The Cdc14p phosphatase affects late cell-cycle events and morphogenesis in *Candida albicans*

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Summary

We have characterized the *CDC14* gene, which encodes a dual-specificity protein phosphatase in *Candida albicans*, and demonstrated that its deletion results in defects in cell separation, mitotic exit and morphogenesis. The *C. albicans cdc14*Δ mutants formed large aggregates of cells that resembled those found in *ace2*-null strains. In *cdc14*Δ cells, expression of Ace2p target genes was reduced and Ace2p did not accumulate specifically in daughter nuclei. Taken together, these results imply that Cdc14p is required for the activation and daughter-specific nuclear accumulation of Ace2p. Consistent with a role in cell separation, Cdc14p was targeted to the septum region during the M-G1 transition in yeast-form cells. Interestingly, hypha-inducing signals abolished the translocation of Cdc14p to the division plate, and this regulation depended on the cyclin Hgc1p, since *hgc1*Δ mutants were able to accumulate Cdc14p in the septum region of the germ tubes. In addition to its role in cytokinesis, Cdc14p regulated mitotic exit, since synchronous cultures of *cdc14*Δ cells exhibited a severe delay in the destruction of the mitotic cyclin Clb2p. Finally, deletion of *CDC14* resulted in decreased invasion of solid agar medium and impaired true hyphal growth.

Key words: Cytokinesis, Cell separation, Mitotic exit, *ACE2*, *HGC1*

Introduction

The opportunistic human pathogen *Candida albicans* exhibits considerable morphogenetic plasticity in response to environmental cues (reviewed by Berman and Sudbery, 2002; Ernst, 2000). It can grow as a unicellular yeast or in multicellular form (e.g. as pseudohyphae or true hyphae), both of which are distinct morphological states (Sudbery et al., 2004). The yeast form closely resembles the budding yeast *Saccharomyces cerevisiae*. The pseudohyphal form consists of chains of elongated yeast cells that have a constriction at the junctions between adjacent cells, whereas hyphae are cylinders with the sides parallel along their entire length. It has been suggested that this morphogenetic plasticity is important for virulence, because mutants that are unable to switch morphologies are less virulent in mouse models (Braun et al., 2000; Lo et al., 1997). However, the precise contribution of morphogenesis to the pathogenesis of this microorganism remains highly controversial (Gow et al., 2002), because the same signals that trigger the morphogenetic switch also induce the expression of virulence factors such as adhesins and the SAP family of aspartyl proteases.

In the dimorphic yeast *S. cerevisiae*, the cell-cycle machinery regulates cell shape (Pruyne and Bretscher, 2000). The G1-phase-cyclin–Cdk1 complex promotes apical and tubular growth, whereas complexes of B-type Clb and Cdk1 activate isotropic growth (Lew and Reed, 1993; Rua et al., 2001). Mutations that delay the apical/isotropic switch confer an enhanced pseudohyphal growth phenotype, whereas premature activation of the switch results in a round morphology. Correlating well with these data, the G2-M transition is delayed in *S. cerevisiae* pseudohyphal cells, although through an unknown mechanism (Ahn et al., 2001; Kron et al., 1994).

In *C. albicans*, the link between the cell cycle and morphogenesis is complex. Asynchronous cultures of yeast cells exposed to serum at high temperature can induce germ tubes throughout the cell cycle (Hazan et al., 2002), although it is not clear whether the germ-like tubes emerging from G2-M cells have the hallmarks of true hypha. By contrast, when *C. albicans* cells are challenged with high temperature and high pH, there is a restriction point after which a budding cell cannot produce hyphal projections until the next cell cycle (Soll et al., 1985). In the *C. albicans* genome, there are three gene homologs to the *S. cerevisiae* G1 cyclins: *CCN1*/*CLN1*, *CLN3* and *HGC1*. Ccn1p/Cln1p does not play a major role in morphogenesis (Loeb et al., 1999), whereas Hgc1p regulates hyphal growth under all hypha-inducing conditions (Zheng and Wang, 2004). Intriguingly, it has been shown that Cln3p-depleted cells spontaneously form hyphae under yeast growth conditions, suggesting that its activity negatively regulates the yeast-hyphal transition (Bachewich and Whiteway, 2005; Chapa y Lazo et al., 2005). Deletion of the forkhead transcription factor Fkh2p is associated with increased levels of the B-type cyclin CaClb4p and constitutive pseudohyphal growth (Bensen et al., 2002). In addition, overexpression of B-type cyclins reduces the extent of filamentous growth (Bensen

et al., 2005), suggesting that Clb cyclins might act as antagonists of hyphal growth.

The conserved Cdc14p family of dual-specificity protein phosphatases function to downregulate mitotic Clb/Cdk1 activities. In *S. cerevisiae*, Cdc14p plays an essential role in the inactivation of mitotic Cdk at the end of mitosis by promoting the proteolysis of mitotic cyclins and by allowing the accumulation of the Cdk inhibitor Sic1p (Visintin et al., 1998). Regulation of Cdc14p in *S. cerevisiae* is achieved by three complex mechanisms controlling its subcellular localization. During most of the cell cycle, Cdc14p is sequestered in the nucleolus in an inactive state by the RENT complex through its interaction with the anchor protein Net1p (Shou et al., 1999; Traverso et al., 2001). Upon entry into anaphase, the FEAR network promotes a transient release of Cdc14 from the nucleolus during early anaphase (Stegmeier et al., 2002), after which the MEN network maintains Cdc14p in this released state until telophase (Jaspersen et al., 1998).

Here, we analyzed the function of the *C. albicans CDC14* in the different morphological states, in order to investigate whether its hypothetical activity as a negative regulator of B-type cyclins might influence hyphal morphology. In this study, we demonstrate that Cdc14p affects late cell-cycle events, such as Clb2p destruction and cell separation. In addition, *cdc14*Δ cells showed impaired hyphal and invasive growth, suggesting that Cdc14p activity is also required for morphogenesis in this fungal pathogen.

Results

Cdc14p is not essential for vegetative growth

Scrutiny of the *C. albicans* database (http://www-sequence.stanford.edu/group/candida/index.html) revealed an open reading frame (ORF) (*orf6.2154*) that had significant homology to the *S. cerevisiae CDC14* gene. Structural comparison of the *S. cerevisiae* Cdc14p with the *C. albicans* homolog unveiled a conserved core of 330 amino acids located in the region of the N-terminus (67% identity), where the protein tyrosine phosphatase (PTP) signature motif is located, followed by a more divergent region (24.5% identity). Interestingly, within this region, the *C. albicans* Cdc14p contained two putative Cdk consensus phosphorylation sites (S/TPXK/R), one located in a possible nuclear localization signal (NLS) and the second in a potential PEST sequence (PESTfind score +5.93) that were absent in the *S. cerevisiae* counterpart (Fig. 1A).

In order to study the function of *CDC14* in *C. albicans*, a conditional strain was constructed in which a single remaining copy of *CDC14* was placed under the control of the *MET3* promoter (Care et al., 1999). *MET3-CDC14* cells grew normally under repressing conditions (not shown), suggesting that *CDC14* was not an essential gene in *C. albicans*, in contrast to its *S. cerevisiae* counterpart (Wan et al., 1992). To confirm this observation, a homozygous *cdc14*Δ*/cdc14*Δ mutant was constructed using the *URA3* Blaster method (Fonzi and Irwin, 1993) in two wild-type backgrounds: CAI4 and

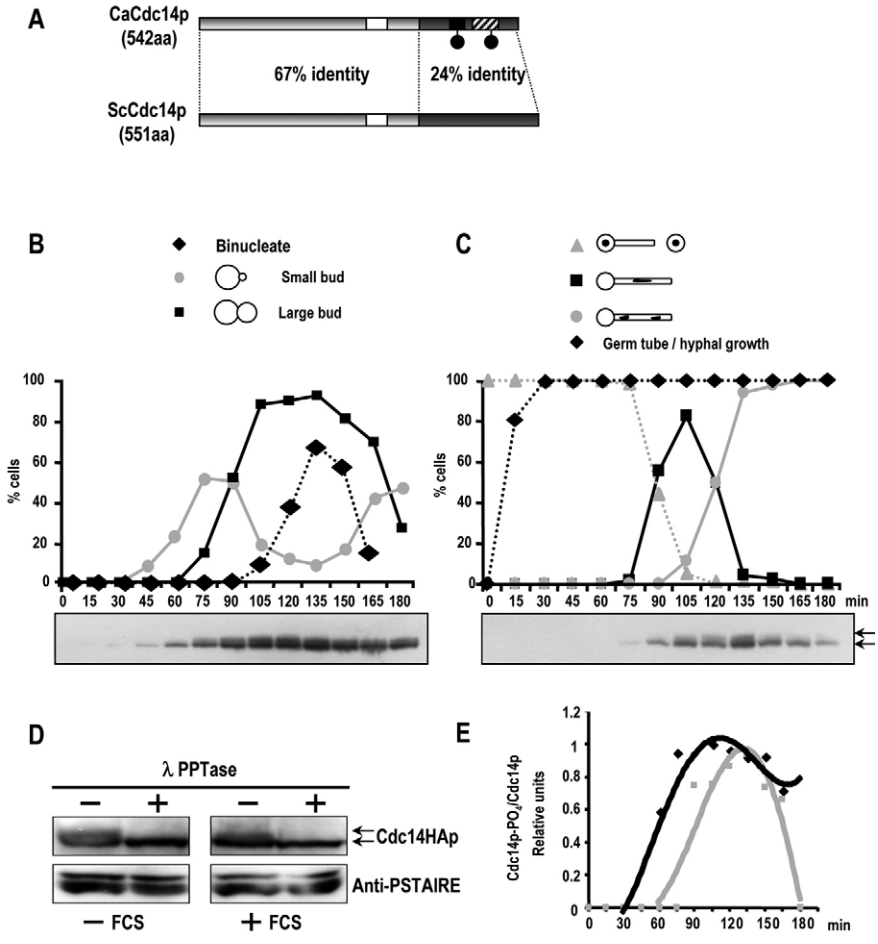

Fig. 1. Cdc14p levels are cell-cycle regulated. (A) Structural comparison of the *S. cerevisiae* Cdc14p (ScCdc14p) phosphatase with the *C. albicans* Cdc14p homolog (CaCdc14p). White box, PTP motif; black box, putative NLS; hatched box, PEST sequence; black dots, Cdc28 phosphorylation sites. (B,C) Small G1 cells carrying Cdc14p-HA (JC94) were isolated by elutriation and released into (B) YPD at 30°C (yeast growth) or (C) YPD + 5% FCS at 37°C (hyphal growth). Samples were collected at intervals of 15 minutes after release and were assayed by western blotting for Cdc14p-HA levels. (D) Mitotic extracts corresponding to sample at 120 minutes from experiment B (– FCS) or sample at 135 minutes from experiment C (+ FCS) were treated with (+) or without (–) λ phosphatase (λ PPTase) for 30 minutes. Samples were separated by SDS-PAGE and probed with anti-HA antibodies (12CA5). Anti-PSTAIRE antibody to detect Cdc28p was used as loading control. (E) Ratio of phosphorylated (Cdc14p-PO_4) versus unphosphorylated (Cdc14p) forms of Cdc14p during yeast (black line) and hyphal growth (gray line). The ratio was calculated using Adobe Photoshop 7.0 software to quantify the intensity of the signals of the blots.

BWP17. The *cdc14Δ* mutants obtained in both genetic backgrounds displayed similar phenotypes, as described below. The growth rate of *cdc14Δ/cdc14Δ* cells, as determined by dry weight curves, was similar to that of wild-type cells, suggesting no obvious defect in cell growth (not shown). Therefore, these observations indicated that *CDC14* is not essential for cell viability in *C. albicans*.

Cdc14p levels are regulated throughout the cell cycle

To study Cdc14p regulation in *C. albicans*, a *CDC14-HA/CDC14* strain was constructed. Addition of the HA epitope did not interfere with Cdc14p activity, since *CDC14-HA/cdc14::HIS1* cells behaved as *CDC14/cdc14::HIS1* control cells (not shown). To examine Cdc14p levels along the cell cycle during budding and hyphal growth, small G1 cells carrying the *CDC14-HA* allele were isolated by elutriation and released into liquid YPD medium at 30°C (yeast-inducing conditions) or YPD plus 5% FCS at 37°C (hypha-inducing conditions). Samples were collected every 15 minutes after release to prepare protein extracts and analyzed by western blotting, using anti-HA antibodies (12CA5). A dramatic change in Cdc14p protein levels was observed, both during budding (Fig. 1B) and hyphal growth (Fig. 1C). In both cases, no detectable levels of Cdc14p-HA were observed in G1 cells but, as cells passed through S-phase, the protein began to accumulate, its highest level being reached during mitosis. In addition, there was a significant alteration in the molecular weight of the protein at the time when cells were undergoing mitosis and cytokinesis (Fig. 1B, 105-135 minutes). To determine whether these slower-migrating forms were a result of phosphorylation, protein extracts from mitotic cells were treated with λ phosphatase (λ PPTase). Addition of λ PPTase to the extracts produced a shift in Cdc14p to the faster-migrating form (Fig. 1D), indicating that Cdc14p had indeed been phosphorylated. The ratio of phosphorylated to unphosphorylated forms of Cdc14p showed that the protein was mainly phosphorylated as the cells were passing through mitosis (Fig. 1E). In comparison with yeast form cells, the accumulation and phosphorylation of Cdc14p was delayed in hyphal-induced cells, indicating that the timing of cell-cycle progression occurred with different kinetics in hyphae and in yeast cells. Thus, these observations show that the levels of Cdc14p are cell-cycle-regulated during budding and hyphal growth in *C. albicans*, and that the protein is phosphorylated during mitosis.

Localization of Cdc14p

To study the in vivo localization of Cdc14p, a plasmid carrying a *CDC14-GFP* construct under the *ACT1* promoter (pJJ1) was transformed into the CAI4 strain. In *S. cerevisiae*, strong expression of *CDC14* promotes mitotic exit and G1 arrest (Visintin et al., 1998), whereas increased expression of *Schizosaccharomyces pombe flp1*$^{+}$, the fission yeast *CDC14* ortholog, blocks cells in G2 (Cueille et al., 2001; Trautmann et al., 2001). Flow cytometry analysis indicated that *C. albicans* cells carrying the pJJ1 plasmid did not show any delay in cell-cycle progression as compared with *CAF2* control cells (not shown). The absence of cell-cycle arrest was not a result of the inactivation of Cdc14p function by the insertion of green fluorescent protein (GFP), since plasmid pJJ1 was able to complement a *cdc14Δ/cdc14Δ* strain (not shown). These results indicate that the expression of *CDC14* under the *ACT1* promoter does not interfere with cell-cycle progression.

Time-lapse microscopy images of unbudded yeast cells bearing plasmid pJJ1 showed that the fluorescent signal was cell-cycle regulated. In good correlation with the variation in protein levels, no GFP signal was detected in small unbudded G1 cells, whereas Cdc14p-GFP started to accumulate in the nuclei of the cells at G1-S transition (small budded cells) and reached a maximum during mitosis (Fig. 2A). When the cells had completed cytokinesis and entered the next cell cycle, the Cdc14p-GFP signal disappeared from the nucleus. Interestingly, we noticed that a fraction of Cdc14p-GFP also localized to the bud neck at the end of mitosis (see arrow, Fig. 2A). To confirm this observation further, Calcofluor White was added to cells carrying *CDC14-GFP* to stain the chitin present in the septum region. As shown in Fig. 2B, the Calcofluor signal and the green fluorescence of Cdc14p-GFP colocalized, indicating that Cdc14p was indeed present in the septum region of yeast cells. This localization was dependent on septins since *cdc10Δ* cells bearing the pJJ1 plasmid did not show any GFP signal at the bud neck (not shown).

A key mechanism by which Cdc14p is regulated both in *S. cerevisiae* and in *S. pombe* involves shuttling of the protein between the nucleolus and the cytoplasm (reviewed by

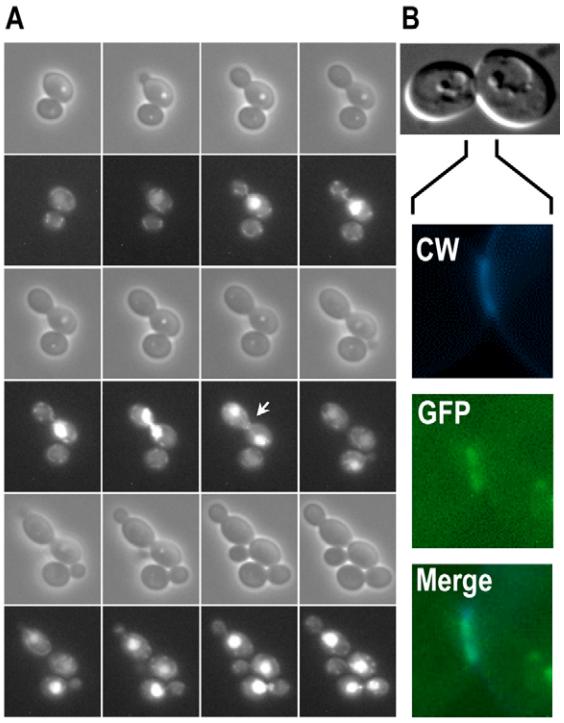

Fig. 2. Localization of Cdc14p during yeast growth. (A) Time-lapse fluorescence microscopy of unbudded yeast cells transformed with a plasmid containing the *pACT1-CDC14-GFP* construct (JJ22). Cells were mounted on glass slides containing 2% agar and differential interference contrast (DIC) (rows 1, 3 and 5) or GFP (rows 2, 4 and 6), and pictures were taken every 15 minutes. Note the presence of the GFP signal at the bud neck after the nucleus has migrated to the daughter cell (arrow). (B) Images of a single cell that was simultaneously stained with Calcofluor White (CW) and GFP are shown, and the overlap of both images (Merge).

Stegmeier and Amon, 2004). To examine the localization of Cdc14p at physiological levels, we constructed a strain in which the gene encoding yellow fluorescent protein (YFP) was fused to one allele of *CDC14* in a *NOP1-CFP* (nucleolar marker) background. Observation of living *CDC14-YFP NOP1-CFP* cells showed that Cdc14p did not accumulate in the nucleolus in interphase cells. At the G1-S transition, Cdc14p-YFP started to accumulate both in the nucleolar-staining (Nop1 signal) and the 4′,6-diamidino-2-phenylindole (DAPI)-staining region of the nucleus (Fig. 3A, cells 1-3). At the S-G2 transition, Cdc14p-YFP started to concentrate as faint foci on the nuclear periphery (Fig. 3B, cell 1). In early mitosis, the Cdc14p nuclear signal diminished and Cdc14p-YFP concentrated most intensely as two strong dots (Fig. 3B, cells 2-3) that persisted until late mitosis (cell 4 in Fig. 3A,B). These foci on the nuclear periphery could correspond to the spindle pole body (SPB), as judged by colocalization with Tub2-CFP (Fig. 3C). During cytokinesis, the Cdc14p-YFP signal at the SPBs was fainter and the protein was preferentially found at the septum region (Fig. 3D). Thus, all these results stress a key difference between *C. albicans* and other eukaryotes in Cdc14p regulation, since in *C. albicans* the protein is degraded at the end of mitosis, instead of being sequestered into the nucleolus.

In *C. albicans*, asymmetric cell division leads to hyphal forms, where subapical compartments are often arrested in G1, whereas apical cells are actively dividing (Barelle et al., 2003). During this process, cytokinesis occurs in the absence of cell separation. To monitor the localization of Cdc14p during hyphal growth, yeast cells carrying pJJ1 were grown in liquid YPD with 5% FCS at 37°C, and samples were collected for fluorescence microscopy. We found that Cdc14p-GFP was detected only at the nucleus of the apical compartment of the hypha, whereas no signal was present in any of the G1-arrested subapical compartments (Fig. 4, see lines indicating the septa). Moreover, in striking contrast to yeast forms, Cdc14p-GFP was never observed at the septum region in any of the compartments of the hypha (*n*=30 GFP-positive hyphae). Together, these data show that the main difference in the spatial regulation of Cdc14p-GFP during yeast and hyphal growth is the localization of the protein at the septum region during cytokinesis. In yeast forms, in which cell separation takes place after cytokinesis, Cdc14p-GFP is present at the bud neck; in

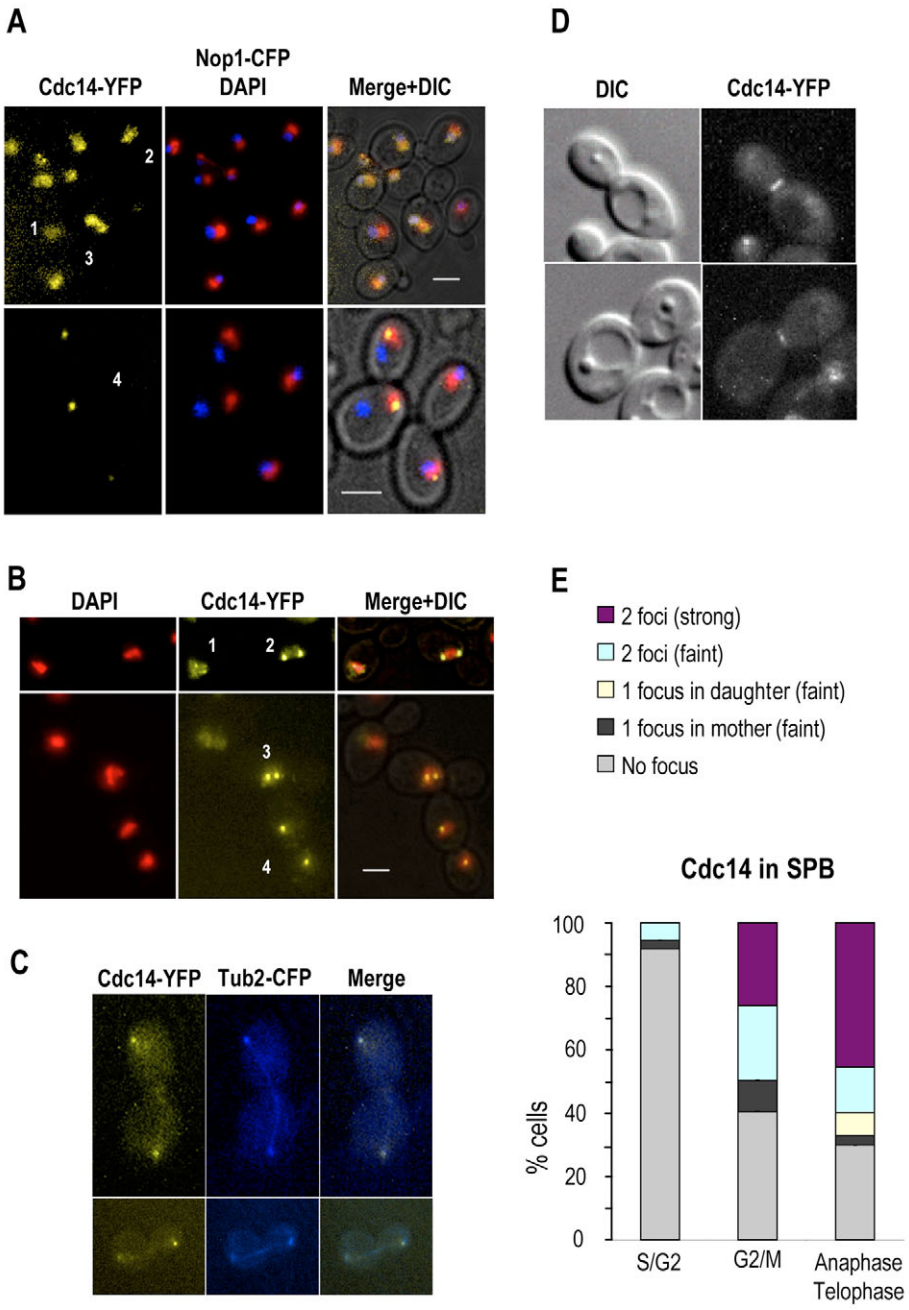

Fig. 3. Localization of Cdc14p-YFP expressed from its endogenous promoter. (A) Cdc14p is not tethered to the nucleolus during interphase. Exponentially growing cells expressing *CDC14-YFP NOP1-CFP* (JC252) were prepared for microscopy. Images of Cdc14p-YFP, Nop1p-CFP (blue) plus DAPI (red) and the merge of the three channels plus DIC (Merge+DIC) are shown. Representative photographs with different cell-cycle stages are shown (see text). (B) Cdc14p is located at the SPB. Exponentially growing cells expressing *CDC14-YFP/CDC14-YFP* (JC189) were grown in YPD at 25°C and scored for Cdc14p-YFP at foci (*n*>100; representative photographs with faint and strong foci signal are shown). (C) Micrograph of a cell in anaphase from strain JC310 (*CDC14-YFP TUB2-CFP*). (D) Cdc14p is localized to the bud neck in a fraction (35% of binucleated cells) of large budded cells (JC189). (E) Quantification of the localization of Cdc14p at the SPB at different stages of the cell cycle from the experiment shown in B. Bars, 2 μm.

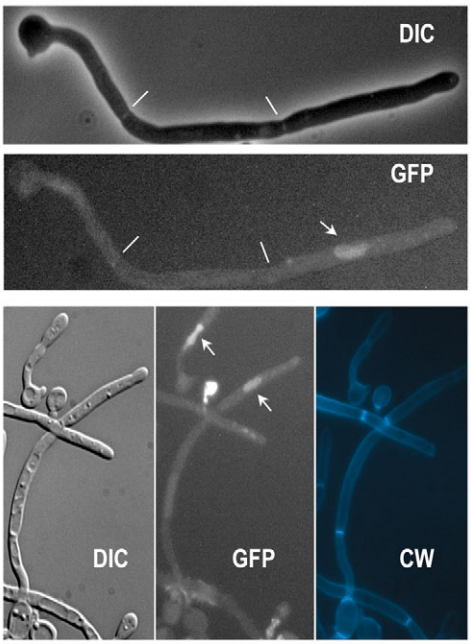

Fig. 4. Localization of Cdc14p-GFP in hyphal cells. Cells expressing Cdc14p-GFP (JJ22) were grown in YPD plus 10% FCS at 37°C to induce hypha formation before images were taken. Images using DIC, GFP staining or Calcofluor White staining (CW) to visualize septa are shown. Note that Cdc14p is located in the nucleus of the apical cell (arrows). Lines: position of septa.

hyphal forms, in which cell separation is inhibited, no fluorescent signal is detected.

Cdc14p is required for cell separation

To investigate whether the presence of Cdc14p in the septum of yeast cells has any biological significance, cell morphology was analyzed during vegetative growth. In contrast to the wild-type strain, in which yeast cells readily separated after cytokinesis, we found that exponentially growing *cdc14*Δ cultures formed large clumps of cells that could be dispersed by sonication (Fig. 5A). To confirm that mutant cells had completed cytokinesis but had a defect in septum degradation, we used electron microscopy. This confirmed that *cdc14*Δ cells have assembled normal septa, in which primary and secondary septa can be seen (Fig. 5B). Thus, these results indicate that the absence of Cdc14p does not interfere with cytokinesis and septation, but results in defective cell separation.

In *S. cerevisiae*, cell separation requires the controlled action of at least two hydrolytic enzymes: the chitinase encoded by the *CTS1* gene (Kuranda and Robbins, 1991) and the endo-β-1,3-glucanase encoded by *ENG1* (Baladrón et al., 2002). In *C. albicans*, three chitinase genes (*CHT1*, *CHT2* and *CHT3*) have been cloned and it has been proposed that Cht2p has a similar function to ScCts1p (McCreath et al., 1995). It has also been shown recently that disruption of the *ENG1* gene results in the formation of chains of cells (Esteban et al., 2005). To investigate whether the cell separation defect observed in *cdc14*Δ mutants might be a result of a decrease in the transcription of these hydrolase genes, we analyzed the expression of *CHT3* and *ENG1* by northern blot. The results indicated that, in contrast to wild-type cells, *cdc14*Δ mutants showed a dramatic decrease in the expression of these genes (Fig. 6B). Introduction of a wild-type copy of *CDC14* into the *cdc14*Δ/*cdc14*Δ mutant restored the transcription of these genes (Fig. 6B), and also suppressed the separation defect (not shown).

Expression of Ace2p-regulated genes is downregulated in *cdc14*Δ mutant cells

In *S. cerevisiae*, a daughter-specific transcription program induces mother-daughter separation (Colman-Lerner et al., 2001; Nelson et al., 2003). This genetic program depends on the transcription factor Ace2p, which regulates the expression of a group of genes involved in cell separation, including *CTS1*, *ENG1* and *DSE1* (Baladrón et al., 2002; Colman-Lerner et al., 2001; Doolin et al., 2001). Thus, *ace2* mutants grow as clumps of cells (Racki et al., 2000). A search of the *C. albicans* database revealed the presence of an ORF (*orf6.5105*) with strong similarity to ScAce2p. To investigate whether the product of *orf6.5105* could also be involved in cell separation, we deleted both copies of the gene in the BWP17 strain. Exponentially growing cultures of the homozygous mutant formed large cell aggregates that clearly resembled those previously described for *S. cerevisiae ace2* mutants (Fig. 6A). Moreover, expression of *DSE1*, *CHT3* and *ENG1* was very reduced in this mutant, as assessed by northern blot (Fig. 6B). Taken together, these observations indicate that *orf6.5105* could be the functional homolog of *S. cerevisiae ACE2* and henceforth will be referred to

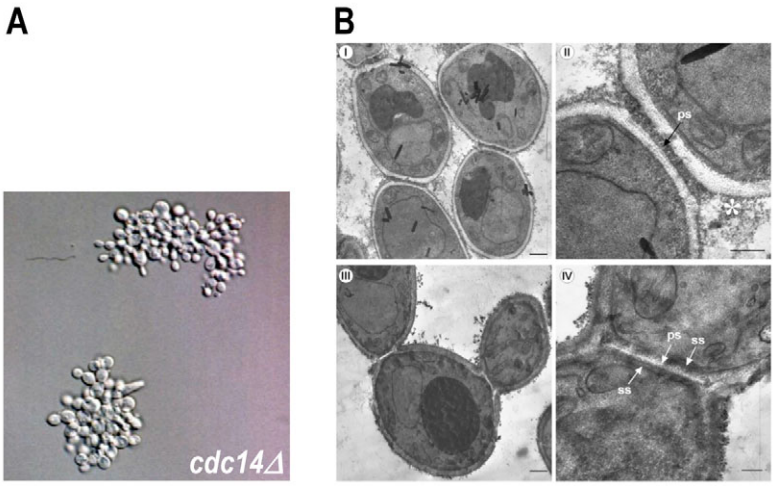

Fig. 5. *cdc14*Δ cells have severe defects in cell separation. (A) Morphology of *cdc14*Δ/*cdc14*Δ mutant cells grown in YPD at 30°C (JC13). (B) Electron microscopy of wild-type (BWP17) and *cdc14*Δ mutant (JC13) cells. Wild-type (panels I and II) and *cdc14*Δ mutant (panels III and IV) cells were grown to mid-log phase before preparation for electron microscopy. The neck region of *cdc14*Δ mutants (IV) shows the presence of normal septum, in which the primary (ps) and secondary septa (ss) can be easily visualized. Note that cell separation has already started in the septum of the wild-type cell shown in II, and the cell wall has already been dissolved, but the primary septum is still present (white *). Bars: I and III, 0.5 μm; II and IV, 0.2 μm.

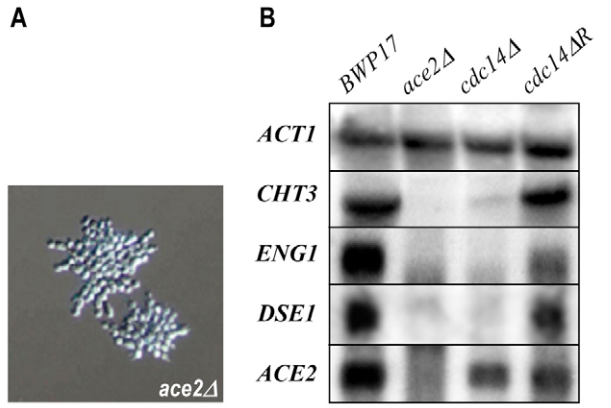

Fig. 6. (A) Morphology of *ace2*Δ/*ace2*Δ mutant cells (JC99) grown in YPD at 30°C. (B) *cdc14*Δ cells show reduced expression of cell-wall-degrading enzymes. Polyadenylated RNA isolated from exponentially growing cells of the indicated strains was probed with specific probes for *ACT1*, *CHT3*, *ENG1*, *DSE1* and *ACE2* genes.

as *ACE2*. Similar results have been described recently (Kelly et al., 2004).

The reduction in the transcription of Ace2p target genes observed in *cdc14*Δ cells was not due to a defect in *ACE2* expression, since *ACE2* transcript levels were similar to that of wild-type cells (Fig. 6B). These results therefore suggest that Cdc14p is necessary for full activation of the Ace2p transcriptional programme responsible for cell separation during yeast growth.

Cdc14p is required for localization of Ace2p to the daughter nucleus

Similar to *S. cerevisiae*, Ace2p accumulates in the nucleus of the daughter yeast cell (Colman-Lerner et al., 2001; Kelly et al., 2004). To investigate further the nature of the cell separation defect of *cdc14*Δ mutants, the localization of Ace2p was analyzed in wild-type and mutant cells using an Ace2p-GFP fusion protein. In contrast to the wild-type strain, where Ace2-GFP was mainly detected in the daughter nuclei as described (Kelly et al., 2004), we never found Ace2p nuclear accumulation in *cdc14*Δ cells, and only a faint cytoplasmic fluorescence could be seen in many of the cells (Fig. 7B). Therefore, this result shows that Cdc14p is required for daughter-specific nuclear accumulation of Ace2p in *C. albicans.*

Hgc1p inhibits Cdc14p septum localization during hyphal growth

Our findings indicate that Cdc14p is an activator of cell separation, and that it is targeted to the septum region specifically during growth in the yeast form, since this recruitment is abolished during hyphal growth, a process in which cell separation after cytokinesis is inhibited. To gain further insight into the signals that prevent Cdc14p accumulation in the septum region in response to hypha-inducing signals, we analyzed the subcellular localization of Cdc14p-YFP in a *hgc1* mutant strain. *HGC1* encodes a G1-like cyclin that plays an important role in preventing cell separation during hyphal growth (Zheng and Wang, 2004). In exponentially growing *hgc1*Δ *CDC14-YFP* cells at 30°C (yeast

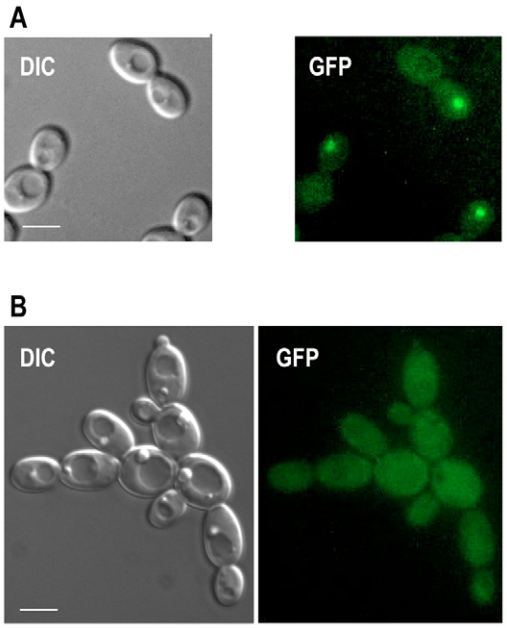

Fig. 7. Localization of Ace2p in the daughter nucleus depends on Cdc14p. Exponentially growing cells expressing Ace2p-GFP in wild-type (A, SCAY1) or *cdc14*Δ mutant (B, JC198) cells were visualized using DIC and fluorescence (GFP) microscopy. Bars, 5 μm.

growth conditions), the localization of Cdc14p-YFP was similar to that of control cells (not shown). However, when these cells were incubated in hypha-inducing conditions (YPD plus 10% FCS at 37°C), cell separation occurred in germ tubes, as previously described. Interestingly, we found that Cdc14p-YFP was present in the septum region in these actively separating cells (Fig. 8). This observation thus suggests that Hgc1p activity is required to prevent the localization of Cdc14p at the septum region in response to hypha-inducing signals.

The phosphatase Cdc14p is required for mitotic exit

In *S. cerevisiae*, Cdc14p is a component of the MEN pathway and plays an essential role in downregulating B-type cyclins during mitotic exit (Schwab et al., 1997; Schwob et al., 1994; Visintin et al., 1998). To test whether the *C. albicans* Cdc14p might have a similar role in the regulation of mitotic cyclin levels, we determined the amount of Clb2p-HA and Clb4p-HA in asynchronous *cdc14*Δ cultures. Relative to wild-type levels, Clb2p and Clb4p were detected at 2.15-fold and 1.95-fold higher levels in *cdc14*Δ cells, respectively (Fig. 9A). Moreover, flow cytometry analysis of exponentially growing *cdc14*Δ cultures revealed a marked increase in the number of cells with a 2C DNA content, as compared with the wild-type strain grown in YPD or SC medium (Fig. 9B). Both results indicate that Cdc14p also plays a role in cell-cycle progression in *C. albicans*. To study this possibility further, wild-type and *cdc14*Δ cells carrying an HA-tagged version of the *CLB2* gene were synchronized by elutriation and used to compare Clb2p protein levels during the first cell cycle (Fig. 9C). In the wild-type strain, Clb2p-HA levels underwent a periodic oscillation, increasing to a maximum at 75-90 minutes and then decreasing to its lowest level (120-135 minutes). However, in the *cdc14*Δ mutant, the reduction in Clb2p-HA levels was not observed during the course of the

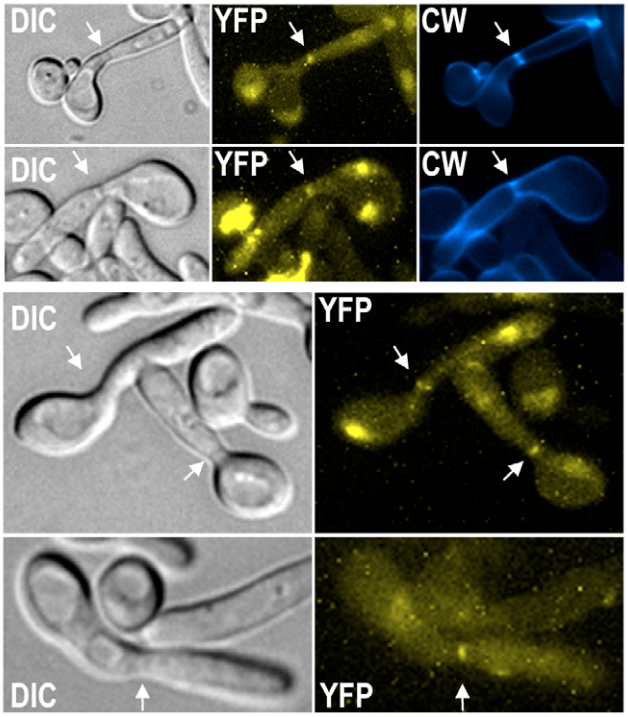

Fig. 8. Localization of Cdc14p-YFP in *hgc1*Δ cells. Exponentially growing cells of strain JC194 (*hgc1*Δ *CDC14-YFP/CDC14*) were induced for hyphal growth in YPD plus 10% FCS for 120 minutes and cells were stained with Calcofluor White (CW). DIC, CW and YFP fluorescence images are shown. Arrows indicate position of the septum.

experiments, suggesting a long delay in mitotic exit. Consistent with this lag, asynchronously growing *cdc14*Δ cells were 1.8-fold larger than BWP17 cells (84.3±25 versus 49.01±20.4 relative units; n=100). Despite the delay in the cell-cycle progression of *cdc14*Δ cells, their larger size could explain why the increase in dry weight in *cdc14*Δ and wild-type cells was similar. Thus, in addition to its role in cell separation, Cdc14p is also required for the degradation of Clb2p and timely mitotic exit in *C. albicans.*

Cdc14p is required for invasive and hyphal growth

In *S. cerevisiae*, cell-cycle regulators are involved in morphogenesis, controlling the apical/isotropic switch (Rua et al., 2001). In *C. albicans*, it has been shown very recently that strains overexpressing either *CLB2* or *CLB4* produce hyphae less efficiently (Bensen et al., 2005). Since Cdc14p depletion produced an increase in the levels of mitotic cyclins, we examined whether invasive and hyphal growth was also affected in the *cdc14*Δ mutant. To study invasive growth, we

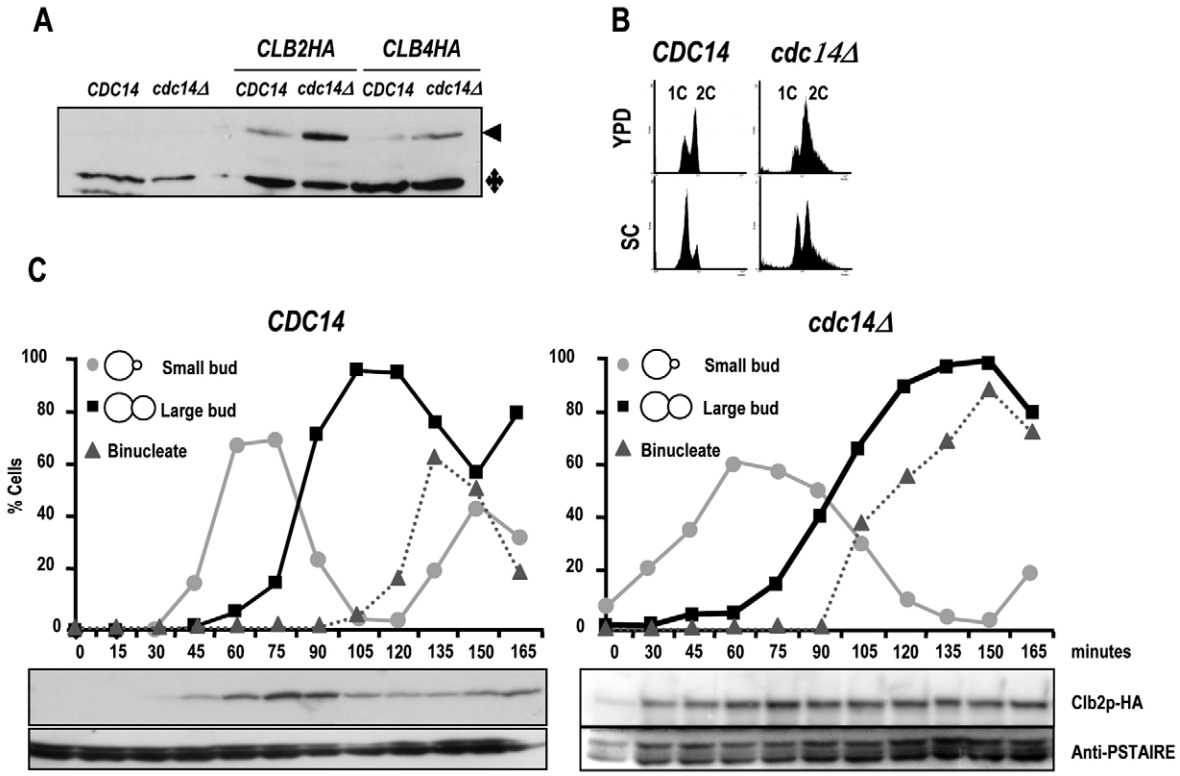

Fig. 9. *cdc14*Δ cells show upregulated levels of B-cyclins. (A) Protein extracts from asynchronous cultures of the indicated strains grown in YPD were prepared and analyzed by western blot using 12CA5 antibodies to detect HA-tagged proteins. Triangle, Clbs-HA; diamond, a cross-reacting band used as loading control. (B) Flow cytometry analysis of asynchronous cultures of strains *CDC14* and *cdc14*Δ grown in YPD or SC media at 30°C. (C) G1 cells from strains JC64 (*CDC14/CDC14*; *CLB2HA/CLB2*) and JC70 (*cdc14*Δ*/cdc14*Δ; *CLB2HA/CLB2*) were isolated by elutriation and released into YPD at 30°C. Samples were collected at intervals of 15 minutes after release, and protein extracts were prepared and assayed by western blotting for Clb2p-HA levels. Cdc28p (anti-PSTAIRE antibody) was used as loading control. Samples were also monitored for bud morphology and DAPI staining.

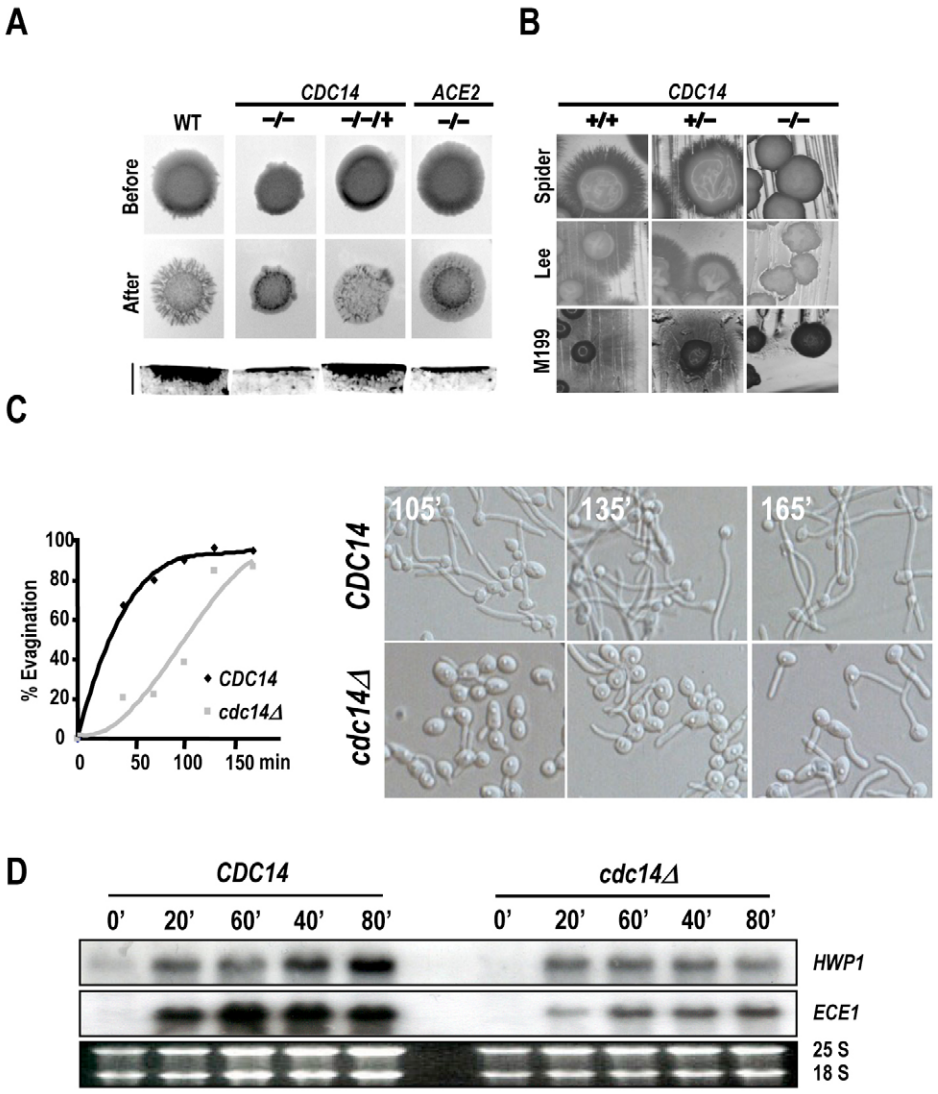

Fig. 10. Cells lacking Cdc14p display impaired invasive and hyphal growth. (A) Cells of the indicated genotype were grown on YPD agar plates at 30°C for 7 days. Colonies were photographed before and after the plates had been washed. After washing, transverse sections (TS) were cut from the plates and photographed. Bar, 5 mm. (B) Colony morphology of the indicated strains grown on different hypha-inducing media after 5 days at 30°C. (C) Overnight cultures of strains JC190 (*CDC14/CDC14*) and JC12 (*cdc14*Δ/*cdc14*Δ) were diluted in pre-warmed YPD plus 5% serum at 37°C. At the indicated times, cells were removed and fixed to monitor response (% evagination) and the length of germ tubes. (D) Analysis of *ECE1* and *HWP1* expression by northern blot in *CDC14/CDC14* and *cdc14*Δ/*cdc14*Δ strains at different times after cells had been transferred to YPD plus 5% FCS at 37°C.

analyzed the ability to invade the agar substrate by plating wild-type and *cdc14*Δ mutants on YPD agar and incubating the plates at 30°C for 7 days before the plates were washed. Frontal views of the washed plates did not reveal large differences between the two strains (Fig. 10A). However, transverse sections of the plate showed that the degree of penetration of both strains was significantly different, the *cdc14*Δ mutant being considerably less invasive than the wild-type strain. Introduction of the wild-type *CDC14* gene into the *cdc14/cdc14* mutant restored the invasive growth defect. The *ace2*Δ strain showed the same phenotype as that of the *cdc14*Δ strain. Since Cdc14p is required for nuclear accumulation of Ace2p (Fig. 7), this observation suggests that the invasive growth defect observed in *cdc14*Δ cells could be due to their inability to activate the transcription of Ace2p target genes, some of which might be required for invasive growth.

To examine whether *cdc14*Δ cells might have some hyphal growth defect, we analyzed the morphology of the *cdc14*Δ strain under different hypha-inducing conditions. On Spider, Lee or M199 solid media, the *cdc14*Δ mutant strain was unable to form hyphae (Fig. 10B), whereas the wild-type strain generated extensive hyphal colonies. The heterozygous *CDC14/cdc14* strain produced intermediate hyphal colonies. To study the response to serum, a strong inducer of hyphal growth, wild-type and *cdc14*Δ cells were incubated in liquid YPD medium containing 5% FCS at 37°C, and samples were taken at different intervals and fixed to monitor the response. Cells without Cdc14p showed a substantial delay in their response to serum as compared with wild-type cells, producing shorter germ tubes (Fig. 10C). To determine whether these germ tubes gave rise to true hyphae, we used the criteria proposed by Sudbery et al. (Sudbery et al., 2004) in G1 elutriated *cdc14*Δ cells in liquid YPD containing 5% FCS at 37°C. In these cells, the first mitosis occurred within the germ tube and the septa localized 15 μm (±1, *n*=20) from the germ tube neck (not shown), indicating that *cdc14*Δ cells develop true hyphae.

The defect in response to serum observed in the *cdc14*Δ mutant strain could be due either to a defect in sensing the hyphal induction signal or to an inability to initiate/maintain polarized growth. A conserved Ras-cAMP signaling pathway activates hyphal growth in response to serum. The transcription factor Efg1p is a key component of this pathway, which activates the expression of several hypha-specific genes, such

as *ECE1* (Birse et al., 1993), *HWP1* (Sharkey et al., 1999; Staab et al., 1996), *HYR1* (Bailey et al., 1996; Birse et al., 1993), *RBT1* and *RBT4* (Braun et al., 2000). To determine whether this signaling pathway was fully activated in *cdc14*Δ mutants, we analyzed the expression of *ECE1* and *HWP1* as markers of activation of the Ras-cAMP signaling pathway. Asynchronous cultures were diluted in YPD plus 5% FCS at 37°C and samples were taken every 20 minutes for northern analysis. As shown in Fig. 10D, the kinetics of *ECE1* and *HWP1* induction was similar in wild-type and *cdc14*Δ cells. However, the level of expression in the *cdc14*Δ mutant was 3.5-fold lower than that of the wild-type cells, suggesting that only a small population of cells of asynchronous *cdc14*Δ cultures could sense the signals from serum. Thus, this subset of cells was responding with the same kinetics as wild-type cells, even though their germ tubes were shorter. This suggests that serum-sensitive *cdc14*Δ cells would be unable to initiate or maintain the polarized growth required for true hyphal growth.

Discussion

Here, we have shown that the protein phosphatase Cdc14p is not essential for cell-cycle progression in *C. albicans*, but that its absence produces a significant delay in the degradation of Clb2p during mitotic exit. In addition to this role, Cdc14p also plays an essential function in the activation of the Ace2p transcriptional program required for cell separation. Finally, by regulating the ability to maintain polarized growth, Cdc14p activity is required for true hyphal growth and invasion, traits that are important for the virulence of this fungal pathogen.

Dual regulation of Cdc14p

We have shown that Cdc14p protein levels are cell-cycle regulated in *C. albicans*, peaking at the G2-M transition and declining during the G1 phase of the cell cycle (Fig. 1). It is not clear how this periodic oscillation is regulated. Although *CDC14* transcription could be cell-cycle regulated, the fact that the expression of *CDC14-GFP* under the *ACT1* promoter showed a cell-cycle-regulated fluorescence signal (Fig. 2A) suggested that Cdc14p levels would mainly be regulated by post-transcriptional mechanisms. In this context, we observed that Cdc14p was modified, probably by phosphorylation, in a cell-cycle-dependent fashion (Fig. 1B-D). Similar to *S. pombe* (Cueille et al., 2001) and *S. cerevisiae* (Visintin et al., 2003), Cdc14p became phosphorylated during mitosis. The existence of Cdc28p phosphorylation consensus sites at the putative NLS and PEST sequences present at the C-terminus of Cdc14p suggests that the subcellular distribution and/or stability of Cdc14p could be regulated by phosphorylation. Indeed, we observed that the subcellular localization of Cdc14p varied during the cell cycle, suggesting that the spatial regulation of its activity could be important for progression through the cell cycle.

In yeast, humans and *Xenopus*, the nucleolus serves as a storage of inactive Cdc14p during interphase, from where it is released at the end of the cell cycle to perform different cellular functions (Cueille et al., 2001; Gruneberg et al., 2002; Jaspersen et al., 1998; Kaiser et al., 2004; Kaiser et al., 2002; Shou et al., 1999; Stegmeier et al., 2002; Trautmann et al., 2001; Traverso et al., 2001). Surprisingly, our results have shown that this is different in *C. albicans*, since Cdc14p is degraded at the end of mitosis instead of being sequestered in the nucleolus. Moreover, nuclear-located Cdc14p is progressively concentrated at both SPBs during interphase, reaching the strongest signal in late mitosis. During cytokinesis, the Cdc14p signal at the spindle poles becomes faint and the protein is mainly located at the bud neck. It is tempting to draw an analogy with other systems, where Cdc14 is kept inactive by tethering to the nucleolus, to suggest that the gradual accumulation of Cdc14p at both SPBs might be an alternative mechanism to restrain the activity of this phosphatase. It is also possible that the localization of Cdc14p at the SPBs might reflect a role in spindle function. However, *cdc14*Δ cells are not hypersensitive to benomyl nor have detectable defects in nuclear segregation (A.C.-B. and J.C.-B., unpublished).

Cdc14p is not essential for mitotic exit

Exit from mitosis requires inactivation of mitotic Cdks. In *S. cerevisiae*, the *CDC14* gene is essential for cell viability (Wan et al., 1992). Cdc14p inactivates mitotic Cdks by promoting the APC/C-Hct1p-dependent destruction of B-type cyclins and the accumulation of the Cdk inhibitor Sic1p (Jaspersen et al., 1999; Visintin et al., 1998). Unlike *ScCDC14*, several observations suggest that *CaCDC14* plays a non-essential role in mitotic exit. First, in our hands, *cdc14*Δ cells were viable. The increase in dry weight in *cdc14*Δ and wild-type cells was similar, presumably because, despite the delay in cell-cycle progression, individual *cdc14*Δ cells were 1.8-fold larger than wild-type cells. Second, in asynchronous cultures of *cdc14*Δ cells, the levels of Clb2p and Clb4p were elevated as compared with that of the wild type. Third, analysis of synchronous cultures of *cdc14*Δ cells revealed a severe delay in the degradation of Clb2p.

Whether Cdc14p regulates mitotic exit through Hct1p and Sic1p-like-proteins in *C. albicans* is currently unknown. Since Hct1p is involved in regulating cyclin levels (A.C.-B. and J.C.-B., unpublished), Cdc14p could play a minor role in the regulation of Hct1p. However, the *C. albicans* genome does not contain a protein related in sequence to Sic1p, suggesting that either Cdk inhibitors are not important in bringing about the drop in mitotic kinase activity required for exiting mitosis or that this function is performed by a protein that is not related in sequence to Sic1p.

Cdc14p is required for cell separation

In yeast, cell separation after cytokinesis requires localized degradation of the components of the septum at the mother-daughter junction. In both *S. cerevisiae* and fission yeast, this process depends on the transcription factor Ace2p, which activates the expression of several genes involved in cell wall hydrolysis, such as *ENG1*, *DSE1*, *CTS1* and *SCW11* in *S. cerevisiae* (Baladrón et al., 2002; Colman-Lerner et al., 2001; Doolin et al., 2001) or *eng1*, *agn1*, *adg1*, *adg2* and *adg3* in *S. pombe* (Alonso-Nuñez et al., 2005; Dekker et al., 2004; Martín-Cuadrado et al., 2003; Rustici et al., 2004). In *S. cerevisiae*, Ace2p is regulated at multiple levels. Nuclear import is inhibited by CDK phosphorylation (O'Conallain et al., 1999). In addition, a signaling network (termed RAM) is required to regulate Ace2p activity that includes the proteins Cbk1p, Mob2p, Hym1p, Tao3p, Sog2p and Kic1p (Nelson et al., 2003). Ace2p activation cannot be regulated solely by nuclear import, because Ace2p can enter nuclei in RAM

deletion mutants and yet remain inactive as a transcription factor (Nelson et al., 2003). At the end of mitosis, the Mob2p/Cbk1p kinase complex, a downstream effector of the RAM network, is required for the specific accumulation and activation of Ace2p in daughter nuclei (Bidlingmaier et al., 2001; Colman-Lerner et al., 2001; Nelson et al., 2003; Weiss et al., 2002). It has been suggested that Mob2/Cbk1p-mediated phosphorylation of Ace2p would inhibit its nuclear export in daughter nuclei, thereby allowing its asymmetric accumulation and activation (Jensen et al., 2000; Weiss et al., 2002). We observed that deletion of the *ACE2* gene led to the formation of large aggregates of yeast cells and to a dramatic drop in the expression of the genes required for cell separation in *C. albicans* (Fig. 6). These phenotypes closely resembled those described for *S. cerevisiae ace2*Δ mutants, suggesting that both proteins would be true functional homologs. While this work was in preparation, it was reported that the product of the *ACE2* gene is involved in cell separation and that Ace2p-GFP localizes to the daughter nucleus (Kelly et al., 2004).

*C. albicans cdc14*Δ cells have severe defects in cell separation (Fig. 5), suggesting that this protein phosphatase must play an important role in the last steps of cytokinesis during growth in the yeast form. Indeed, we have shown that Cdc14p is required for accumulation of Ace2p in daughter nuclei (Fig. 7) and, as a consequence, the expression of Ace2p target genes was strongly reduced in *cdc14*Δ cells (Fig. 6B). However, since we were unable to detect Ace2p in cell extracts, we cannot rule out the possibility that Cdc14p might regulate Ace2p stability. In good correlation with its function in cell separation, Cdc14p is targeted to the bud neck at the M-G1 transition during growth in the yeast form (Fig. 2B, Fig. 3D). It is interesting that a similar neck localization has been recently described for *S. cerevisiae* Cdc14 (Bembenek et al., 2005). What might the role of Cdc14p be in cytokinesis upon reaching the neck? Our current model is that translocation of this protein phosphatase to the septum region could activate the Ace2-dependent pathway of the Cbk1p kinase. In *S. cerevisiae*, Cbk1p and Mob2p localize to the bud neck region at the end of mitosis (Bidlingmaier et al., 2001; Racki et al., 2000; Weiss et al., 2002) and MEN signaling might control the ability of Mob2p/Cbk1p to promote the nuclear localization and activity of Ace2p (Weiss et al., 2002). In *C. albicans*, *cbk1*Δ cells form large aggregates of round cells and lack the ability to undergo the yeast-hypha transition (McNemar and Fonzi, 2002).

Inhibition of cell separation during hyphal growth

During hyphal growth, cytokinesis takes place but cell separation is inhibited. In *C. albicans*, *HGC1* encodes a G1 cyclin-related protein that forms a complex with Cdc28p and plays a role in hyphal morphogenesis. *HGC1* expression is under the control of hypha-inducing signals, ensuring that Hgc1p is expressed at all times during the cell cycle as long as inducing conditions are maintained. In response to serum, *hgc1*Δ cells produce germ-tube-like cells that separate after cytokinesis, giving rise to elongated unicellular compartments (Zheng and Wang, 2004).

Our results have revealed an important difference in the spatial regulation of Cdc14p during the yeast and hyphal growth of *C. albicans*, since hypha-inducing conditions abolished the translocation of Cdc14p to the septum region (Fig. 4). Interestingly, this difference was found to be dependent on Hgc1p, since *hgc1*Δ mutants were able to accumulate Cdc14p to the site of cytokinesis in the presence of serum (Fig. 8). In this context, it is noteworthy that Cdc14p is a phosphoprotein (Fig. 1C) that has two Cdc28p consensus sites (S/TPXK/R) at its C-terminus (Fig. 1A). Therefore, the Hgc1p/Cdc28p-dependent phosphorylation of Cdc14p might play a role in preventing the translocation of this protein phosphatase to the septum in the presence of serum. The involvement of Cdks in the inhibition of cytokinesis is a common mechanism conserved in yeast and animal cells. Inactivation of mitotic Cdk is required for translocation to the division site of different proteins necessary for cytokinesis (Echard and O'Farrell, 2003; Frenz et al., 2000; Hwa Lim et al., 2003; Luca et al., 2001; Wheatley et al., 1997; Xu et al., 2000). The dependence of this translocation on Cdk inactivation could be a core mechanism that couples exit from mitosis and cytokinesis in eukaryotic cells (Seshan and Amon, 2004). *C. albicans* might have modified this conserved mechanism by controlling the expression of the Hgc1p cyclin through the cAMP/PKA pathway. We propose that the hypha-specific expression of Hgc1p would guarantee the inhibition of cell separation after mitosis by sequestering cell separation activators, such as Cdc14p, from the site of cytokinesis.

Cdc14p and morphogenesis

We found that *cdc14*Δ cells were poorly able to invade the agar substrate under conditions that stimulate invasive growth in wild-type cells. Since the invasiveness defect of *cdc14*Δ cells was similar to that of *ace2*Δ mutants, we suggest that this might be due to a failure in the activation of the Ace2p target genes required for invasive growth. In *C. albicans*, Ace2p is needed for biofilm formation and virulence (Kelly et al., 2004), suggesting that this transcription factor would play an important role in the modification of the cell wall during differentiation.

Lack of Cdc14p also impaired true hyphal growth, suggesting that Cdc14p contributes to filamentous growth. In response to serum, only one subset of asynchronous *cdc14*Δ cells responded with the same kinetics as wild-type cells, and those cells gave rise to shorter and wider germ tubes. The serum-sensitive *cdc14*Δ cells were probably those with low levels of B-type cyclins that corresponded to cells passing through G1 (Fig. 9C, 0 minutes). This is consistent with the observation that strains overexpressing either Clb2p or Clb4p exhibit the same serum response as *cdc14*Δ mutants (Bensen et al., 2005). In addition, we found that serum induced a delay in G2/M in germ tubes when compared with yeast-form cells (Fig. 1C), suggesting that the cell cycle might not be regulated in the same way in these two cell types. Consistent with this idea, upon hypha induction, the accumulation of the G1 cyclin Cln1p is prolonged, whereas the accumulation of both Clb2p and Clb4p is delayed when compared with yeast-form cells (Bensen et al., 2005). Thus, our observations suggest that the regulation of the B-cyclins by Cdc14p is important at the onset of germ tube formation. In this context, it is noteworthy that the mitotic Clb2p plays an important role in morphogenesis and pathogenesis in the fungus *Ustilago maydis* (Garcia-Muse et al., 2004).

Materials and Methods

Strains and growth conditions

Strains used are listed in Table 1. YEPD, synthetic complete media (SDC) and synthetic minimal medium (SC) lacking specific nutrients have been described previously (Sherman, 1991). Hyphal growth on solid medium was induced by using

Table 1. Strains used in this study

Strain	Genotype	Ref.
BWP17	*ura::imm434/ura3::imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*	(Enloe et al., 2000)
CAI4	*ura3::imm434/ura3::imm434*	Fonzi and Irwin (1993)
CAF2	*URA3/ura3::imm434*	Fonzi and Irwin (1993)
JC4	*CAI4 CDC14/ cdc14::hisG-URA3-hisG*	This study
JC5	*CAI4 CDC14/ cdc14::hisG*	This study
JC6	*CAI4 cdc14::hisG/cdc14::hisG-URA3-hisG*	This study
JC7	*CAI4 cdc14::hisG/cdc14::hisG*	This study
YAC1	*CAI4 MET3-CDC14::URA3 / cdc14::hisG*	This study
JC12	*BWP17 cdc14::hisG/cdc14::hisG-URA3-hisG*	This study
JC13	*BWP17 cdc14::hisG/cdc14::hisG*	This study
JC51	*BWP17 cdc14::hisG/cdc14::hisG, CDC14 URA3*	This study
JC64	*BWP17 CLB2-3HA URA3*	This study
JC70	*BWP17 cdc14::hisG/cdc14::hisG CLB2-3HA URA3*	This study
JC77	*BWP17 CLB4-3HA URA3*	This study
JC82	*BWP17 cdc14::hisG/cdc14::hisG CLB4-3HA URA3*	This study
JC90	*BWP17 CDC14-YFP HIS1*	This study
JC94	*BWP17 CDC14/ CDC14-3HA URA3*	This study
JC99	*BWP17 ace2::URA3/ace2::HIS1*	This study
JC115	*BWP17 cdc14::HIS1/CDC14*	This study
JC128	*BWP17 cdc14::HIS1/CDC14-3HA URA3*	This study
JC189	*BWP17 CDC14-YFP HIS1/CDC14-YFP ARG4*	This study
JC190	*BWP17 RP10::URA3*	This study
JC194	*WYZ12 hgc1::ARG4/hgc1::HIS1 CDC14-YFP URA3*	This study
JC198	*CAI4 cdc14::hisG/cdc14::hisG, ACE2-GFP URA3/ACE2*	This study
JC310	*BWP17 CDC14-YFP HIS1, TUB2/TUB2-CFP URA3*	This study
JC252	*BWP17 CDC14-YFP URA3/CDC14 NOP1-CFP HIS1/NOP1*	This study
JJ22	*CAI4* (pJJ1-*pACT-CDC14-GFP*)	This study
JJ23	*JC7* (pJJ1-*pACT-CDC14-GFP*)	This study
AG10	*BWP17 cdc10::HIS1/cdc10::ARG4*	This study
AG11	*BWP17 cdc10::HIS1/cdc10::ARG4 (pJJ1-pACT-CDC14-GFP)*	This study
SCaY1	*CAI4 ACE2-GFP::URA3/ACE2*	Kelly et al. (2004)
WYZ12	*BWP17 ura3::imm434/ura3::imm434 hgc1::ARG4/hgc1::HIS1*	Zheng et al. (2004)
YJB8911	*BWP17 NOP1-CFP HIS1/NOP1*	J. Berman (University of Minnesota, Minneapolis, MN)

M199 (Life Technologies), Spider (Liu et al., 1994) or Lee's media (Buffo et al., 1984). Hyphal growth in liquid medium was induced by growing the cells in YEPD plus 5% fetal calf serum (FCS) (Sigma) at 37°C. Ura$^-$ auxotrophs were selected on SDC plates containing 0.1% 5-fluororotic acid (5-FOA) and 80 mg/l uridine. *C. albicans* cells were transformed by the lithium acetate procedure (Walther and Wendland, 2003). Dry weight measurements were performed as described previously (Kelly et al., 2004).

Strain constructions

All strains were checked for correct genome integration by PCR or Southern analysis (data not shown). Disruption of both alleles of *CDC14* was performed as described by Fonzi and Irwin (Fonzi and Irwin, 1993) using plasmid pMB7-*cdc14*Δ digested with both *Pst*I and *Sac*I. Disruption strains were also constructed using a previously described PCR-mediated gene disruption system (Gola et al., 2003). Disruption cassettes, consisting of either *URA3*, *ARG4* or *HIS1* selection markers, flanked by 100 bp of the target gene were generated using pFA plasmids (Gola et al., 2003) and the primers listed in Table 2. In-frame insertion of a C-terminal XFP tag to the *CDC14* and *ACE2* genes was performed as described previously (Gola et al., 2003) using the primers listed in Table 2. The *CDC14* gene was HA-tagged by transforming BWP17 strain with pCaHA-CDC14 linearized with *Pst*I, generating strain JC94. The *CDC14* gene was reconstituted by transforming strain JC13 with pMB7-RCDC14 digested with both *Hin*dIII and *Sac*I. The *MET3* promoter was inserted 5′ to *CDC14* by transforming strain JC5 with pCaDIS-CDC14-5′ partially digested with *Sca*I.

Plasmids

A *CDC14* disruption cassette using the *URA3* Blaster method was constructed as follows. First, a 2.1 kb *Sal*I fragment (–1691 to +373) from a genomic clone of *CDC14* was subcloned into the *Sal*I site of the pMB7 vector, generating pMB7-CDC14-5′. Then, a 1 kb region after the stop codon was amplified by PCR using primers AC3 (engineered to include a *Kpn*I site) and AC4 (including a *Sac*I site). The PCR product was digested with *Kpn*I and *Sac*I, and ligated into pMB7-CDC14-5′ digested with *Kpn*I and *Sac*I to generate pMB7-*cdc14*Δ. To tag *CDC14* with HA, the *CDC14* ORF was PCR amplified from genomic DNA isolated from strain *BWP17* using primers AC1 and CDC14-3HA, which included a *Eco*RV site. The PCR product was subcloned into pGEM digested with *Eco*RV, generating pGEM-CDC14RV. The *Sal*I-*Eco*RV fragment (1.1 kb) from pGEM-CDC14RV was subcloned into pCaHA (a gift from P. Sudbery, University of Sheffield, Sheffield, UK), and digested with *Sal*I and *Eco*RV to generate pCaHA-CDC14. To construct *MET3-CDC14*, the first 1000 nucleotides were PCR amplified using primers AC1 (which included a *Bam*HI site) and AC2. The PCR product was digested with *Bam*HI and *Pst*I, and the 600 bp fragment was subcloned into pCaDIS (Care et al., 1999) digested with *Bam*HI and *Pst*I to generate pCaDIS-CDC14-5′. To construct a reconstituted strain with one copy of the *CDC14* gene, we first obtained a modified pMB7 in which one copy of *hisG* had been eliminated by digestion with *Xba*I followed by religation. Then, a 1.8 kb fragment containing the *CDC14* gene, was PCR amplified using primers RCDC14-1 (engineered to include a *Xba*I site) and RCDC14-2 (which included a *Xba*I site). The PCR product was digested with *Xba*I and ligated into the *Xba*I site of the modified pMB7 to generate pMB7-RCDC14. To construct *CDC14-GFP* under the *ACT1* promoter, the *CDC14* ORF lacking the stop codon was PCR amplified and subcloned into the *Sma*I site of pAG1 (González-Novo et al., 2004) to generate pJJ1.

Microscopy

For light microscopy, cells were fixed with 70% ethanol and stained with DAPI or Calcofluor White as previously described (Sherman, 1991). Samples were viewed and photographed as wet mounts, using a Leica DMXRA microscope equipped for Nomarski optics and epifluorescence. Pictures were taken with a Photometrics Sensys CCD camera. To observe XFP fluorescence, 50 ml of cells (A_{600}=0.05) grown in SC media were washed twice with PBS and resuspended in 50 μl of PBS. A 5 μl aliquot of the cell suspension was mounted on glass slides for microscopic examination. Time-lapse microscopy (for Fig. 2) was carried out with the same microscope, to which a thermostatic device (supplied by Linkam) had previously been fitted in order to keep the cells at 28°C. For Fig. 3, *C. albicans* cells with CFP- and/or YFP-tagged proteins were analyzed by fluorescence microscopy after DAPI staining. For fluorescence microscopy, a series of z-focal plane images were collected on a Leica IRB using 63×/1.4 or 100×/1.35 lenses, a Hamamatsu C4742-95 digital camera and OpenLab software (Improvision). A tuneable light source (Polychrome IV) with a Xenon lamp was used. Images in different z-axis planes were flattened into a two-dimensional projection and processed in Openlab. DNA was stained using 4′,6-diamidino-2-phenylindole (DAPI/antifade; Qbiogene) at 1 μg/ml final concentration after short treatment of the cells with 1% Triton X-100. Cells were prepared for electron microscopy as described previously (Bensen et al., 2002).

Table 2. Oligonucleotides used in this study

Name	Sequence (5′ to 3′)
Strain construction	
AC1	GGGGATCCATGGATTTCGGACCTTTGAAC
AC2	GGGGATCCGCCAAAGCACCACTCACCTTACG
NAC3	GGGGTACCCACGAATGTTTTGCGCGTGTC
AC4	CGGGAGCTCGACCATATCTAGATGCCGATTCC
CDC14 3HA	GCTTGATATCATGTTTCTTTCCACTTAACTT
*cdc14*Δ delete forward primer	CAGATAGAATATACCTTGGCGCTTATGATCATCACAAAAGGGATACAGAAGATTTGGCATATTTCACAGTTGAAG-ACGCATTACCATATAATGCATTTTACGAAGCTTCGTACGCTGCAGGTC
*cdc14*Δ delete reverse primer	GTATACATTGATTTAATGAAGGTGAAGCAAAAAAGAAAGAATAACAAATAACAAATAAAAACAGGTTATCAAA-AGAAATGAACTTAACATACATGTACAATCTGATATCATCGATGAATTCGAG
*ace2*Δ delete forward primer	ATGCATTGGAAATTTCTGAACTTTCGAAAGTACCATCTTTCTTTCCATTTAAATTTATTTGATCTTTCTGTGGAAT-TGTGAGCGGATA
*ace2*Δ delete reverse primer	ACTGAAATGCAATCTTCTCTCCCACTCGAGCAAACACTCTCTCCATGAAAAACCAACTTGTGGCAGAAGTTTCC-CAGTCACGACGTT
*cdc10*Δ delete forward primer	TTATTTCCTTCTTTTCGTTTACGTTTTCGTTTTCGTTTTTTTTTAGGGTTGATTGATTCAAACTAATTCAAACACAA-GTGTTCTTATAAGCCAAAACATCGAAGCTTCGTACGCTGCAGGTC
*cdc10*Δ delete reverse primer	ATTTTTGTTCATACGCGTTTTGCTTTTCAACAAACACACAAAAGAAGAGGAATACAAAAAAGTAAAATCACATT-ATATCAATAACAAACATTATTTATCTTCTGATATCATCGATGAATTCGAG
CDC14-XFP forward primer	GCGTGTCTAAAGCCAGATCCAAAAATAGGATCGCTTCTGGAAACTCACAAACATCAAGAGCACACTCTGGTGG-TGTGAGAAAGTTAAGTGGAAAGAAACATGGTGCTGGCGCAGGTGCTTC
CDC14-XFP reverse primer	TGATATTGCTGAATTAAGTTTAAAGGATTTCGATATATTGGCTTTTGCATATGGTTCGGAAGAACAAATTGAAATT-GTTGAACCAGCTTATGAAGAAAACTTCTGATATCATCGATGAATTCGAG
ACE2-GFP forward primer	GGGAACTAATAACACTCAACAACAATTGCTTAAAACAGATACAGTGGTTGAGAGGATAGAAAAACAGTTGCTAC-AGGAAGATAAGAGTGTTACTGAGGAGTTTTTAATGTTGCAAGGTGCTGGCGCAGGTGCTTC
ACE2-GFP reverse primer	CGATATAAAATAAAAAAGAATAATAGTATAATTTTGAAAAGGGTAAAATGAAATGAACTGAAATGCAATCTTCTC-TCCCACTCGAGCAAACACTCTCTCCATGAAAAACCAACTTGTGGCAGAAGTTCTGATATCATCGATGAATTC-GAG
TUB2-XFP forward primer	TGATTTGGTTAGTGAATACCAACAATACCAAGAAGCTAGTATTGATGAAGAAGAATTAGAATATGCCGATGAAAT-CCCATTAGAAGATGCCGCCATGGAAGGTGCTGGCGCAGGTGCTTC
TUB2-XFP reverse primer	GTTGATTTAGTTGGTGTATATATTTCATTTTTATTTTTGTAAATTATTATTGATATCAGTAGTGGTAAAGAATAGGAC-ATCGTTAAATATATAAAAGTATTCTGATATCATCGATGCATTCGAG
H2FA	CAACGAAATGGCCTCCCCTACCACAG
A2FA	AATGGATCAGTGGCACCGGTG
ARG1	CAATGGATCAGTGGCACCGG
H3FA	GGACGAATTGAAGAAAGCTGGTGCAACCG
ACE2-5SB	CGAGTATATTTGCCACC
URA1	GGTATAGAAATGCTGGTTGG
RCDC14-1	GCTCTAGAATGGATTTCGGACCTTTGAAC
RCDC14-2	CGTCTAGACGAGTGGCCTATCCAAAAGAT
CaACE5	TTCCATGCATTTATCACC
GFP	TCACCATCTAATTCAACC
PCaHA-3	ACACATGCATGCTTAACCGGCATAGTCTGG
G1-CDC14	CCAATTAACTCAACGTCTATC
G4-TUB1	CGGTTTGAACAAAAGTAGGGATGC
U2FA	GCTGTAGTGCCATTGATTCGTAACAC
Northern probes	
ACT1-5	GGTTTTGCCGGTGACGAC
ACT1-3	TCGAAATCCAAAGCAACG
ECE1-1	TGGCAACATTCCACAAGTAATC
ECE1-2	AGCCGGCATCTCTTTTAACTGG
HWP1-1	TCAATTGGGGCCACTGTC
HWP1-2	TGGAATCCAATCGGTTGG
DSE1-5	CAAGGTGGGAATCCTAT
DSE1-3	CTGTAATTGTTATGACC
ACE2-5	GGTGGCAATTCTTACTC
ACE2-3	GCATGGAACCTACATTTG
CHT3-1	TTGTTTGTTGGTGTACCTGC
CHT3-2	ATCACTAGTGGAAGCACTGG
ENG1-5	GCACACTAATAGCCGTTCAAG
ENG2-3	CTTTGTTGTAGAGACTAAGGA

Flow cytometry

For the analysis of DNA contents, samples were prepared as described previously (Jiménez et al., 1998) on a FACScan® (Becton Dickinson).

Cell synchronization

To isolate unbudded G1 cells, strains were grown at 30°C in YPD to A_{600}=1.5, sonicated, and then loaded into the separation chamber of a JE-5.0 elutriation system (Beckman Instruments) maintained at 2500 ***g*** and a flow of 32 ml/minute. After loading, fresh YPD medium was used to recover the cells. To collect the cells from the chamber, the speed was gradually reduced to 2000 ***g*** and the outflow was gradually increased. Unbudded cells were collected, concentrated by centrifugation, and then released into fresh pre-warmed medium. Samples were taken every 15 minutes and protein extracts were prepared.

Protein extracts and western blotting

Total protein extracts were prepared from 1.6×10^8 frozen cells. Cells were resuspended in 20 μl of RIPA buffer (10 mM sodium phosphate, 1% Triton X-100, 0.1% SDS, 10 mM EDTA, 150 mM NaCl, pH 7) and 200 μl of glass beads (0.4

mm; Sigma) were added. Cells were broken for 20 seconds in a Ribolyser machine (Hybaid) and the crude extract was recovered by washing with 200 μl of RIPA. Soluble proteins were obtained by centrifugation of total extracts at 13,400 ***g*** over 10 minutes at 4°C. For western blots, 30 μg of protein extracts were separated on 8% SDS-PAGE, transferred to Hybond-P (Amersham Biosciences) membranes, and probed with anti-HA (12CA5, 1:500) or anti-PSTAIRE (Santa Cruz Biotechnology, Sc53, 1:3000) antibodies. Secondary antibodies conjugated to horseradish peroxidase were diluted 1:15,000. Immunoblots were developed using the Supersignal West Pico kit (Pierce Biotechnology). Quantification of the blots was performed by densitometric analysis (Quantity One Software. Bio-Rad Laboratories). Phosphatase treatment of cell extracts was performed using λ PPTase from New England Biolabs.

RNA isolation and northern blot analysis

Total RNA from exponentially growing cultures was obtained as described (De Las Peñas et al., 2003). Polyadenylated RNA was isolated with the mRNA purification kit (Amersham Biosciences). For northern blot analysis, 5 μg of RNA poly(A^+) was used for each sample. The DNA probes used to detect the different transcripts were obtained by PCR using the primers listed in Table 2.

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