A *Candida albicans* Temperature-Sensitive cdc12-6 Mutant Identifies Roles for Septins in Selection of Sites of Germ Tube Formation and Hyphal Morphogenesis

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Septins were identified for their role in septation in *Saccharomyces cerevisiae* and were subsequently implicated in other morphogenic processes. To study septins in *Candida albicans* hyphal morphogenesis, a temperature-sensitive mutation was created that altered the C terminus of the essential Cdc12 septin. The *cdc12-6* cells grew well at room temperature, but at 37°C they displayed expected defects in septation, nuclear localization, and bud morphogenesis. Although serum stimulated the *cdc12-6* cells at 37°C to form germ tube outgrowths, the mutant could not maintain polarized hyphal growth and instead formed chains of elongated cell compartments. Serum also stimulated the *cdc12-6* mutant to induce a hyphal reporter gene (HWP1-GFP) and a characteristic zone of filipin staining at the leading edge of growth. Interestingly, *cdc12-6* cells shifted to 37°C in the absence of serum gradually displayed enriched filipin staining at the tip, which may be due to the altered cell cycle regulation. A striking difference from the wild type was that the *cdc12-6* cells frequently formed a second germ tube in close proximity to the first. The mutant cells also failed to form the diffuse band of septins at the base of germ tubes and hyphae, indicating that this septin band plays a role in preventing proximal formation of germ tubes in a manner analogous to bud site selection. These studies demonstrate that not only are septins important for cytokinesis, but they also promote polarized morphogenesis and selection of germ tube sites that may help disseminate an infection in host tissues.

The human fungal pathogen *Candida albicans* is capable of causing severe systemic infections. Although immunocompromised patients are particularly at risk, immunocompetent individuals are susceptible to infection when the inoculum is high, which can occur under circumstances such as biofilm formation on medical devices. The major pathogenic effects of *C. albicans* are due to invasive growth into tissues, which is facilitated in part by the ability of *C. albicans* to switch between different morphologies (29, 36). *C. albicans* can grow as budding cells, chains of elongated cells termed pseudohyphae, or as long filamentous cells with parallel walls called hyphae (36). The filamentous morphology promotes invasive growth into agar *in vitro* and has been linked to invasive growth into tissues *in vivo* (20, 32). Previous studies also indicated that *C. albicans* must be able to switch between different morphologies to be fully pathogenic. Mutants that are locked in either the hyphal or budding form have been shown to be less virulent in models of hematogenously disseminated systemic candidiasis (23, 32, 44).

The septin family of cytoskeletal filament-forming proteins has been shown to contribute to morphogenesis in *C. albicans* (16, 28, 34, 40). The septins were first identified in the yeast *Saccharomyces cerevisiae* as proteins that are needed for septum formation and cytokinesis (15, 25). The septins localize to the bud neck, where they form a scaffold to recruit other proteins that promote septum formation. The septins also act as a boundary domain to restrict proteins involved in septum formation to the proper position in the bud neck and also restrict certain proteins to the daughter cells (9, 30). Deletion analysis of the seven different septin genes in *C. albicans* revealed that their relative importance is similar to the orthologous genes in *S. cerevisiae*. CDC3 and CDC12 are essential, whereas CDC10, CDC11, and SEP7 are not essential but contribute to proper septation and morphogenesis (16, 40). Mutation of the *C. albicans* orthologs of the SPR3 and SPR28 septin genes that are expressed during sporulation in *S. cerevisiae* did not detectably affect *C. albicans* septation or morphogenesis (40).

Septins have also been implicated in other morphogenic events. For example, *S. cerevisiae* septins promote proper pheromone-induced morphogenesis, spore formation, and selection of the site of future bud formation (12). The possibility that the septins may also play special roles during *C. albicans* hyphal growth was suggested by studies that detected septins at sites distal to septum formation, including the base of the initial hyphal outgrowths (known as germ tubes) and at the growing hyphal tips (35, 40). Consistent with this, *cdc10* and *cdc11* mutants exhibited partial defects in hyphal morphogenesis, including a greater frequency of curved hyphae, and slight inconsistencies in cell wall deposition (40). Significantly, the *cdc10*Δ and *cdc11*Δ mutants displayed defects in morphogenesis and invasive growth in a mouse model of systemic *C. albicans* infection (39). These mutants also had a related defect in the morphogenesis of the filamentous cells that produce chlamydospores (26).

In order to obtain a fuller understanding of the role of the *C. albicans* septins in hyphal growth, a new approach was necessary to study the essential septin genes *CDC3* and *CDC12*. Therefore, a temperature-sensitive *CDC12* mutant strain was constructed that was patterned after the well-studied *cdc12-6 S. cerevisiae* mutant. The *C. albicans* *cdc12-6* strain grew well at room temperature but not at 37°C, the temperature at which hyphal growth is induced. It...
also showed temperature-sensitive defects in septation and bud morphogenesis, similar to those reported for the analogous mutation of *S. cerevisiae* (1, 17). Interestingly, the *C. albicans cdc12-6* mutant also revealed important new roles for septins in maintaining highly polarized hyphal growth and for selection of the second site of germ tube formation.

### MATERIALS AND METHODS

#### Strains and media.

The *C. albicans* strains used in the present study are described in Table 1. The cells were propagated in rich YPD medium (2% glucose, 1% peptone, 2% yeast extract) or SD (yeast nitrogen base synthetic medium with dextrose) as described previously (33). Uridine at 80 mg/liter was added to cultures of *ura3* strains.

A *C. albicans cdc12ΔCDC12* heterozygous mutant was created in strain BW17 by homologous recombination as described previously (40, 42). In brief, a deletion cassette was constructed by PCR using primers that contain sequence homology to the 5’ and 3’ ends of the *CDC12* open reading frame to amplify a cassette containing the *ARG4* gene for use as a selectable marker to delete one copy of *CDC12*. The C-terminal coding sequences of the remaining *CDC12* allele were then replaced by an altered sequence that was patterned after the changes found in the *S. cerevisiae cdc12-6* mutant allele (Brian Haarer, unpublished data). The *S. cerevisiae cdc12-6* allele contains mutations that result in a Lys-to-Asn change at position 391, followed immediately by a TAG stop codon, truncating the protein by 16 amino acids. Amino acid sequence alignments indicated that the Lys-391 of *Sc-Cdc12* corresponds to Ser-384 of Ca-Cdc12. Therefore, the analogous indicated changes were used to amplify the *CDC12* gene using pGEM-Ura3 as a template (42), and then the resulting cassette was used to select for integration of the *cdc12-6* allele into *C. albicans*. The primer sequences were 5’-GACTAATTTATTATATTATGAGAAAGAATAGTTGGAA TCAGGAAGGAAAGAAATGCAGTGGAAATGGTATTGTTCCT CAGTTGGTAAACACGCTG-3’ and 5’-GTAACCCAAAACACGAGAATT AAGTCCAGTATATATCAGTGGACAAAAACTAAAAATGTT TAATCTCCTCCCTCCTACATTATAA-3’. Similar temperature-sensitive phenotypes for budding and hyphal morphogenesis were observed for independent transformants that were *cdc12Δ:ARG4/cdc12Δ:Ura3*, so one strain (CZ10) was then used for all subsequent studies. Strain CZ10 was transformed with a *HIS1* gene to complement all of the auxotrophies and create strain LLF016. The *HIS1* DNA fragment was isolated by PCR amplification using genomic DNA from *C. albicans* strain SC5314 as a template.

**TABLE 1 Strains used in this study**

| *C. albicans* strain | Parent strain | Genotype |
|----------------------|---------------|----------|
| BW17                 | Sc5314        | *ura3Δ::imm434/ura3Δ::imm434 his1::hisG/his1::hisG arg4::hisG arg4::hisG |
| DIC185               | BW17          | *ura3Δ::imm434/URA3 his1::hisG/His1::arg4/ARG4 |
| YLS685               | BW17          | *ura3Δ::imm434/URA3 his1::hisG/His1::arg4/ARG4 |
| YAW12                | BW17          | *cdc12::ARG4/CD12 ura3::imm434/ura3::imm434 his1::hisG/His1::arg4/ARG4 |
| CZ10                 | YAW12         | *cdc12-6 strain CZ10, except CDC10-GFP::HIS1 |
| CZ11                 | CZ10          | *cdc12-6 strain CZ10, except NOP1-GFP::HIS1 |
| CZ14                 | CZ10          | *cdc12-6 strain CZ10 |
| LLFO03               | BW17          | BW17, except CDC10-GFP::HIS1 |
| LLFO06               | BW17          | BW17, except NOP1-GFP::URA3 |
| LLFO09               | LLFO03        | CDC10-GFP::strain LLFO03, except URA3/ura3::imm434 |
| LLFO10               | CZ10          | *cdc12-6 strain CZ10, except HWPI-GFP::HIS1 |
| LLFO12               | YLS685        | YLS685, except HWPI-GFP::HIS1 |
| LLFO16               | CZ10          | *cdc12-6 strain CZ10, HIS1/His1::hisG |

**FIG 1** The *C. albicans cdc12-6* septin mutant is temperature sensitive for growth. (A) Alignment of the C-terminal sequences of the *S. cerevisiae* and *C. albicans* Cdc12 proteins. (B) Tenfold dilutions of the wild-type control *CDC12/CDC12* (DIC185), *cdc12-6/cdc12Δ* (LLF016), and heterozygote *CDC12/cdc12Δ* (YAW12) strains were spotted onto rich-medium YPD plates. The plates were incubated for 48 h at the indicated temperature and then photographed.
ular staining indicate of plasma membrane lysis. GFP was analyzed directly in live cells without further processing by using a fluorescence microscope equipped with a fluorescein isothiocyanate (FITC) filter set. This filter set was used to detect GFP, since it was easier to recognize the auto-fluorescence of dead cells as a different color from the true GFP signal. Chitin staining was performed by incubating cells with 10 µg of Calcofluor White/ml in phosphate-buffered saline (PBS) for 5 min, followed by two washes with PBS, and then viewing the cells without fixation using a UV filter set. Filipin staining was carried out essentially as described previously (2, 27). The cells were induced with or without serum for 2 h at 37°C, stained with 10 µg of filipin/ml, and then analyzed immediately by fluorescence microscopy using a UV filter set. The cells were viewed on an Olympus BH-2 microscope, and images were captured with an AxioCam digital camera (Carl Zeiss, Thornwood, NY) operated with Axiovision software. The fluorescence signal intensity for cells expressing HWPI-GFP was quantified using Axiovision software. The results represent the average of three independent experiments in which at least 50 cells were analyzed each time.

Confocal microscopy was used to analyze septin ring structure in cells producing Cdc10-GFP. The cells were cultured overnight in log phase at room temperature, and then aliquots were incubated for 2 h at 23 or 37°C. The aliquot incubated at 37°C was further divided two tubes. One tube was incubated in rich YPD medium to promote growth of budding cells. Bovine calf serum was added to the other tube to a final concentration of 20% to induce hyphal growth. The cells were then analyzed by fluorescence microscopy using a Zeiss LSM510 META NLO two-photon laser scanning confocal microscope at the Stony Brook University Central Microscopy Imaging Center.

RESULTS

Construction of a C. albicans temperature-sensitive cdc12-6 mutant. The essential septin gene CDC12 was examined by creating a mutant allele based on the changes found in the well-studied temperature-sensitive S. cerevisiae cdc12-6 mutant (Haarer, unpublished). The mutation alters the C-terminal sequences of Cdc12 as depicted in Fig. 1A. These alterations may alter septin function because the C-terminal region of Cdc12 contributes to the stability of septin structures by promoting interaction between two septin filaments (4, 5, 37). The other copy of CDC12 was deleted so that only the mutant cdc12-6 allele remained. The C. albicans cdc12-6 strain displayed a strong temperature-sensitive phenotype (Fig. 1B). The cdc12-6 strain grew as well as the wild-type control strain or a cdc12Δ/CDC12 heterozygote when spotted onto solid medium agar plates at temperatures up through 30°C, but it was not viable at 37°C. Thus, although the C termini of Cdc12 from S. cerevisiae and C. albicans are not identical, introduction of the analogous alterations of the Sc-cdc12-6 allele into Ca-CDC12 still resulted in a strong temperature-sensitive phenotype.

cdc12-6 mutant phenotypes during budding. The effects of the cdc12-6 mutation on C. albicans morphogenesis were examined at room temperature (0 h) and at different times after shifting cells to 37°C (Fig. 2). When grown at room temperature, the cdc12-6 cells formed buds, albeit some with a slightly abnormal shape. Many cells were also present in clusters, indicating a partial defect in septation and cytokinesis. After 2 h at 37°C, cdc12-6 cells formed elongated buds (Fig. 2B). After 6 h, continued growth of the mutant cells resulted in highly elongated filamentous cells. At around 6 h the cdc12-6 cells began to lyse near the tips, as evidenced by intracellular staining with trypan blue (Fig. 2D). The highly elongated buds formed by the cdc12-6 mutant are similar to the morphology of septin mutants in S. cerevisiae, which are thought to form due to activation of a cell cycle checkpoint pathway that prolongs apical growth of cells (30).

A hallmark of S. cerevisiae septin mutants is their defect in septation. To assay this in the C. albicans cdc12-6 mutant, log-phase cells shifted to 37°C were stained with Calcofluor White to detect the ring of cell wall chitin that forms at the septum (31). At room temperature, both the wild type and the cdc12-6 mutant showed typical Calcofluor White staining at bud necks (Fig. 3A). Similar results were observed for the wild-type strain at 37°C. In contrast, sites of septation were rarely detected in the cdc12-6 mutant shifted to 37°C (Fig. 3B). This was most evident after 6 h of incubation at 37°C; the cdc12-6 cells formed chains of elongated pseudohyphal-type cells with a few obvious septae (Fig. 3C). There were, however, patches of Calcofluor White staining that could represent aberrant attempts to form septae.

Septins also contribute to proper nuclear segregation in S. cerevisiae by interacting with microtubules to orient nuclear migration into the bud (21). The distribution of nuclei in C. albicans was therefore examined by monitoring the nuclear-localized protein Nop1-GFP. Wild-type cells typically contained one nucleus per cell, as expected. In contrast, the cdc12-6 cell compartments frequently contained multiple nuclei or they lacked a nucleus, especially after the longer 6 h of incubation at 37°C, indicating a defect in nuclear segregation (Fig. 3C).
Septin localization in the cdc12-6 mutant was analyzed by studying the Cdc10 septin fused to GFP. Wild-type cells grown at either room temperature or 37°C exhibited the expected localization of Cdc10-GFP to rings at the bud neck (Fig. 3A). The cdc12-6 cells grown at room temperature also showed bud neck localization of Cdc10-GFP (Fig. 3A). However, after a shift to 37°C for 2 h, approximately half of the cdc12-6 cells lacked detectable Cdc10-GFP, and the others primarily contained faint patches or rings of Cdc10-GFP toward the growing end of the elongated cell cluster (Fig. 3B). Thus, the septins are still capable of forming a complex at 37°C, but it primarily appears near the leading edge of growth and does not stabilize at the pinched zones that correspond to bud necks. Cdc10-GFP was still showed a similar distribution in the cdc12-6 mutant after 6 h at 37°C (Fig. 3C). Although a majority of cells appeared to lack Cdc10-GFP, a patch or ring of septins was frequently detected at the leading edge of growth or at sites of budding off the main filamentous cell clusters.

The three-dimensional structure of the Cdc10-GFP septin rings that formed in the cdc12-6 mutants was analyzed by confocal microscopy (Fig. 4). Wild-type cells grown at room temperature or 37°C showed the expected Cdc10-GFP ring at the bud neck. In contrast, the Cdc10-GFP structures in the cdc12-6 mutant were abnormal. At room temperature, Cdc10-GFP localized in a spectrum of patterns ranging from typical ring structures, to partial rings with a break in the continuity, and very faint rings (Fig. 4). Shifting the cdc12-6 cells to 37°C for 2 h resulted in much more severe defects in Cdc10-GFP localization. Cdc10-GFP most frequently appeared as a series of bars and did not form a contiguous ring. The Cdc10-GFP rings in cdc12-6 cells showed similar defects for cells grown in the presence or absence of serum. Some of these structures appeared to be similar to the types of septin rings seen in S. cerevisiae cells induced with mating pheromone or carrying a mutation in GIN4 (6, 24) and in C. albicans mutants with hyper-active Cdc42 (11).

**Hyphal morphogenesis defects in cdc12-6 mutant cells.** The ability to undergo hyphal morphogenesis was examined by treating cells with 20% bovine calf serum at 37°C. As expected, wild-type cells efficiently formed the initial polarized outgrowths termed germ tubes that continued to elongate in a highly polar-
ized manner to form filamentous cells with multiple cell compart-
ments termed hyphae (Fig. 2 and 5). Serum also induced cdc12-6 cells to form germ tubes at 2 h that were generally similar to the wild-type cells (Fig. 2 and 5). Serum clearly induced a distinct morphogenesis pathway in the cdc12-6 mutant; most cell walls grew parallel and did not display the curvature that was seen in the absence of serum (Fig. 2 and 3). However, Cdc10-GFP localization was abnormal in the cdc12-6 cells induced with

FIG 5 Cell morphology, septin ring, and nuclear localization are altered in cdc12-6 cells under hypha-inducing conditions. Cells were grown to log phase at room temperature, serum was added to 20% final concentration, and then the cultures were shifted to 37°C for 2 h (A) or 6 h (B). As labeled at the top of each column of photos, the cells were analyzed by light microscopy to detect morphology (DIC) or fluorescence microscopy to detect Calcofluor White staining of chitin (CW) or the GFP fusion protein (GFP) as indicated on the left. The strains used were wild-type cells carrying CDC10-GFP (LLF009) or NOP1-GFP (LLF006) and cdc12-6 cells carrying CDC10-GFP (CZ11) or NOP1-GFP (CZ14). Bars, 10 μm.
Serum. About half of the cell clusters lacked detectable Cdc10-GFP, and the Cdc10-GFP structures that were present were typically fainter (Fig. 5A). In addition, the septin rings that formed in cdc12-6 cells had a wider diameter than those detected in the germ tubes and hyphae of wild-type cells (Fig. 4). This is likely due in part to the continued expansion of the width of the cdc12-6 germ tubes (see below). In addition, the Cdc10-GFP rings in cdc12-6 cells typically had breaks in their continuity and some appeared as a series of bars, as was seen for cells grown in the absence of serum (Fig. 4).

After 6 h of incubation at 37°C with 20% serum, the morphology of the cdc12-6 cells was very distinct from the wild type (Fig. 5B). The cdc12-6 cells formed filaments that were wider and curved, indicating the original germ tubes continued to grow in width, whereas new growth in wild-type cells is restricted to the apical tip. The hyphal inducing conditions did not appear to alter the viability of cells at 37°C. Trypan blue staining revealed that dead cells still began to accumulate by 6 h of incubation (Fig. 2). The cdc12-6 cells also showed frequent branching of new offshoots of filamentous outgrowth that was not seen for the wild type. Analysis of Nop1-GFP localization showed that many of these branched regions did not contain nuclei, whereas other regions contained multiple nuclei (Fig. 5). This indicates that nuclear division continued in the absence of septation but that the nuclei were not segregating into the different cell compartments. The cdc12-6 cells rarely formed septa that could be detected by Calcofluor White staining, even at the sites where Cdc10-GFP was localized. Instead, patches of Calcofluor White staining were commonly detected in the new filamentous growth that may represent aberrant attempts to initiate septum formation. After 6 h there were patches or rings of Cdc10-GFP detected in ca. 30% of the cells. Interestingly, the Cdc10-GFP structures in the cdc12-6 mutant were frequently detected in the middle of the elongating germ tube, as seen for wild-type cells, and not at the tip of the filamentous cell as was seen for cdc12-6 cells grown at 37°C in the absence of serum. This suggests that septin localization is affected by the altered cell morphogenesis or by distinct signaling pathways activated in hyphae.

Hypha-induced responses. The ability of the mutant cells to induce hyphal genes was assayed by quantifying the expression of a HWP1-GFP gene fusion. This reporter gene was constructed by placing GFP expression under the control of the hypha-induced HWP1 promoter. Cells carrying this reporter gene were grown in the presence or absence of the hyphal inducers serum or GlcNAc, and the relative induction was assessed by quantifying the signal intensity of GFP using fluorescence microscopy. Although the wild-type and mutant cells strongly induced HWP1-GFP (Fig. 6A), the cdc12-6 cells were slightly less efficient than the wild type (P < 0.003). Thus, septin function is not essential for induction hyphal genes.

Another hallmark of hyphal cells is that the apical region stains more readily with the ergosterol-binding agent filipin (27). As expected, essentially 100% of the wild-type cells induced with serum showed increased staining with filipin at hyphal tips (Fig. 6B). Similar results were observed for cdc12-6 cells induced with serum. Surprisingly, control studies showed that ca. 31.4% (n = 191) of the cdc12-6 cells shifted to 37°C for 1.5 h in the absence of serum also showed stronger filipin staining at the tips. This increased over time to 41.8% at 2 h (n = 110) and 63.3% at 3 h (n = 128). These results for filipin staining contrasted with the expression of HWP1-GFP, which required serum to be induced. Thus, this characteristic of hyphae could be induced in cdc12-6 cells in the absence of serum.

Altered position of second germ tubes in cdc12-6 mutant. The cdc12-6 cells induced to form hyphae for an extended time frequently formed a second germ tube very close to the first one, which was rarely observed in the wild type (Fig. 7) (10). To quantify the difference, the relative positions of the sites where the first and second germ tubes initiated were scored as one of six patterns: 150° to 180° apart, 120° to 150° apart, 60 to 120° apart, <60° apart, two germ tubes in contact, or a second germ tube that emerged from the first germ tube rather than the mother cell. Interestingly, cdc12-6 cells showed significantly increased frequency of cells forming a second germ tube proximal to the first (Fig. 7A). The majority of cdc12-6 cells formed a second germ tube within a 60° angle of the first, whereas essentially all of the wild-type cells formed a second germ tube that was more than 60° from the first. The cdc10Δ and cdc11Δ mutants occasionally formed proximal germ tubes (40), but this defect was more extreme in the cdc12-6 cells.

The altered site selection for germ tube outgrowth in the cdc12-6 mutant indicates that septins influence this process. An interesting possibility is that the basal band of septins may play a role in determining the site of the second germ tube (Fig. 7B). The basal band is a more diffuse type of septin ring that is located at the junction between the mother cell and the germ tube (35, 40). The function of this basal band of septins is not clear, since cyto-
kinesis does not occur at this site. However, its location suggests that the basal septin band may function in germ tube site selection analogous to the role of the septin ring in bud site selection (24). Consistent with this, the basal band of septins was not detected in cdc12-6 cells (Fig. 7B). Thus, proper Cdc12 function is required to form both the basal septin band and the septin rings that form at sites of cytokinesis.

A relationship between bud site and germ tube site selection is also supported by previous studies which showed that the bud sites of wild-type C. albicans were clustered at an axial site at room temperature but were primarily not adjacent at 30 and 37°C (10). The budding mode of cdc12-6 cells could not be assessed at an elevated temperature, which is not permissive for this mutant. Therefore, we analyzed the nonessential cdc10/H9004, cdc11/H9004, and sep7/H9004 septin mutants. These mutants also showed defects at 37°C that prevented accurate assessment of bud site selection but could be examined at 30°C. Interestingly, all three nonessential cdc10Δ, cdc11Δ, and sep7Δ septin mutants. These mutants also showed defects at 37°C that prevented accurate assessment of bud site selection but could be examined at 30°C. Interestingly, all three nonessential septin mutants budded primarily at a cluster of axial sites at room temperature (>90%; n = >200), similar to the wild type. In contrast, at 30°C only 33% of wild-type cells budded in an axial manner (n = 202), whereas the cdc10Δ, cdc11Δ, and sep7Δ mutants all still budded primarily in an axial manner (>60%; n = >144). The effect was most obvious for the cdc11Δ mutant (76% axial budding at 30°C), a finding consistent with the cdc11Δ mutant having the morphogenesis phenotype of the three. This suggests that the temperature-related switch in bud-site selection underlies the mechanisms that promote germ tube formation at distal rather than axial sites.

DISCUSSION

Temperature-sensitive septin mutants have played a valuable role in S. cerevisiae for identifying the function of septins in septation and other morphogenic events, including mating and sporulation (12, 25). Although hyphal morphogenesis could not be examined in S. cerevisiae, studies of the nonessential septin genes CDC10, CDC11, and SEP7 indicated that they are important for normal hyphal morphogenesis in C. albicans (16, 34, 40) and in filamentous fungi (7, 14, 22). Therefore, in the present study a temperature-sensitive cdc12-6 mutant was created to carry out the first analysis of an essential septin gene in C. albicans. Shifting the C. albicans cdc12-6 strain to 37°C caused a rapid defect in morphogenesis and septation, similar to those seen in S. cerevisiae. The cdc12-6 mutation likely causes a temperature-sensitive phenotype because it alters the C-terminal region of Cdc12 that is important for stabilizing connections between two septin filaments (4, 5, 37).

Induction and maintenance of hyphal morphogenesis. The cdc12-6 cells formed buds at room temperature and could be stim-
ulated with serum to form germ tubes at 37°C (Fig. 2 and 5). The initial germ tube outgrowths in the cdc12-6 mutant cells at 2 h did not appear to be significantly more defective than the wild type, whereas bud morphogenesis was clearly affected by 2 h (Fig. 2). This suggests that septin function is not required to initiate germ tubes. Previous studies showed that the C. albicans cdc10Δ and cdc11Δ mutants frequently formed curved germ tube necks (26, 40), but this phenotype was not exacerbated in the cdc12-6 mutant.

Although the cdc12-6 mutant formed germ tubes, highly polarized hyphal morphogenesis was not maintained. The filamentous outgrowths became wider over time and took on the characteristics of pseudohyphal cells. The cdc12-6 mutant phenotype was more extreme than the mutants lacking the nonessential septin genes CDC10 and CDC11, which showed subtler defects in maintaining polarized morphogenesis (40). This indicates that the septins have a special function in maintaining highly polarized growth. Altered septin localization is therefore likely to contribute to the abnormal hyphal morphogenesis of mutants that display defects in targeting septins to appropriate sites (11, 18, 41). However, some phenotypes of cdc12-6 cells may be due to the activation of stress pathways. The defects of cdc12-6 cells in septation and cell wall biogenesis (Fig. 2 and 5) should activate cell wall stress pathways that could indirectly affect actin localization and morphogenesis. Activation of stress pathways could also account from some of the altered patches of Calcofluor White staining in cdc12-6 cells at 37°C (Fig. 3 and 5). Unusual patches of Calcofluor White staining were also detected after treatment of cells with caspofungin, an inhibitor of cell wall β-glucan synthesis (3).

Regulation of the Cdc11 septin by phosphorylation has also been implicated in proper C. albicans hyphal morphogenesis. Mutation of a site in Cdc11 to prevent phosphorylation by Cdc28 (S994A) caused a defect in maintaining highly polarized hyphal growth (34). This defect in maintaining polarized growth was more extreme than the defects seen for cdc12-6 cells. Mutation of a different site in Cdc11 to prevent phosphorylation by Gin4 (S995A) caused cells to sequentially initiate multiple short germ tubes, suggesting a role for septins in stabilizing the active site of polarized morphogenesis (34). However, the cdc10Δ, cdc11Δ, and cdc12-6 mutants rarely produce the multiple germ tube protruberances seen in the wild type or that were seen so frequently in the cdc11-S995A mutant (34). Thus, some phenotypes caused by mutating Cdc11 phosphorylation sites are likely due to the dominant activity of a misregulated septin rather than the absence of septin function.

The cdc12-6 mutant at 37°C also showed more frequent branching of the filamentous outgrowths. This phenotype likely relates to altered cell cycle regulation due to the failure of the germ tubes to undergo septation to form hyphae with different cell compartments. In wild-type cells, the mother cell vacuole swells to force most of the cytoplasmic constituents to the daughter cell compartment at the leading edge of growth (38). Consequently, the mother cells and subsequent subapical cells are delayed in initiating second germ tubes or branches until they can restore the cytoplasmic components. Consistent with this, septin rings persist in the hyphal cells at sites of septation and do not disassemble quickly after septation as they do in budding cells (35, 40). More frequent branching was also seen for septin in mutants of the filamentous fungus Aspergillus nidulans (19, 22).

**Hypha-induced gene expression and filipin staining.** The ability of the cdc12-6 mutant to induce hyphal responses was confirmed by showing that serum and GlcNAc also induced expression of the HWPI-GFP reporter gene (Fig. 6A). In addition, serum also induced a domain at the tips of the germ tubes in both the wild type and the cdc12-6 mutant that stained more readily with the ergosterol binding agent filipin (Fig. 6B). The increased filipin staining is indicative of an altered lipid content in the plasma membrane at hyphal tips (27). Surprisingly, a high proportion (63%) of the cdc12-6 cells shifted to 37°C in the absence of serum for 3 h also showed increased tip staining. This was unexpected because previous studies indicated that this filipin-staining domain was only detected in hyphal and not pseudohyphal cells (27). These results could suggest that cdc12-6 cells induce this response in the absence of hyphal inducers. However, enriched filipin staining is also transiently observed at sites of cytokinesis (2, 27). Thus, a likely alternative possibility is that the filipin staining relates to altered cell cycle regulation in cdc12-6 cells. In support of this latter possibility, enriched filipin staining of the tips of cdc12-6 cells increased over time rather than coinciding with the induction of the initial germ tube outgrowth as seen with serum induction (13, 27).

**Site of second germ tube formation.** A striking defect of the cdc12-6 mutant is that the second germ tube usually formed proximal to the first germ tube (Fig. 7). This contrasts with wild-type cells in which the second germ tube emerges at a distal position. The basal band of septins that forms at the junction between the mother cell and the germ tube is therefore implicated in this process. The role of the basal band of septins is not well understood; septation does not occur at this site, and the septins are detected in a more diffuse pattern than the septin ring seen at sites of septation (18, 35, 40). This raises the possibility that the function of the basal band is to prevent the initiation of second germ tubes proximal to the first. Consistent with this, the basal septin band is stably maintained after the germ tube has undergone cytokinesis and turned into a hyphal cell. Thus, it is stably maintained until later stages when the second germ tube initiates. The formation of the second germ tube at a distal site is significant, since it would help disseminate an infection by promoting growth in a new direction.

This role for septins in germ tube site selection is likely related to their role in bud site selection. In C. albicans, wild-type cells form buds in an axial manner at room temperature but switch to forming buds at nonadjacent bipolar sites at 37°C (10). In contrast, the C. albicans septin mutants primarily budded in an axial manner at 30°C, where wild-type cells had mostly switched to budding at bipolar sites. Thus, septins play roles in preventing the formation of a new site morphogenesis adjacent to an existing bud or germ tube in C. albicans. In contrast, the S. cerevisiae septins are needed for proper axial budding of haploid cells (8), which means they act in a distinct manner to recruit the morphogenesis machinery to an adjacent site.

Altogether, these studies demonstrate that, in addition to their essential role in septum formation, the septins are needed for maintenance of the highly polarized morphology and proper selection of sites of germ tube formation. Both of these are important for dissemination of an infection in host tissues. These conclusions are supported by the defect of cdc10Δ and cdc11Δ mutants in invasive growth into tissues and virulence in a mouse model of candidiasis (39).
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