In many organisms, the G₁ phase of the cell cycle acts as an interface between environmental conditions and the decision to grow or differentiate. In mammalian cells, cyclin D is responsive to the environment (20), while in Saccharomyces cerevisiae, the cyclin Cln3p regulates the length of G₁ in response to multiple environmental signals (32, 38) and is regulated by many signaling pathways (3, 8, 14, 15, 17, 22, 31, 33). The absence of Cln3p causes a delay in G₁ and an increase in cell size, while overexpression of a stabilized form decreases the length of G₁, resulting in smaller cells (7, 30). Cln3p functions with the cyclin-dependent kinase Cdc28p to activate the transcription complexes SBF (Swi4-Swi6 cell cycle box binding factor) and MBF (MluI binding factor) (6, 10), which in turn stimulate transcription of G₁-specific genes such as the cyclins CLN1 and CLN2. Cln1p and Cln2p control the G₁/S transition and associated bud emergence and spindle pole body duplication. In the absence of Cln3p, cells eventually reenter the cell cycle and bud, due in part to the action of Bck2p (11, 37). G₁ phase and Cln3p activity are also modulated during cell differentiation in S. cerevisiae but only under appropriate environmental inducing conditions. In response to mating pheromone, the G₁ phase and Cdc28/Cln activity are blocked by Far1p, and mental inducing conditions. In response to mating pheromone, by deletion of S. cerevisiae response to the environment (20), while in mammalian cells, cyclin D is responsive to the environment (20), while in Saccharomyces cerevisiae, the cyclin Cln3p regulates the length of G₁ in response to multiple environmental signals (32, 38) and is regulated by many signaling pathways (3, 8, 14, 15, 17, 22, 31, 33). The absence of Cln3p causes a delay in G₁ and an increase in cell size, while overexpression of a stabilized form decreases the length of G₁, resulting in smaller cells (7, 30). Cln3p functions with the cyclin-dependent kinase Cdc28p to activate the transcription complexes SBF (Swi4-Swi6 cell cycle box binding factor) and MBF (MluI binding factor) (6, 10), which in turn stimulate transcription of G₁-specific genes such as the cyclins CLN1 and CLN2. Cln1p and Cln2p control the G₁/S transition and associated bud emergence and spindle pole body duplication. In the absence of Cln3p, cells eventually reenter the cell cycle and bud, due in part to the action of Bck2p (11, 37). G₁ phase and Cln3p activity are also modulated during cell differentiation in S. cerevisiae but only under appropriate environmental inducing conditions. In response to mating pheromone, the G₁ phase and Cdc28/Cln activity are blocked by Far1p, and mental inducing conditions. In response to mating pheromone, by deletion of S. cerevisiae response to the environment (20), while in mammalian cells, cyclin D is responsive to the environment (20), while in Saccharomyces cerevisiae, the cyclin Cln3p regulates the length of G₁ in response to multiple environmental signals (32, 38) and is regulated by many signaling pathways (3, 8, 14, 15, 17, 22, 31, 33). The absence of Cln3p causes a delay in G₁ and an increase in cell size, while overexpression of a stabilized form decreases the length of G₁, resulting in smaller cells (7, 30). Cln3p functions with the cyclin-dependent kinase Cdc28p to activate the transcription complexes SBF (Swi4-Swi6 cell cycle box binding factor) and MBF (MluI binding factor) (6, 10), which in turn stimulate transcription of G₁-specific genes such as the cyclins CLN1 and CLN2. Cln1p and Cln2p control the G₁/S transition and associated bud emergence and spindle pole body duplication. In the absence of Cln3p, cells eventually reenter the cell cycle and bud, due in part to the action of Bck2p (11, 37). G₁ phase and Cln3p activity are also modulated during cell differentiation in S. cerevisiae but only under appropriate environmental inducing conditions. In response to mating pheromone, the G₁ phase and Cdc28/Cln activity are blocked by Far1p, and mating projections develop (21). Under nitrogen starvation conditions, cells differentiate into pseudohyphae with a diminished G₁ phase and an elongated G₂ phase, which is enhanced by deletion of CLN3 (26). However, absence of CLN3 alone does not trigger differentiation.

Candida albicans is a pathogenic fungus that exists in multiple cell forms, including yeast, pseudohyphae, and hyphae (35). High temperature (37°C) and serum, for example, stimulate hyphal growth, while low temperature (30°C) and the absence of serum favor yeast cell development. Many signaling pathways link environmental cues to hyphal growth (4), but the relationship between the G₁ phase of the yeast cell cycle and differentiation of hyphae is complex. Nuclear division and separation occur with similar kinetics in hyphae and in yeast cells, and hyphal growth can be induced at any cell cycle stage (19), but germ tubes show hypha-specific localizations in septation and mitosis (19, 36). In addition, C. albicans contains homologues of the S. cerevisiae G₁ cyclins, including Cen1p, Hgc1p, and Cln3p, and two of these factors influence hyphal growth. Deletion of CCN1 results in the inability to maintain hyphal growth under certain conditions (27), while deletion of HGCl1 prevents hyphal growth under all hypha-inducing conditions (40). Neither factor is essential for the cell cycle, suggesting that G₁ cyclin homologues in C. albicans have evolved important roles in hyphal morphogenesis as opposed to cell cycle progression (40). The function of Cln3p (27, 34), however, has not been explored.

We investigated the roles of Cln3p in growth and differentiation in C. albicans and found that it is essential for the yeast but not the hyphal cell cycle. Strikingly, Cln3p can also directly link G₁ progression to differentiation in the absence of environmental inducing signals, in contrast to that demonstrated with Cln3p in S. cerevisiae and G₁ cyclins in other organisms. Thus, an important regulatory relationship may exist between the G₁ phase of the cell cycle and development in C. albicans.

**MATERIALS AND METHODS**

Strains and oligos are listed in Table 1. Growth media included liquid and solid SD media with or without 2.5 mM methionine and 0.5 mM cysine to repress or induce the MET promoter, respectively (5). To determine the effects of CLN3 repression, cells were grown overnight in repressing medium at 30°C, diluted to an optical density at 6,000 nm of 0.2 in either repressing or inducing medium, and examined at different times. For RNA extraction, strains were inoculated into 250 ml of inducing or repressing medium with the addition of 10% fetal calf serum (FCS) (Invitrogen, Burlington, Ontario, Canada) at 37°C. Alternatively,
cells were incubated in IMDM (Invitrogen) supplemented with methionine and cysteine at 37°C.

To delete \( CLN3 \) from strain CAI4, a 3-kb fragment containing the \( CLN3 \) open reading frame and 1 kb of 3' and 5' flanking sequence were amplified by PCR with oligos CB70F and CB70R, respectively, and cloned into pUC18, creating plasmid pCB138. The \( CLN3 \) open reading frame was replaced with a \( hisG-URA3-hisG \) cassette (13), creating plasmid pCB139. The \( CLN3 \) deletion construct was liberated and transformed into strain CAI4. \( Ura^- \) colonies were plated onto 5-fluoroorotic acid, creating the \( Ura^- \) strain CB435. Since the second copy of \( CLN3 \) could not be deleted, it was placed under the control of the \( MET3 \) promoter (16) with oligos CB99F and CB99R. The promoter replacement product was transformed into strain CB435, creating strain CB488. Control strain CB504 was made by transforming strain CAI4 with empty vector pCaDIS (5), which contained the \( URA3 \) marker and the \( MET3 \) promoter to regulate \( CLN3 \) expression in strains lacking \( RAS \), \( CPH1 \), and \( EFG1/CPH1 \), the same strategy was used, creating strains CB498, CB499, and CB501 from strains CDH108, CdDH25, and HLC69, respectively.

To analyze expression of other \( G_1 \) cyclin homologues during repression of \( CLN3 \), Northern analysis was performed. One-kilobase probes for \( HIS1 \), PCK1, and \( CTC1 \) were used, creating strains CB498, CB499, and CB501 from strains CDH108, CdDH25, and HLC69, respectively.

To determine the function of Cln3p, one allele was deleted from strain CAI4 and the second allele was placed under control of the \( MET3 \) promoter. Turning off the only copy of \( CLN3 \) on solid repressing medium under yeast growth conditions of 30°C caused the yeast cells to enlarge and switch to a filamentous morphology (Fig. 1A). The filamentous cells were predominantly unbudded during the first 3 to 4 h and then developed polar evaginations (Fig. 1B; Fig. 2). At 6 h of repression, cells resembled unconstituted filaments (50.2%, \( n = 227 \)), constricted and elongated pseudohyphae (12%), or enlarged unbudded cells (49.8%). By 24 h, the majority of cells were in a filamentous form (Fig. 1B). Since \( CLN3 \) was still repressed at this time (Fig. 1C), it may be essential (9) but only for budding and perhaps not for hyphal and pseudohyphal growth. In contrast, cells grew in the yeast form under inducing conditions, and the control strain grew as yeast cells in both inducing and repressing medium (Fig. 1B). DAPI staining of \( CLN3 \)-repressed cells demonstrated that the enlarged, unbudded cells contained a single nucleus (Fig. 2), consistent with a block in the cell cycle at \( G_1 \). However, nuclear division resumed with formation of filaments. In some cells, the nucleus traveled out into the filament prior to dividing (Fig. 2), as observed in true hyphae (35, 36). Calcofluor staining demonstrated that nuclear division was coupled with septation (Fig. 2). The first septum was positioned distal to the neck in cells that did not contain any constrictions, similar to serum-induced hyphae (4), but the distance between septa in the unconstituted filaments was greater than that observed in hyphae produced in serum (57.1 ± 1.0 \( \mu \)m, \( n = 24 \) versus 22.2 ± 0.3 \( \mu \)m, \( n = 23 \); mean ± standard error of the mean [SEM]). The data suggest that the unconstituted cells are true hyphae. Therefore, Cln3p has a conserved role in regulating the length of \( G_1 \) and cell size during yeast growth, but it also has a novel function in influencing hyphal and pseudohyphal development in the absence of hypha-inducing environmental signals. The fact that cells increase in size prior to differentiation suggests that cell size may be linked to cell differentiation in \( C. albicans \).

### RESULTS AND DISCUSSION

Repression of \( CLN3 \) results in cell enlargement and production of true hyphae and pseudohyphae. To determine the function of Cln3p, one allele was deleted from strain CAI4 and the second allele was placed under control of the \( MET3 \) promoter. Turning off the only copy of \( CLN3 \) on solid repressing medium under yeast growth conditions of 30°C caused the yeast cells to enlarge and switch to a filamentous morphology (Fig. 1A). The filamentous cells were still growing after 24 h and formed wrinkled colonies (data not shown). In contrast, cells plated on solid inducing medium grew normally as yeast cells (Fig. 1A). In liquid repressing medium at 30°C, yeast cells enlarged and were predominantly unbudded during the first 3 to 4 h and then developed polar evaginations (Fig. 1B; Fig. 2). At 6 h of repression, cells resembled unconstituted filaments (50.2%, \( n = 227 \)), constricted and elongated pseudohyphae (12%), or enlarged unbudded cells (49.8%). By 24 h, the majority of cells were in a filamentous form (Fig. 1B). Since \( CLN3 \) was still repressed at this time (Fig. 1C), it may be essential (9) but only for budding and perhaps not for hyphal and pseudohyphal growth. In contrast, cells grew in the yeast form under inducing conditions, and the control strain grew as yeast cells in both inducing and repressing medium (Fig. 1B). DAPI staining of \( CLN3 \)-repressed cells demonstrated that the enlarged, unbudded cells contained a single nucleus (Fig. 2), consistent with a block in the cell cycle at \( G_1 \). However, nuclear division resumed with formation of filaments. In some cells, the nucleus traveled out into the filament prior to dividing (Fig. 2), as observed in true hyphae (35, 36). Calcofluor staining demonstrated that nuclear division was coupled with septation (Fig. 2). The first septum was positioned distal to the neck in cells that did not contain any constrictions, similar to serum-induced hyphae (4), but the distance between septa in the unconstituted filaments was greater than that observed in hyphae produced in serum (57.1 ± 1.0 \( \mu \)m, \( n = 24 \) versus 22.2 ± 0.3 \( \mu \)m, \( n = 23 \); mean ± standard error of the mean [SEM]). The data suggest that the unconstituted cells are true hyphae. Therefore, Cln3p has a conserved role in regulating the length of \( G_1 \) and cell size during yeast growth, but it also has a novel function in influencing hyphal and pseudohyphal development in the absence of hypha-inducing environmental signals. The fact that cells increase in size prior to differentiation suggests that cell size may be linked to cell differentiation in \( C. albicans \).
FIG. 1. Repression of *CLN3* results in hyphal and pseudohyphal growth under yeast growth conditions. (A) Strains CB488 and CB504 grown on solid SD inducing or repressing medium for 24 h at 30°C; (B) strains grown in liquid inducing or repressing medium at 30°C; (C) Northern blot of *CLN3* expression in strains CB488 and CB504 grown in the presence (+) or absence (−) of methionine and cysteine (MC) for the indicated times in liquid SD media. *ACT1* was used as a loading control. Bar, 10 μm.
In contrast, cln3 mutants in *S. cerevisiae* arrest but resume yeast budding due to the activity of Bck2p (11). Since *C. albicans* does not contain a sequence homologue of Bck2p, other factor(s) must be involved in resuming the cell cycle and in triggering cell differentiation as opposed to budding. CLN3 transcripts are not repressed in serum-induced hyphae (29), but Cln3p could be modulated at other levels. Similar findings from Peter Sudbery’s lab (5a) demonstrate that the type of cell produced from *CLN3* repression can vary depending on medium composition, suggesting that Cln3p is responsive to nutrients and may be capable of regulating hyphal growth under certain conditions. Since the timing of cell cycle stages is similar in yeast and apical hyphal cells of *C. albicans* (19), it was surprising that Cln3p was essential for the yeast but not the hyphal cell cycle, suggesting that they may not be regulated in the same manner. Although other G1 cyclin homologues in *C. albicans* have acquired novel functions associated with the *Candida*-specific ability to form true hyphae, Cln3p is distinct in that it has also retained a critical role in regulating yeast cell cycle progression.

**Development of hyphae and pseudohyphae through repression of *CLN3* differentially requires Efg1p, Cph1p, and Ras1p.** To identify other factors that may be important for the Cln3p-depleted phenotype, we constructed a series of mutants where *CLN3* could be shut off in strains lacking *RAS, CST20*, and *EFG1/CPH1* (Fig. 3A). These mutants do not affect yeast growth but impair hyphal formation under different inducing conditions (12, 23–25). Absence of *CST20* did not affect *CLN3*-repressed filamentous growth on solid or in liquid medium (Fig. 3B to D). The absence of *EFG1* and *CPH1* did not affect *CLN3*-repressed filamentation on solid medium, but reduced filamentous growth in liquid medium (Fig. 3B to D). Since many mutants of *C. albicans* show a different ability to form filaments on solid versus liquid hypha-inducing media, the results imply that different signals from the solid versus liquid environment can influence the *CLN3*-repressed phenotype. These results suggest that the absence of Cln3p can trigger differentiation of true hyphae as well as pseudohyphae either through or independent of the Efg1p/Cph1p hyphal signaling pathways, depending on the external conditions. Absence of *RAS*, however, dramatically reduced filament formation and enhanced cell size in both liquid and solid medium (Fig. 3C and D). In addition, cell growth on solid media was significantly impaired, even after 72h (Fig. 3B), suggesting that *CLN3* may be synthetically lethal with *RAS*. The single *RAS* homologue in *C. albicans* is not essential. The deletion mutant demonstrates a slight reduction in growth rate under yeast growth conditions but can only form pseudohyphae under hypha-inducing conditions (12, 24). Therefore, Ras1p clearly has an additional and different role associated with Cln3p function compared to Efg1p/Cph1p, which may involve contributing to G1 progression. Consistent with this, Ras2p in *S. cerevisiae* has been linked to the G1/S transition (33).

**The G1 cyclin homologue Pcl2p is down-regulated in *CLN3*-repressed cells.** In *S. cerevisiae*, a burst in *PCL1, PCL2, CLN1*, and *CLN2* G1 cyclin expression is required for bud morphogenesis and occurs when G1-arrested cells reenter the cell cycle (11, 28). To investigate the expression patterns of G1 cyclin homologues during *CLN3* repression and during resumption
FIG. 3. CLN3 repression in the absence of RAS, EFG1/CPH1, and CST20. (A) Northern analysis of CLN3 expression in ras/ras (CB498), cst20/cst20 (CB499), and efg1/efg1 cph1/cph1 (CB501) backgrounds. CLN3 expression in strains CB504 (CLN3/CLN3) and CB488 (cln3/MET3::CLN3) is also indicated for comparison. Cells were grown for 6 h in repressing or inducing liquid SD medium. (B) Twenty-five microliters of 5 x 10^5 cells of each strain/ml was spread on plates and incubated for 72 h at 30°C. (C, D) Strains grown on solid or in liquid media, respectively, for 24 h at 30°C. Bars, 20 μm. MC, methionine and cysteine; +, present; −, absent.
of the cell cycle in CLN3-repressed filaments, Northern analysis was used with probes to CCN1, HGC1, PCL1, and PCL2 (Fig. 4). CCN1 was up-regulated at 6 and 24 h of CLN3 repression, after filaments had formed, but expression of HGC1 and PCL1 was not detected. However, a homologue of the cyclin PCL2 was down-regulated during all stages of CLN3 repression, even when the cell cycle resumed in the differentiated cells (Fig. 4), suggesting that Pcl2p may be a yeast cell cycle and budding-specific factor, similar to Cln3p.

**Cln3p is required but not essential for cell cycle progression and morphogenesis in environment-induced hyphae.** We next determined whether the absence of CLN3 had any effect on environment-induced hyphal formation. Strain CB488 and control strain CB504 were incubated into liquid repressing medium and incubated at 30°C for 1 h to shut off CLN3 expression and then transferred to repressing medium containing 10% FCS and incubated at 37°C for 90 min. In the absence of CLN3, cells formed germ tubes with normal kinetics, but nuclear movement into the tube was delayed (Table 2; Fig. 5A). This effect was not due to slower growth, since germ tube lengths were similar in Cln3p-depleted and control cells (Table 2). Incubation in inducing medium supplemented with FCS resulted in normal germ tube emergence and nuclear migration (data not shown). When cells were depleted of CLN3 for 3 h to increase size and then transferred to another hypha-inducing medium, IMDM supplemented with methionine and cysteine, hyphae developed but were swollen (Fig. 5B). The cells also demonstrated greater interseptal distances than control hyphae (33.9 ± 0.8 μm, n = 22 versus 22.2 ± 0.3 μm, n = 23; mean ± SEM), and the first septum was placed farther away from the mother yeast cell (18.7 ± 0.2 μm, n = 39 versus 12.2 ± 0.2 μm, n = 30; mean ± SEM) (Fig. 5B). After overnight incubation, the hyphae appeared more swollen and not as long as control cells (Fig. 5B), demonstrating that hyphal morphogenesis was increasingly perturbed with prolonged repression of CLN3. Septation and nuclear division were not blocked, supporting the notion that CLN3 is not essential for the cell cycle in hyphae. However, the greater distances between septa suggest that Cln3p may be required for cell cycle timing. Since morphogenesis and timing of cell cycle progression were both affected by the absence of Cln3p in hyphae, these processes may be linked. It is not clear whether the increase in hyphal size in turn affects the timing and placement of septation, but a relationship between hyphal size and septa deposition has been reported for the fungus Aspergillus nidulans (39).

Specific G1 cyclins act as environment-sensing regulatory modules that influence cell growth and differentiation in many organisms (18, 20). In C. albicans, the G1 cyclin Cln3p is critical for G1 progression during yeast growth but also has a novel function in influencing cell differentiation in the absence of hypha-inducing, environmental cues. These results are unique in that absence of Cln3p in S. cerevisiae or cyclin D in mammalian cells does not trigger differentiation and uncouple developmental progression from normal environmental controls. In addition, blocking the yeast cell cycle of C. albicans at stages other than G1 does not result in true hyphal growth but the development of distinct, checkpoint-associated filaments (1, 2). Therefore, the hyphal regulatory networks in C. albicans may be specifically linked to the G1 phase of the cell cycle through Cln3p function. Although serum can induce germ tubes from preformed buds during later stages of the yeast cell cycle (19), it is not clear whether these represent true hyphae. The results also demonstrate a differential requirement for a cell cycle factor in yeast versus hyphal cells, suggesting that the cell cycle may not be regulated in the same manner in these two cell types. Elucidating the molecular pathways that connect G1 phase and Cln3p function to cell differentiation, including the involvement of Ras1p and Pcl2p, will increase our understanding of the many strategies by which C. albicans can manipulate its cell fate and demonstrate how the cell cycle may be directly coupled to development.
FIG. 5. Repression of CLN3 under hypha-inducing conditions delays nuclear migration and influences hyphal morphology and positioning of septa. (A) Cells were incubated in repressing medium for 1 h at 30°C, transferred to repressing medium containing 10% FCS at 37°C for 1.5 h, fixed, and stained with DAPI and calcofluor; (B) cells were incubated in repressing medium for 3 h at 30°C and then inoculated into IMDM supplemented with methionine and cysteine at 37°C for the times indicated. Bars, 10 μm.
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