Given that the rDNA locus is deacetylated by Sir2 in C. albicans (25, 26), presumably to suppress unbalanced recombination as in S. cerevisiae (49, 50), one possibility is that the defect in hypha formation in the sir2 mutant is a result of unbalanced recombination within the tandem rDNA array. Consistent with this idea, in Candida parapsilosis changes in colony morphology are accompanied by size alterations of the chromosome containing the rDNA gene cluster (51). Another possibility is that Sir2 promotes hyphal growth through regulating the expression of telomere-associated (TLO) genes, which can enhance filamentation and resistance to oxidative stress (24, 52, 53). It is also possible that Sir2 regulates expression of hypha-specific genes by deacetylating nonhistone proteins. A well-studied example of such regulation is the deacetylase Hda1, which helps maintain hyphal development in C. albicans (14). In the initiation phase of hyphal development, the NuA4 histone acetyltransferase complex binds to the promoters of hypha-specific genes to activate their transcription. However, in the maintenance phase, the deacetylase Hda1 removes the NuA4 complex from these promoters by deacetylating the subunit Yng2, thereby allowing reassembly of nucleosomes that block the repressor Nrg1.

Therefore, to understand the role of Sir2 in regulating hyphal growth, it will be necessary to identify the protein targets of Sir2.

Hyphal growth can be triggered by a variety of host environmental conditions that C. albicans encounters during infection. These include elevated temperature, nitrogen starvation, serum, CO2, and alkaline pH (32). However, factors involved in sensing these environmental cues still need to be uncovered. Since the deacetylation activity of Sir2 requires NAD+, which is a key redox carrier in central metabolism, environmental conditions that impact intracellular [NAD+] may be interpreted by Sir2 and passed on to its downstream targets that influence filamentation. For example, changes in metabolic flux due to nutrient perturbations could impact NAD+ availability. In disseminated candidiasis, C. albicans gains access to the bloodstream, where glucose is plentiful (47). When glucose is oxidized for energy through glycolysis, NAD+ is reduced to NADH. However, if C. albicans is engulfed by macrophages or neutrophils, the environment has less optimal nutrient sources, such as lipids and amino acids (33). These non-sugar compounds enter metabolic pathways that require glycolysis to run in reverse (gluconeogenesis). Thus, yeast may reach different steady-state [NAD+]/[NADH] ratios depending on the available carbon sources, which could in turn impact sirtuin activity. Indeed, in another pathogenic yeast, C. glabrata, NAD+ levels regulate expression of virulence factors through Sir2 and Hst1 (21, 22). In addition, in the yeast Kluyveromyces lactis, NAD+ and Sir2 regulate stress response genes that mitigate the effects of oxidative stress and DNA damage (27). Therefore, sirtuins help yeast species sense stresses and activate appropriate responses. Thus, our study is consistent with C. albicans using Sir2 to modulate filamentation in response to environmental cues during infection.

Our study also indicates that Sir2, Hst1, and Hst2 have distinct roles in C. albicans, even though Sir2 and Hst1 are paralogs resulting from an ancient gene duplication. This result is in agreement with previous studies that supported the distinct roles of Sir2 and Hst1 in C. albicans (26). For example, silencing at the rDNA locus and subtelomeres is performed by Sir2 rather than Hst1 (26). Hst2 is evolutionarily more divergent
from Sir2 and Hst1 (54), and thus its function is expected to be different. For instance, in S. cerevisiae, Hst2 cannot complement the silencing deficiency caused by Sir2 deletion (47). In addition, the different subcellular localizations of Sir2, Hst1, and Hst2 observed in this study also suggested their distinct functions. Our study did not provide evidence that Hst1 and Hst2 function in filamentation of C. albicans, so their functions remain to be uncovered.

In this study, we explored the roles of three sirtuins in hyphal growth of C. albicans. We found that Sir2 is critical for hypha formation and moreover, its function in hypha formation depends on its deacetylase activity. Since morphological plasticity is an important virulence trait of C. albicans, our study lays the groundwork for discovering novel targets for antifungal therapies.

**MATERIALS AND METHODS**

**Media and growth conditions.** C. albicans strains were stored in 25% glycerol stocks at −80°C. For routine manipulation during strain construction, strains were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) at 30°C. Transformants were selected on YPD with 0.2 mg/ml nourseothricin (ClonNAT). Cells in which the CRISPR cassette had been excised were selected on CSM-leucine (0.67% glucose, 0.69 g/liter CSM-Leu powder [Sunrise Biosciences, catalog no. 1005-100]). For filamentation assays, cells were grown in YPD or spider medium (1% mannitol, 1% nutrient broth, 0.2% K2HPO4 [pH 7.2]) at 37°C.

**Plasmid construction.** To make plasmids (Table 1) that contain repair templates for tagging genes, we first amplified SIR2 (C2_01330C), HST1 (C1_09050W), HST2 (CR_01800C), and NOP1 (C4_06720W) with ~500 bp upstream and downstream of the reading frame using primers with restriction sites (Table 2). The PCR products were then digested and ligated into plasmid pRS414 (55). We next amplified GFP (pLR1221), RFP (yEpGAP-Cherry), or MYC (pWZV87) tags from plasmids using primers whose 5’-ankling sequence matched the intended insertion sites in the genes to be tagged (Table 2). Finally, these tags were incorporated at the 3’ ends of the target genes through PCR stitching (17 cycles of 40 s at 95°C, 50 s at annealing temperature specified by the New England Biolabs Tm calculator, and 20 min at 68°C). Correct stitching of the tag was confirmed by Sanger sequencing.

To create plasmid pLR1221 bearing the GFP gene, we started with a C. albicans Sir2-GFP strain (YJB1305, Judith Berman). GFP was amplified from YJB13051 and ligated into the SpeI and SacI sites of pAE28 (Aisha Ellahi and Jasper Rine), yielding pLR1209. Finally, the sequence encoding three amino acids in GFP (L64 [TTC], T65 [GGT], and S72 [GCG]) was corrected by site-directed mutagenesis to generate pLR1221.

**Strain construction.** C. albicans strains (Table 3) were constructed using a LEU2/LEU2 derivative of SC5314 to facilitate CRISPR-mediated genome editing using described procedures (31). Briefly, a CRISPR “LEU2 Out” cassette expressing Cas9, a specific guide RNA, and a nourseothricin (ClonNAT) resistance gene was assembled by PCR stitching and homologous recombination. This cassette, which was flanked by fragments of LEU2, was integrated at the LEU2 locus, generating a strain that was auxotrophic for leucine and resistant to nourseothricin. Cells were simultaneously transformed with two halves of the CRISPR LEU2 Out cassette and a repair template directed to the same locus as the guide RNA. For each transformation, a minimum of 1 μg of repair template and 2 μg of CRISPR cassette was used. After

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**TABLE 1 Plasmids used in this study**

| Plasmid    | Description                                                                 | Source or reference |
|------------|------------------------------------------------------------------------------|---------------------|
| pADH110    | Universal template for fragment A for cloning-free stitching of gRNA expression cassette | 31                  |
| pADH119    | C. albicans LEU2OUT template for fragment B for cloning-free stitching of gRNA expression cassette | 31                  |
| pADH137    | C. albicans LEU2OUT CAS9 expression plasmid                                  | 31                  |
| pRS414     | Empty vector used to make repair template for GFP-, Myc-, or RFP-tagged genes | 55                  |
| pWZV87     | MYC                                                                          | 58                  |
| pAE28      | pRS316 with centromere 3 of Torulaspora dellbruckei                         | Jasper Rine         |
| pLR1221    | GFP in pAE28                                                                | This study          |
| yEpGAP-Cherry | RFP                                                     | 59                  |
| pLR1257    | HST1 with flanking sequence                                                  | This study          |
| pLR1260    | SIR2 with flanking sequence                                                  | This study          |
| pLR1268    | SIR2-MYC                                                                    | This study          |
| pLR1269    | SIR2-GFP                                                                    | This study          |
| pLR1271    | HST1-GFP                                                                    | This study          |
| pLR1309    | HST2 with flanking sequence                                                  | This study          |
| pLR1310    | HST2-GFP                                                                    | This study          |
| pLR1311    | NOP1 with flanking sequence                                                  | This study          |
| pLR1312    | NOP1-RFP                                                                    | This study          |
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**TABLE 2** Oligonucleotides for amplifying genes and tags for cloning

| Description | Sequences |
|-------------|-----------|
| GFP with flanking SpeI/SacI sites | F: **ACTAGT**TCTAAAGGTGAAATATTTC  
R: **GAGCTCT**TTGTAACATTTGCATGCATTTGG |
| **HST1** with flanking SacII/SacII sites | F: **GGCGGGCCGCGGGAAATTCGCCCCTC**CAAC  
R: **GGCGGGACGCTCGGTTGATGGAGCAACATAG**C |
| **SIR2** with flanking SpeI/SacII sites | F: **GGCGGA**CATTGTGAGTGGTGACACAATATAC  
R: **GGCGGGCCGCGGGGTGTTATGCTGATTA**CACCC |
| **HST2** with flanking SalI/SacII sites | F: **GGCGG**TCCAGGCTTTAGCTATTTATATGG  
R: **GGCGGAGGTGCCTTTGACTTATTTATATGG**CACCCAT |
| **MYC** to be integrated at the 3’ end of **SIR2** | F: **GGGAAATTGTCAAGAAATCACTTCGACAAAAAAAGCTGCTAGTGGTGAACAAAAG**  
R: **CACAAAAGATACCCAAACTCTCATTTGAGATCCG**TCATTTCA |
| **GFP** to be integrated at the 3’ end of **SIR2** | F: **GGGAAATTGTGAAAGAACATCGACAAAAAGCTGCTAGTGGTGAACAAAAG**  
R: **CACAAAAGATACCCAAACTCTCATTTGAGATCCG**TCATTTCA |
| **GFP** to be integrated at the 3’ end of **HST1** | F: **GGGAAATTGTGAAAGAACATCGACAAAAAGCTGCTAGTGGTGAACAAAAG**  
R: **CACAAAAGATACCCAAACTCTCATTTGAGATCCG**TCATTTCA |
| **GFP** to be integrated at the 3’ end of **HST2** | F: **GGGAAATTGTGAAAGAACATCGACAAAAAGCTGCTAGTGGTGAACAAAAG**  
R: **CACAAAAGATACCCAAACTCTCATTTGAGATCCG**TCATTTCA |
| **NOP1** with flanking SpeI/SacII sites | F: **GGCG**GATACGGCTTTTCTCTTCTCATTTGAGATCCG**TCATTTCA |
| **RFP** to be integrated at the 3’ end of **NOP1** | F: **GGGAAATTGTGAAAGAACATCGACAAAAAGCTGCTAGTGGTGAACAAAAG**  
R: **CACAAAAGATACCCAAACTCTCATTTGAGATCCG**TCATTTCA |

*Sir2, Hst1, and Hst2, either singly or in combination, were amplified with primers containing restriction sites (underlined). MYC, GFP, and RFP were amplified with chimeric primers that annealed to the tag (underlined) and the gene into which it was inserted (not underlined).*

confirming by PCR that the desired genome editing had occurred, cells were plated on medium lacking leucine to select for the excision of the CRISPR cassette from the LEU2 locus.

To delete **SIR2, HST1,** and **HST2,** either singly or in combination, we used 20-bp guide RNAs (Table 4) targeting the open reading frame of each gene. The repair templates (Table 5) were generated using oligonucleotides with 49 bases of homology both upstream and downstream of the target gene. A GG di-nucleotide was introduced at the junction between the upstream and downstream sequences to function as PAM for a guide RNA in a subsequent genome addback. To create a double-stranded repair template, two complementary oligonucleotides were heated to 99°C for 30 s and then ramped down to 65°C at a rate of 0.1°C/s.

The addback strains were generated by incorporating the wild-type **HST1** or **HST2** genes back into

**TABLE 3** Yeast strains used in this study

| Strain | Genotype | Source or reference |
|--------|----------|---------------------|
| **AHY940** | SC5314 LEU2/leu2Δ (used as wild-type in this study) | 31 |
| YJB13051 | **SIR2**-GFP::URA3/SIR2-GFP::HIS1 URA3/URA3 his1::HISG his1::HISG | Judith Berman |
| LRY3225 | **AHY940** sirlα/sir1Δ-1 | This study |
| LRY3226 | **AHY940** sirlα/sir1Δ-2 | This study |
| LRY3235 | **AHY940** sirlα::SIR2-GFP/sir1Δ::SIR2-GFP | This study |
| LRY3236 | **AHY940** sirlα::SIR2-MYC/sir1Δ::SIR2-MYC | This study |
| LRY3366 | **AHY940** sirlα::SIR2(N329A)-MYC/sir1Δ::SIR2(N329A)-MYC-1 | This study |
| LRY3367 | **AHY940** sirlα::SIR2(N329A)-MYC/sir1Δ::SIR2(N329A)-MYC-2 | This study |
| LRY337 | **AHY940** hst1Δ/hst1Δ-1 | This study |
| LRY338 | **AHY940** hst1Δ/hst1Δ-2 | This study |
| LRY339 | **AHY940** hst1Δ::HST1-GFP/hst1Δ::HST1-GFP | This study |
| LRY3368 | **AHY940** hst1Δ::HST1/hst1Δ::HST1 | This study |
| LRY3341 | **AHY940** hst2Δ/hst2Δ-1 | This study |
| LRY3342 | **AHY940** hst2Δ/hst2Δ-2 | This study |
| LRY3323 | **AHY940** hst2Δ::HST2/hst2Δ::HST2-1 | This study |
| LRY3324 | **AHY940** hst2Δ::HST2/hst2Δ::HST2-2 | This study |
| LRY3357 | **AHY940** hst2Δ::HST2-GFP/hst2Δ::HST2-GFP | This study |
| LRY3241 | **AHY940** sirlα/sir1Δ hst2Δ/hst2Δ | This study |
| LRY3242 | **AHY940** hst2Δ/hst2Δ sirlα/sir1Δ | This study |
| LRY3243 | **AHY940** sirlα/sir1Δ hst1Δ/hst1Δ-1 | This study |
| LRY3244 | **AHY940** sirlα/sir1Δ hst1Δ/hst1Δ-2 | This study |
| LRY3245 | **AHY940** hst2Δ/hst2Δ hst1Δ/hst1Δ-1 | This study |
| LRY3246 | **AHY940** hst2Δ/hst2Δ hst1Δ/hst1Δ-2 | This study |
| LRY3247 | **AHY940** sirlα/sir1Δ hst2Δ/hst2Δ hst1Δ/hst1Δ | This study |
| LRY3451 | **AHY940** NOP1-RFP/NOP1 | This study |
| LRY3453 | **AHY940** sirlα::SIR2-GFP/sir1Δ::SIR2-GFP NOP1-RFP/NOP1 | This study |
TABLE 4 Oligonucleotides used to create gRNAs*

| Mutation | Oligonucleotide used to create guide RNA |
|----------|----------------------------------------|
| hst1Δ    | CGTAAGCTTCTTTTATTTGGGACACACGAAAGGAGTGTATTAGCTAGAAATAGC |
| sir2Δ    | CGTAAGCTTCTTTTATTTGGCACACGCAAGGAGTGTATTAGCTAGAAATAGC |
| hst2Δ    | CGTAAGCTTCTTTTATTTGATACGGGTCTTTATGCAAACGTTTTAGAGCTAGAAATAGC |
| HST2 addback | CGTAAGCTTTTTTATTTGTTTGAGAGAAATCCTCTAGTGTTTTAGAGCTAGAAATAGC |
| SIR2 addback | CGTAAGCTTTTTTATTTTGAATAACAAATAACAATCAATTGTTTTAGAGCTAGAAATAGC |
| HST1 addback | CGTAAGCTTCTTTTATTTGTAGCTGTTTTAGAGCTAGAAATAGC |
| SIR2-N329A | CGTAAGCTTCTTTTATTTGATACGGGTCTTTATGCAAACGTTTTAGAGCTAGAAATAGC |
| NOP1-RFP | CGTAAGCTTTTTTATTTTGGACACACGAAAGGAGTGTATTAGCTAGAAATAGC |

*To generate guide RNA expression cassettes, the oligonucleotides were used to amplify “fragment B” from pADH119. This PCR product was then stitched to “fragment A,” as described previously (31). The underlined portion represents the cut site within the C. albicans genome.

The endogenous loci in the deletion strains. The repair templates were generated by amplifying the HST1 and HST2 genes, including ~500 bp upstream and downstream. The guide RNAs targeted the deletion site, using a PAM site that had been incorporated into the deletion repair template.

To tag SIR2, HST1, HST2, or NOP1, tagged alleles were first generated on plasmids (Table 1). The tagged alleles were released from the plasmids by restriction digestion, extracted from an agarose gel, and then used for transformation. The SIR2-GFP, SIR2-MYC, HST1-GFP, and HST2-GFP repair templates were integrated into the corresponding knockout strains, generating strains in which both alleles were tagged. The NOP1-RFP repair template was transformed into wild-type NOP1 strain, since NOP1 is an essential gene. We were only able to generate heterozygous NOP1-RFP strains, suggesting that the RFP interferes with the function of Nop1.

Table 1 shows the integration of the tag was confirmed by Sanger sequencing, and expression was confirmed by immunoblotting.

To create the catalytically inactive Sir2 allele, we generated a 140-bp repair template changing AAT (Asn) to GCC (Ala) at amino acid position 329. We also introduced synonymous mutations at positions 331, 332, 333, and 336 to prevent the gRNA from recutting Sir2 after gene editing. To generate the 140-bp repair template, we first created a 100-bp PCR product using two overlapping oligonucleotides (Table 1). This product was then extended by 40 bp in a second PCR. This repair template was integrated into the Sir2-Myc strain. Integration of the mutations was confirmed by Sanger sequencing, and expression was confirmed by immunoblotting.

The strains and plasmids used in this study are available upon request.

Immunoblotting. Immunoblots were performed as previously described (56) with modifications. C. albicans cells were grown to an optical density at 600 nm (OD600) of 2.5 to 3.0, and 50 OD cells were used to prepare cell lysate. Cells were lysed by bead beating for 30 min at 4°C in 500 μl of lysis buffer (10% glycerol, 150 mM KCl, 10 mM HEPES [pH 7.9], 1.5 mM MgCl2, 0.5 mM dithiothreitol, 5 mg/ml chymostatin, protease inhibitors). Proteins were then denatured, resolved on a 10% acrylamide-SDS gel, transferred to a nitrocellulose membrane, and probed with mouse anti-Myc (Millipore, 05-724), anti-GFP (Roche, 1181446001), or anti-Pgk1 (Invitrogen, 459250) antibodies. Immunoblots were quantified using ImageJ.

Liquid filamentsation assay. Strains were grown overnight with shaking at 30°C in liquid YPD. Overnight cultures (10 OD) were pelleted, washed once with phosphate-buffered saline (PBS [8 g of NaCl, 200 mg of KCl, 1.44 g of Na2HPO4, and 240 mg of KH2PO4], adjusted pH to 7.4, and filled to 1 liter with water), and resuspended in 1 ml of PBS. We then inculcated 40 μl of the washed cells into 2 ml of YPD or spier medium in 24-well plates. Plates were incubated for 3 h at 37°C with shaking, and then the cells were imaged on a Zeiss AxioVision microscope at 20× magnification. The cells were scored into three categories: yeast, pseudohyphae, and true hyphae (as shown in Fig. 1A and B). The percentages of each cell type were determined by counting at least 200 cells/strain. The averages and standard deviations of the mean of three independent experiments were calculated.

RNA isolation and quantitative RT-PCR. To measure the expression of hyphal-specific genes, cells were harvested before (overnight cells grown in YPD) and after hyphal induction (cells grown in spider medium at 37°C for 3 h in 24-well plates). RNA was isolated as previously described (57) with modifications.

The cells were harvested before (overnight cells grown in YPD) and after hyphal induction (cells grown in spider medium at 37°C for 3 h in 24-well plates). RNA was isolated as previously described (57) with modifications in the cell lysate preparation step: cells were lysed by a bead-beating method for 30 min at 4°C, incubated at 65°C for 20 min, chilled in dry ice until frozen solid, and then centrifuged for 10 min at maximum speed in a microcentrifuge to separate the aqueous phase, which contains the RNA, and the phase.

For qRT-PCR, 5 μg of RNA was DNase treated for 30 min at 37°C using Turbo DNase (Invitrogen) to eliminate genomic DNA. cDNA was synthesized using the DNase-treated RNA as the template with the iScript reverse transcription supermix (Bio-Rad), and qPCR was performed using SYBR green Supermix (Bio-Rad). The qPCR was performed on a CFX384 real-time PCR machine (Bio-Rad). The hyphal-specific genes (HWP1, ECE1, and ALS3) and a control gene (ACT1) were quantified relative to a standard curve prepared from genomic DNA. The primers are listed in Table 6. RNA from four separate cultures was prepared for each strain. The ratio of the hyphal-specific gene to the control gene was determined for each replicate. The ratios for the four replicates were averaged, and this value was normalized to the average ratio in wild-type cells not undergoing hyphal induction. Error bars represent the standard errors of the mean (SEM) of four independent qRT-PCR replicates.

Fluorescence microscopy. Cells were grown as described for the filamentation assay. After a 3-h incubation in YPD at 37°C, 1 ml of cell culture was harvested, pelleted, and resuspended in 0.5 ml of PBS.
| Mutation | Oligonucleotides used to create repair template |
|----------|-----------------------------------------------|
| hst1Δ    | Top strand: TATATTCTATTCTATTCTATCAATTGTTAC  |
|          | AACAAAAACAAGTGAGCAACCGGACAAAGGAAGAGAA       |
|          | Bottom strand: TTTCTCTCTCTTGTGCTCGTCTCT    |
|          | TCTATAGATGTTATTGTTATTGAGTAAATTTGGA         |
| sir2Δ    | Top strand: TTTCTTTATTATATTGACTTGTTGTCAG  |
|          | TTATGAGAGTACCTCTGTTGTTAAATTAATTTG          |
| hst2Δ    | Top strand: ATACGTACACTTCTTACAACAGACTATA  |
|          | ACTTTAAACCCGAGACGAGTGCTGGTCACTCGTCTC      |
| HST1 addback | F: CCCCTCACCCTCTCTCTC |
|          | R: CTTAGCCTAATAACCTAATACCTAT               |
| HST2 addback | F: GATGTAGTCTCAGAC |
|          | R: CCTCAGGTAAAATAATACTAG                    |
| SIR2-N329A | 1: TAGTTTTATCATGCAATTGAAATTACCTACAAGATAAA |
|          | CACAAAAATTTTATGATTTATGATTTATG             |

*Repair templates for gene deletions were prepared by annealing the two oligonucleotides. Repair templates for gene addbacks were amplified from genomic DNA using the indicated oligonucleotides. The repair template for mutating SIR2, was constructed through two successive PCRs, as described in Materials and Methods. F, forward; R, reverse.*
**REFERENCES**

1. Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB. 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin Infect Dis 39:309–317. https://doi.org/10.1086/421946.

2. Pfaffer MA, Diekema DJ. 2010. Epidemiology of invasive mycoses in North America. Crit Rev Microbiol 36:1–53. https://doi.org/10.1080/14625822.2009.1082555.

3. Moran C, Grussemeyer CA, Spalding JR, Benjamin DK, Jr, Reed SD. 2010. A Candida albicans secreted aspartyl proteinase inhibits Candida albicans adherence. Microb Pathog 48:80–85. https://doi.org/10.1016/j.micpath.2009.08.008.

4. Gow NA, Brown AJ, Odds FC. 2002. Fungal morphogenesis and host interaction. Research in Microbiology 153:833–842. https://doi.org/10.1051/rmic:2002076.

5. Bassetti M, Righi E, Ansaldi F, Merelli M, Scarparo C, Antonelli M, Garnacho-Montero J, Diaz-Martin A, Palacios-Garcia I, Luzziat R, Rosin C, Lagunes L, Rello J, Almirante B, Castells M, Mozo C, Pastor A, saliva. 2015. Multicenter multinational study of abdominal candidiasis: epidemiology, outcomes and predictors of mortality. Intensive Care Med 41:1601–1610. https://doi.org/10.1007/s00134-015-3866-2.

6. Gow NA, Brown AJ, Odds FC. 2002. Fungal morphogenesis and host invasion. Curr Opin Microbiol 5:366–371. https://doi.org/10.1016/S1369-5274(02)00338-7.

7. Kumamoto CA, Vinces MD. 2005. Contributions of hyphae and hyphae-core-gulated genes to Candida albicans virulence. Cell Microbiol 7:1546–1554. https://doi.org/10.1111/j.1462-5822.2005.00616.x.

8. Saville SP, Lazzell AL, Monteagudo C, Lopez-Ribot JL. 2003. Engineered control of cell morphology in vivo reveals distinct roles for yeast and filamentous forms of Candida albicans during infection. Eukaryot Cell 2:1035–1060. https://doi.org/10.1128/EC.2.5.1035-1060.2003.

9. Phan QT, Myers CL, Fu Y, Sheppard DC, Yeaman MR, Welch WH, Ibrahim AS, Edwards JE, Jr, Filler SG. 2007. ALS3 is a Candida albicans invasin that binds to cadherins and induces endocytosis by host cells. PLoS Biol 5:e64. https://doi.org/10.1371/journal.pbio.0050064.

10. Staab JF, Bradway SD, Fidel PL, Sundstrom P. 1999. Adhesive and mammalian transglutaminase substrate properties of Candida albicans Hwp1. Science 283:1535–1538. https://doi.org/10.1126/science.283.5407.1535.

11. Naglik JR, Challacombe SJ, Hube B. 2003. Candida albicans secreted aspartyl proteinases in virulence and pathogenesis. Microbiol Mol Biol Rev 67:400–428. https://doi.org/10.1128/MMBR.67.4.400-428.2003.

12. Moyes DL, Wilson D, Richardson JP, Mogavero S, Tang SX, Werneck J, Hofs S, Gratacap RL, Robbins J, Runglali M, Murciano C, Blagojevic M, Tavarez J, Förster TM, Hebecker B, Kasper L, Vizcay J, Lancau SJ, Kichik N, Härder A, Kurza O, Luo T, Krüger T, Kniemeyer O, Cota E, Bader O, Wheeler RT, Güttermann T, Hube B, Naglik JR. 2016. Candidalysin is a fungal peptide toxin critical for mucosal infection. Nature 532:64–68. https://doi.org/10.1038/nature17625.

13. Birse C, Irwin M, Fonzi W, Sypherd P. 1993. Cloning and characterization of ECE1, a gene expressed in association with cell elongation of the dimorphic pathogen Candida albicans. Infect Immun 61:3648–3655. https://doi.org/10.1128/IAI.61.9.3648-3655.1993.

14. Lu Y, Su C, Wang A, Liu H. 2011. Hyphal development in Candida albicans requires two temporally linked changes in promoter chromatin for initiation and maintenance. PLoS Biol 9:e1001105. https://doi.org/10.1371/annotation?7b97b9ec-881a-4940-83ab-15ff318f8d19.

15. Hnisz D, Bardet AF, Nobile CJ, Petryshyn A, Glaser W, Schöck U, Stark A, Kucher K. 2012. A histone deacetylase adjuts transcription kinetics at coding sequences during Candida albicans morphogenesis. PLoS Genet 8:e1003118. https://doi.org/10.1371/journal.pgen.1003118.

16. Lee J-E, Oh J-H, Ku M, Kim J, Lee J-S, Kang S-O. 2015. Snx6 has dual roles in Candida albicans filament development through the interaction with Rpd31. FEMS Lett 589:513–520. https://doi.org/10.1007/s00648-015-5488-3.

**TABLE 6 Oligonucleotides used for quantitative PCR**

| Gene   | Sequence(s) |
|--------|-------------|
| HWP1   | F: GCTGGTCCAGATCATCCATGC  
|        | R: AAGGTTGAGTGCCAGAGACGTG |
| ECE1   | F: TGCCATGTTCGTCAGGTCGT  
|        | R: TAGCTTGTTGACAGTTCAGG |
| ALS3   | F: CCACCTCACAATCCCACTC  
|        | R: CAGCAATGATGAACTAGATGATCTTCATC |
| ACT1   | F: TGTTGATGGTTGATCTCACG |
|        | R: GACAATTTCCTTTTACCAAC |

*F, forward; R, reverse.*
18. Houtkjaerger RH, Pirinen E, Auwerx J. 2012. Sirtuins as regulators of metabolism and health. Nat Rev Mol Cell Biol 13:225–238. https://doi.org/10.1038/nrm3293.

19. Mei Z, Zhang Y, X. J Huang, H, J. He, J Tao Y. 2016. Sirtuins in metabolism, DNA repair, and cancer. J Exp Clin Cancer Res 35:1–14. https://doi.org/10.1186/s13046-016-0461-5.

20. Kupis W, Palajga J, Tomal E, Niewiadomska E. 2016. The role of sirtuins in cellular homeostasis. J Physiol Biochem 72:371–380. https://doi.org/10.1007/s10059-016-0492-6.

21. Orta-Zavala E, Guerrero-Serrano G, Gutiérrez-Escobedo G, Cañas-Villamar I, Juárez-Cepeda J, Castaño I, De Las Peñas A. 2013. Local silencing controls the oxidative stress response and the multidrug resistance in Candida glabrata. Mol Microbiol 88:1135–1148. https://doi.org/10.1111/mmi.12247.

22. Domergue R, Castaño I, De Las Peñas A, Zupancic M, Lockatell V, Hebel JR, Johnson D, Cormack BP. 2005. Nicotinic acid limitation regulates the oxidative stress response and the multidrug resistance in Candida albicans. Mol Microbiol 88:1135–1148. https://doi.org/10.1111/mmm.12247.

23. Sudbery P, Gow N, Berman J. 2004. The distinct morphogenic states of Candida cells undergoing the yeast-to-hyphal transition. Mol Biol Cell 16:2903–2912. https://doi.org/10.1091/mbc.e05-01-0073.

24. Anderson MZ, Gerstein AC, Wigen L, Baller JA, Berman J. 2014. Silencing is experienced functional changes in the AAA homolog Nrg1. Curr Genet 56:771–787. https://doi.org/10.1007/s00294-0009-0268-4.

25. Nunn S, Holesova Z, Kosa P, Gacser A, Tomaska L. 2009. Biology and genetics of the pathogenic yeast Candida albicans by the transcriptional repressor TUP1. Curr Genet 55:497–509. https://doi.org/10.1007/s00294-009-0268-4.

26. Flanagan PR, Fletcher J, Boyle H, Sulea R, Moran GP, Sullivan DJ. 2018. Expansion of the TLO gene family enhances the virulence of Candida species. PLoS One 13:e0208852. https://doi.org/10.1371/journal.pone.0208852.

27. Dunn MJ, Kinney GM, Washington PM, Berman J, Anderson MZ. 2013. Functional diversification accompanies gene family expansion of MED2 homologs in Candida albicans. Mol Microbiol 51:1159–1173. https://doi.org/10.1111/j.1365-2958.2009.06772.x.

28. Nguyen N, Quail MM, Hernad SJ. 2017. An efficient, rapid, and recyclable system for CRISPR-mediated genome editing in Candida albicans. MSphere 2:e00149-17. https://doi.org/10.1128/msphere.00149-17.

29. Sudbery PE. 2011. Growth of Candida albicans hyphae. Nat Rev Microbiol 9:737–744. https://doi.org/10.1038/nrmicro2636.

30. Sudbery P, Gow N, Berman J. 2004. The distinct morphogenic states of Candida albicans. Trends Microbiol 12:317–324. https://doi.org/10.1016/j.tim.2004.05.008.

31. Kadosh D, Johnson AD. 2000. The NRG1 gene expression in Candida albicans is downregulated during filamentation. EMBO J 19:4753–4761. https://doi.org/10.1093/emboj/19.23.4753.

32. Braun BR, Johnson AD. 1997. Control of filament formation in Candida albicans by the transcriptional repressor TUP1. Science 277:105–109. https://doi.org/10.1126/science.277.5332.105.

33. Braun BR, Kadosh D, Johnson AD. 2001. NRG1, a repressor of filamentous growth in C. albicans, is downregulated during filament formation. EMBO J 20:4753–4761. https://doi.org/10.1093/emboj/20.17.4753.

34. Morad AMA, Leng P, Straffon M, Wishart J, Macskill S, MacCallum D, Schnell N, Talib N, Marechal D, Tekiwa F. 2001. NRG1 represses yeast-hyphae morphogenesis and hypha-specific gene expression in Candida albicans. EMBO J 20:4742–4752. https://doi.org/10.1093/emboj/20.17.4742.

35. Keppler-Ross S, Noffz C, Dean N. 2008. A new purple fluorescent color marker for genetic studies in Saccharomyces cerevisiae and Candida albicans. Genetics 179:705–710. https://doi.org/10.1534/genetics.108.087800.