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 cdc14Δ* mutants formed large aggregates of cells that resembled those found in *ace2*-null strains. In *cdc14Δ* 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 *hgc1Δ* 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 *cdc14Δ* cells exhibited a severe delay in the destruction of the mitotic cyclin *Cln2p*. 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., 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 *cln* 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 forhead transcription factor *Fkh2p* is associated with increased levels of the B-type cyclin *CcClb4p* and constitutive pseudohyphal growth (Bensen et al., 2002). In addition, overexpression of B-type cyclins reduces the extent of filamentous growth (Bensen...
The conserved Cdc14p family of dual-specificity protein phosphatases function to downregulate mitotic Cld/Cdk 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 Cib2p destruction and cell separation. In addition, cdc14Δ 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/H9004/cdc14/H9004 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. 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-PO4) 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 CAI2 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

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**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.](image-url)

(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 faint and strong foci signal are shown. (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.
We used electron microscopy. This confirmed that completed cytokinesis but had a defect in septum degradation, by sonication (Fig. 5A). To confirm that mutant cells had cultures formed large clumps of cells that could be dispersed cytokinesis, we found that exponentially growing type strain, in which yeast cells readily separated after analysis during vegetative growth. In contrast to the wild-type strain, in which 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 analysis during vegetative growth. In contrast to the wild-type strain, in which yeast cells readily separated after analysis during vegetative growth. In contrast to the wild-type strain, in which yeast cells readily separated after analysis during vegetative growth.

To investigate whether the presence of Cdc14p in the septum Cdc14p is required for cell separation 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 analysis during vegetative growth. In contrast to the wild-type strain, in which yeast cells readily separated after analysis during vegetative growth. In contrast to the wild-type strain, in which yeast cells readily separated after analysis during vegetative growth. In contrast to the wild-type strain, in which yeast cells readily separated after analysis during vegetative growth. In contrast to the wild-type strain, in which yeast cells readily separated after analysis during vegetative growth. In contrast to the wild-type strain, in which yeast cells readily separated after analysis during vegetative growth. In contrast to the wild-type strain, in which yeast cells readily separated after analysis during vegetative growth. In contrast to the wild-type strain, in which yeast cells readily separated after analysis during vegetative growth. In contrast to the wild-type strain, in which yeast cells readily separated after analysis during vegetative growth. In contrast to the wild-type strain, in which yeast cells readily separated after analysis during vegetative growth.

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 cdc14Δ 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 cdc14Δ 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 (McCreadh 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 cdc14Δ mutants might be a result of a decrease in the expression 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, cdc14Δ mutants showed a dramatic decrease in the expression of these genes (Fig. 6B). Introduction of a wild-type copy of CDC14 into the cdc14Δ/cdc14Δ 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 cdc14Δ 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...
Cdc14p function in Candida albicans

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 cdc14Δ mutants, the localization of Ace2p was analyzed in wild-type and mutant cells using an Ace2-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 cdc14Δ 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 hgc1Δ CDC14-YFP cells at 30°C (yeast 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 cdc14Δ cultures. Relative to wild-type levels, Clb2p and Clb4p were detected at 2.15-fold and 1.95-fold higher levels in cdc14Δ cells, respectively (Fig. 9A). Moreover, flow cytometry analysis of exponentially growing cdc14Δ 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 cdc14Δ 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 cdc14Δ mutant, the reduction in Clb2p-HA levels was not observed during the course of the

Fig. 6. (A) Morphology of ace2Δ/ace2Δ mutant cells (JC99) grown in YPD at 30°C. (B) cdc14Δ 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, ENGI, DSE1 and ACE2 genes.

Fig. 7. Localization of Ace2p in the daughter nucleus depends on Cdc14p. Exponentially growing cells expressing Ace2p-GFP in wild-type (A, SCAY1) or cdc14Δ mutant (B, JC198) cells were visualized using DIC and fluorescence (GFP) microscopy. Bars, 5 μm.

Fig. 8. Localization of Cdc14p-YFP in a hgc1 mutant strain during hyphal growth. Exponentially growing cells expressing Cdc14p-YFP in wild-type (A, SCAY1) or cdc14Δ mutant (B, JC198) cells were visualized using DIC and fluorescence (GFP) microscopy. Bars, 5 μm.
experiments, suggesting a long delay in mitotic exit. Consistent with this lag, asynchronously growing cdc14 cells were 1.8-fold larger than BWP17 cells (84.3±25 versus 49.0±20.4 relative units; n=100). Despite the delay in the cell-cycle progression of cdc14 cells, their larger size could explain why the increase in dry weight in cdc14 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 mutant. To study invasive growth, we

Fig. 8. Localization of Cdc14p-YFP in hgc1Δ cells. Exponentially growing cells of strain JC194 (hgc1Δ 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.

Fig. 9. cdc14Δ 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 cdc14Δ grown in YPD or SC media at 30°C. (C) G1 cells from strains JC64 (CDC14/CDC14; CLB2HA/CLB2) and JC70 (cdc14Δ/cdc14Δ; 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.
analyzed the ability to invade the agar substrate by plating wild-type and cdc14Δ 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 cdc14Δ mutant being considerably less invasive than the wild-type strain. Introduction of the wild-type CDC14 gene into the cdc14Δ/cdc14Δ mutant restored the invasive growth defect. The ace2Δ strain showed the same phenotype as that of the cdc14Δ strain. Since Cdc14p is required for nuclear accumulation of Ace2p (Fig. 7), this observation suggests that the invasive growth defect observed in cdc14Δ 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 cdc14Δ cells might have some hyphal growth defect, we analyzed the morphology of the cdc14Δ strain under different hypha-inducing conditions. On Spider, Lee or M199 solid media, the cdc14Δ mutant strain was unable to form hyphae (Fig. 10B), whereas the wild-type strain generated extensive hyphal colonies. The heterozygous CDC14/cdc14Δ strain produced intermediate hyphal colonies.

To study the response to serum, a strong inducer of hyphal growth, wild-type and cdc14Δ 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 cdc14Δ 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 cdc14Δ cells develop true hyphae.

The defect in response to serum observed in the cdc14Δ 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), *HWPI* (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 *cdc14Δ* mutants, we analyzed the expression of *ECE1* and *HWPI* 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. 1D, the kinetics of *ECE1* and *HWPI* induction was similar in wild-type and *cdc14Δ* cells. However, the level of expression in the *cdc14Δ* mutant was slightly lower than that of the wild-type cells, suggesting that only a small population of cells in asynchronous *cdc14Δ* 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 *cdc14Δ* 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, *cdc14Δ* 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 Cdns. In *S. cerevisiae*, the *CDC14* gene is essential for cell viability (Wan et al., 1992). Cdc14p inactivates mitotic Cdns 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, *cdc14Δ* cells were viable. The increase in dry weight in *cdc14Δ* and wild-type cells was similar, presumably because, despite the delay in cell-cycle progression, individual *cdc14Δ* cells were 1.8-fold larger than wild-type cells. Second, in asynchronous cultures of *cdc14Δ* cells, the levels of Clb2p and Clb4p were elevated as compared with that of the wild type. Third, analysis of synchronous cultures of *cdc14Δ* 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 *engl, agn1, adg1, adg2* and *adg3* in *S. pombe* (Alonso-Núñez 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 Csk1p, Moh2p, 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 ace2Δ 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 cdc14Δ 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 cdc14Δ 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 activation of Mob2p/Cbk1p to promote the nuclear localization and activity of Ace2p (Weiss et al., 2002). In C. albicans, cbk1Δ 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, hgc1Δ 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 hgc1Δ 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 Cdns 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 cdc14Δ cells were poorly able to invade the agar substrate under conditions that stimulate invasive growth in wild-type cells. Since the invasiveness defect of cdc14Δ cells was similar to that of ace2Δ 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 cdc14Δ cells responded with the same kinetics as wild-type cells, and those cells gave rise to shorter and wider germ tubes. The serum-sensitive cdc14Δ 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 cdc14Δ 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 (SC) and synthetic minimal medium (SDC) lacking specific nutrients have been described previously (Sherman, 1991). Hyphal growth on solid medium was induced by using
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.

Strain constructions

All strains were checked for correct genome integration by PCR or Southern analysis (data not shown). Disruption of both alleles of CDC41 was performed as described by Fonzi and Irwin (Fonzi and Irwin, 1993) using plasmid pMB7-cdc41Δ digested with both PstI and SacI. Disruption strains were also constructed using a previously described PCR-mediated gene disruption system (Gola et al., 2003).

Table 1. Strains used in this study

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<td>Zheng et al. (2004)</td>
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<td>WP17 NOP1-CFP HIS1/NOP1</td>
<td>J. Berman (University of Minnesota, Minneapolis, MN)</td>
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Plasmids

A CDC41 disruption cassette using the URA3 Blaster method was constructed as follows. First, a 2.1 kb SalI fragment (~1981 to +373) from a genomic clone of CDC14 was subcloned into the SalI site of the pMB7 vector, generating pMB7-cdc14Δ-5’. Then, a 1 kb region after the stop codon was amplified by PCR using primers AC3 (engineered to include a Kpnl site) and AC4 (including a SacI site). The PCR product was digested with Kpnl and SacI, and ligated into pMB7-cdc14Δ-5’ digested with Kpnl and SacI to generate pMB7-cdc14Δ. 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 EcoRI site. The PCR product was subcloned into pGEM digested with EcoRV, generating pGEM-CDC14-RV. The SacI-EcoRV fragment (1.1 kb) from pGEM-CDC14-RV was subcloned into pCAHA (a gift from P. Sudbery, University of Sheffield, Sheffield, UK) and digested with SacI and EcoRV to generate pCAHA-CDC14. To construct MET5-CDC14, the first 1000 nucleotides were PCR amplified using primers AC1 (which included a BamH1 site) and AC2. The PCR product was digested with BamH1 and EcoRV, and the 600 bp fragment was subcloned into pCADIS (Care et al., 1999) digested with BamH1 and PstI, and the 1.3 kb fragment was ligated into 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 XbaI followed by religation. Then, a 1.8 kb fragment containing the CDC14 gene, was PCR amplified using primers RCD14-1 (engineered to include a XbaI site) and RCD14-2 (which included an XbaI site). The PCR product was digested with XbaI and ligated into the XbaI site of the modified pMB7 to generate pMB7-RCD14.

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 DMRXA microscope equipped for Nomarski optics and epifluorescence. Pictures were taken with a Photometrics Sensys CCD camera. To observe XFP fluorescence, 50 ml of cells (A600=0.05) grown in SC media were washed twice with PBS and resuspended in 50 ml 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-focus 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 tunable light source (Polychrome IV) with a Xenon lamp was used. Images in different z-axes planes were flattened into a two-dimensional projection and processed in Openlab. DNA was stained using 4′,6-diamidino-2-phenylindole (DAPI/antifade; Qiogene) 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).
Cdc14p function in Candida albicans

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 l of RIPA buffer (10 mM sodium phosphate, 1% Triton X-100, 0.1% SDS, 10 mM EDTA, 150 mM NaCl, pH 7) and 200 l of glass beads (0.4

Table 2. Oligonucleotides used in this study

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<th>Name</th>
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<td>NAC3</td>
<td>GGGGTAACCAACGAAGTTTTTTGCCGGGTGTC</td>
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<td>AC4</td>
<td>CGGGAGCTGGACAAACTGAGCGGTAGC</td>
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</table>
| CDC14 3HA                   | GCTTGAAATCTGCTGACCTGCTGACCTGACCTGACCTGACCTGACCTGACCTG |}

Northern probes

ACT1-5                        | GGTTTGGCGCGGACGAC                     |
ACT1-3                        | TGCAATTCCTACAAAGAAGAGCTGGCAACCGC     |
ECE1-1                        | TGGCAACATCCCAACAGAATATACG           |
ECE1-2                        | AGCCGGC ATCTCTTTTTACTCGG            |
HWP1-1                        | TCAATTGGGGCCACTGTC                  |
HWP1-2                        | TCAATCCGCACTGCGGT                    |
DSE1-5                        | CAAGTTGGGAATCTCT                  |
DSE1-3                        | CGTCAAATGTTGAGC                      |
ACE2-5                        | CGTGCAATGCGACGT                       |
ACE2-3                        | GCATGGAACCTACAT                 |
CHT3-1                        | TTTTGTGTTTTGTGTTACGTC                |
CHT3-2                        | ATACACTAGGGAACGACTGTC               |
ENG1-5                        | GCACACTAATGCGGTACAG                |
ENG2-3                        | TTGGTGTAGAGACACTAAGAAGAGA             |
mm; Sigma) were added. Cells were broken for 20 seconds in a Ribolyser machine (Hybird) and the crude extract was recovered by washing with 200 µl of RIPA. Soluble proteins were separated 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-β-actin (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 densiometric analysis (Quantity One Software. Bio-Rad Laboratories). Phosphatase treatment of cell extracts was performed using a PPTase from New England Biolabs.

RNA isolation and northern blot analysis
Total RNA from exponentially growing cultures was obtained as described (De Las Chapa y Lazo, B., Bates, S. and Sudbery, P. E.). Total RNA/preceding passage were added. Cells were broken for 20 seconds in a Ribolyser machine and the crude extract was recovered by washing with 200 µl of RIPA. Soluble proteins were separated 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-β-actin (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 densiometric analysis (Quantity One Software. Bio-Rad Laboratories). Phosphatase treatment of cell extracts was performed using a PPTase from New England Biolabs.

References

ECE1, a gene expressed in association with cell elongation of the

Candida albicans


