Regulation of the *Candida albicans* Hypha-Inducing Transcription Factor Ume6 by the CDK1 Cyclins Cln3 and Hgc1

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**ABSTRACT** The ability to switch between proliferation as yeast cells and development into hyphae is a hallmark of *Candida albicans*. The switch to hyphal morphogenesis depends on external inducing conditions, but its efficiency is augmented in stationary-phase cells. Ume6, a transcription factor that is itself transcriptionally induced under hypha-promoting conditions, is both necessary and sufficient for hyphal morphogenesis. We found that Ume6 is regulated posttranslationally by the cell cycle kinase Cdc28/Cdk1, which reduces Ume6 activity via different mechanisms using different cyclins. Together with the cyclin Hgc1, Cdk1 promotes degradation of Ume6 via the SCFCDC4 ubiquitin ligase. Since *HGC1* is a key transcriptional target of Ume6, this results in a negative-feedback loop between Hgc1 and Ume6. In addition, we found that Cln3, a G1 cyclin that is essential for cell cycle progression and yeast proliferation, suppresses hyphal morphogenesis and that Cln3 suppresses Ume6 activity both in the heterologous *Saccharomyces cerevisiae* system and in *C. albicans* itself. This activity of Cln3 may provide the basis for the antagonistic relationship between yeast proliferation and hyphal development in *C. albicans*.

**IMPORTANCE** The yeast to hypha (mold) morphogenetic switch of *Candida albicans* plays a role in its virulence and constitutes a diagnostic trait for this organism, the most prevalent systemic fungal pathogen in industrialized countries. It has long been known that hyphae are most efficiently induced from stationary cultures. Here, a molecular basis for this observation is provided. The G1 cyclin Cln3, an essential promoter of yeast proliferation, was found to suppress hyphal induction. Suppression of hyphal induction is achieved by inhibition of the activity of the central activator of hyphal morphogenesis, the transcription factor Ume6. Thus, levels of Cln3 control the switch between proliferation of *C. albicans* as individual yeast cells and development into extended hyphae, a switch that may preface the proliferation/differentiation switch in multicellular organisms.

**KEYWORDS** *Candida albicans*, Cdc4, Cln3, Hgc1, SCF, morphogenesis

*Candida albicans* is a human commensal fungus that can cause superficial infections in immunocompetent individuals, as well as life-threatening systemic infections in immunocompromised patients (1). *C. albicans* is able to assume different growth forms, most notably, yeast, hyphal, and pseudohyphal morphologies (2). This ability to switch between different modes of growth and proliferation appears to be important for virulence, based on the reduced pathogenicity in a mouse model of infection by mutants locked in the yeast mode (3, 4).

The cellular morphology is heavily dependent on growth conditions: whereas in standard rich medium at 30°C, wild-type *C. albicans* grows usually as yeast, many growth conditions have been identified that induce the switch to hyphal growth. These
include an elevated temperature (37°C), elevated CO₂, neutral pH, addition of serum, or various specific growth media that have little in common except that they often impose a growth limitation (reviewed in reference 5). Additional factors that can influence morphogenetic switching are the quorum-sensing molecules tyrosol, an inducer of hyphal growth (6), and farnesol, a repressor (7).

Genetic analysis has elucidated at least part of the regulatory pathways that link extracellular stimuli to morphogenesis. Several signal transduction pathways, notably, the mitogen-activated protein kinase (MAPK)-dependent (8) and cyclic AMP (cAMP)/protein kinase A (PKA)-dependent (9, 10) pathways, that participate in the induction of filamentation have been identified. A number of transcription factors were identified that can influence filamentous growth, including Cph1 (11), Efg1 (4, 12), Cph2 (13), C. albicans Tec1 (CaTec1) (14), CaRim101 (15), CaTup1 (16), CaNrg1 (17), CaMcm1 (18), CaFkh2 (19), and CaUme6 (20, 21). Some of these transcription factors were found to be targets of hypha-inducing signal transduction pathways (22, 23). Induction of filamentous morphology by extracellular signals is accompanied by a distinct transcription program with, notably, expression of genes encoding cell surface components such as the Hwp1, Ece1, and Als3 proteins (13, 24, 25).

Although several of the transcription factors mentioned above are important for hyphal growth, ectopic expression of any of these transcription factors by itself is unable to induce authentic hyphal growth. An exception is CaUme6, which was reported to induce hyphae upon overexpression (3). Caume6Δ/Δ mutants transiently formed germ tubes when exposed to hypha-inducing conditions but were unable to sustain hyphal elongation and hypha-specific gene expression under all conditions tested (20, 21).

Cell morphogenesis is closely associated with regulation of the cell cycle, a link that is best understood in budding yeasts (26, 27). The morphogenetic switch in C. albicans may therefore be expected to involve regulation at the level of the cell cycle regulatory machinery as well (28, 29). The notion of a link between cell cycle and morphogenesis is supported by the observation that various treatments that inhibit cell cycle progression cause a switch to polarized growth (see, for example, references 30 and 31). Similarly, depletion of the Polo-like kinase CaCdc5, a mitotic regulator (30), and depletion of the C. albicans Cln3 homolog, an essential cyclin, were shown to induce polarized growth (32, 33). Notably, however, in all of the instances mentioned above, the polarized growth assumed pseudohyphal rather than typical hyphal morphologies (2), with the possible exception of Cln3 depletion. One mutation that induces true hyphal growth is the deletion of CaCDC4, which encodes a substrate-recognition subunit of the SCF ubiquitin ligase (34, 35), the homolog of which is required for cell cycle progression in Saccharomyces cerevisiae (36, 37).

We had previously identified the cell cycle inhibitor Sol1 as a substrate of Cdc4. However, deletion of SOL1 failed to suppress the hyphal phenotype of the Cacdc4Δ/Δ deletion, implying that one or more additional SCF^{CaCDC4} substrates are responsible for this phenotype. Here, we identified CaUme6 as the SCF^{CaCDC4} substrate that, together with Sol1, is responsible for the hyphal phenotype of Cacdc4Δ/Δ. SCF^{CDC4} requires phosphorylation of its substrates for recognition (38). In a screen for the kinase responsible for CaUme6 degradation, we identified the CDK1 cyclin CaCln3 as a suppressor of CaUme6 activity. However, CaCln3 activity led to CaUme6 stabilization rather than degradation. In contrast, the CDK1 cyclin Hgc1 was required for CaUme6 degradation. Since HGC1 is a key transcriptional target of CaUme6, this generates a negative-feedback loop in which CaUme6 activity causes its own demise. Suppression of CaUme6 activity by CaCln3 disrupts this feedback loop, leading to stabilization of CaUme6.

RESULTS

CaUme6 is a substrate of SCF^{CaCDC4}. The recognition sequence of Cdc4-type substrate receptors is not well defined but commonly includes a proline residue following the phosphorylated residue and a proline or other hydrophobic amino acid(s)
preceding it (39). Analysis of the CaUme6 sequence identified many such potential recognition sites. To directly test whether CaUme6 degradation depends on the presence of SCFCaCDC4, we first tested the stability of CaUme6 in wild-type versus Ca\(\text{cdc53}\)\(^{\text{ts}}\) mutant cells (40). CaCdc53 encodes the cullin subunit, an essential scaffold subunit common to all SCF complexes. As shown in Fig. 1A, CaUme6 was normally rapidly degraded upon promoter shutoff but was almost totally stabilized in the Ca\(\text{cdc53}\)\(^{\text{ts}}\) mutant, suggesting that SCF activity is required for CaUme6 degradation. We next tested whether the F-box protein CaCdc4 was required for CaUme6 degradation by measuring CaUme6 stability by pulse-chase analysis in a strain depleted for CaCdc4. As shown in Fig. 1B, CaUme6 was rapidly degraded in the control strain, with a half-life of 15 min, whereas the CaCdc4-depleted strain showed stabilization of CaUme6, consistent with CaUme6 being a substrate of SCFCaCDC4.

Both CaUme6 and Sol1 contribute to the hyphal phenotype of Ca\(\text{cdc4}\)\(^{\text{ts}}\). In order to investigate the possibility that CaUme6 is the critical target of SCFCaCDC4, responsible for the hyphal phenotype of the Ca\(\text{cdc4}\)\(^{\text{ts}}\) mutant (34), we performed genetic epistasis analysis using comparisons between the CaCDC4 and CaUME6 deletion mutants. If CaUme6 stabilization indeed caused the constitutively hyphal phenotype of Ca\(\text{cdc4}\)\(^{\text{ts}}\), then deletion of CaUME6 in the Ca\(\text{cdc4}\)\(^{\text{ts}}\) mutant should suppress this phenotype. As shown in Fig. 2, this was not the case: the double Ca\(\text{cdc4}\)\(^{\text{ts}}\) Caume6\(^{\text{ts}}\) mutant was still filamentous, albeit mostly pseudohyphal rather than hyphal. However, we knew from previous work that Sol1, another SCFCaCDC4 substrate, may also be involved in the filamentous growth of the Ca\(\text{cdc4}\)\(^{\text{ts}}\) mutant (34). We therefore tested the deletion of both CaUME6 and SOL1 together in the Ca\(\text{cdc4}\)\(^{\text{ts}}\) background; the triple mutant lost its hyphal growth phenotype at the levels of both cell morphology and colony morphology. Reintroduction of a wild-type CaUME6 allele restored filamentous growth to the mutant (Fig. 2A), consistent with CaUme6 being (to

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**FIG 1** CaUme6 is stabilized in the absence of SCFCaCDC4 activity. (A) CaUme6-6xMyc was expressed from the MAL2 promoter of plasmid KB2147 in either wild-type (WT; KC2) or Ca\(\text{cdc53}\)\(^{\text{ts}}\) (KC363) cells by inducing the cultures for 3 h with maltose. Aliquots were taken at the indicated times after glucose addition, and CaUme6 was visualized by Western blotting. C, no-tag control. (B) The TETp-CaCDC4 strain contains a single copy of the CaCDC4 gene under the regulation of the TEToff promoter; i.e., the promoter is shut off in the presence of tetracycline. Myc epitope-tagged CaUme6 was expressed from the CUP1 promoter of plasmid KB1994 in strain KC200 (TETp-CaCDC4). Degradation of CaUme6 was monitored by [\(^{35}\text{S}\)]methionine pulse-chase analysis in a culture preincubated for 3 h with tetracycline versus a control culture. Both cultures were incubated for 15 min with 0.1 mM copper prior to labeling. For each time point, equal radioactivity counts were subjected to immunoprecipitation with anti-Myc antibodies, and the immunoprecipitate was loaded onto the gel. In the presence of tetracycline, a slower-migrating form of CaUme6 accumulates. The graph indicates the amount of CaUme6 signal at each time point, relative to the 0 time point. C, no-tag control.
a major extent if not exclusively) responsible for the hyphal phenotype of the Cdc4−/− mutant. In addition to this morphological analysis, we also measured expression levels of two hypha-specific genes, HWP1 and ECE-1, in the Cdc4−/− strain versus the Cdc4+/− Caume6+/− strain. In the absence of Caume6, the increase in hypha-specific gene expression in the Cdc4−/− strain was largely abolished, even in the presence of SOL1 (Fig. 2B).

**Recapitulation of Caume6 degradation in S. cerevisiae.** F-box proteins of the Cdc4 type universally require phosphorylation of the substrate at one or multiple sites for recognition (36, 41–43). Consistent with the consequent assumption that Caume6 requires phosphorylation for recognition by the SCF^CaCDC4^ ligase, a lower-mobility species of Caume6 was seen to accumulate in CaCdc4-depleted cells (Fig. 1B). Thus, in order to understand the regulation of Caume6 degradation, it was important to identify the kinase(s) that phosphorylates Caume6 at the CaCdc4 recognition sites. Because of the difficulty of carrying out extensive genetic screens in C. albicans, we tested whether S. cerevisiae could serve as a model organism for the investigation of Caume6 degradation. Strikingly, expression of Caume6 under the control of the strong inducible GAL1 promoter in S. cerevisiae was toxic, and this toxicity was exacerbated in the cdc4-1 mutant and in cdc53-1, a mutant of the SCF cullin subunit (Fig. 3A). This hypertoxicity of Caume6 correlated with partial stabilization of Caume6 in the cdc4-1 hypomorphic mutant (Fig. 3B). Interestingly, substituting CDC4 with CaCDC4 in S. cerevisiae restored the strain to normal sensitivity to Caume6 (Fig. 3A), suggesting that the Caume6-CaCdc4 interaction could be reconstituted in S. cerevisiae.

**Identification of a potential Caume6 kinase in S. cerevisiae.** The degradation of Caume6 by SCF^CaCDC4^ or SCF^CaCDC4^ in S. cerevisiae implies that one or more kinases in that organism are capable of phosphorylating Caume6. We therefore next addressed the identity of the Caume6 kinase, using the toxicity phenotype of Caume6 overexpression in S. cerevisiae as the initial assay. Since Cdc4 substrates are often phosphorylated by cyclin-dependent kinases (43), we started by assaying Caume6 sensitivity in a mutant of the main cell cycle CDK, Cdk1/Cdc28. We found that cdc28-1N cells were hypersensitive to Caume6 overexpression (Fig. 4A). Furthermore, Caume6 was strongly stabilized in the cdc28-1N mutant (Fig. 4B).

Cdc28 in S. cerevisiae is activated by nine cyclins—three G1 cyclins and six B-type cyclins—which also contribute to the substrate specificity of the kinase (44). In other instances of CDK-mediated protein degradation, the overexpression toxicity of SCF substrates could be partially suppressed by co-overexpression of the cyclin required for their degradation (45, 46). We therefore next tested whether Caume6 toxicity could be suppressed by overexpression of a Cdc28 cyclin. Whereas B-type cyclin overexpression...
did not suppress CaUme6 toxicity (data not shown), the G1 cyclin Cln3 (but not Cln2) was able to partly suppress CaUme6 toxicity when overexpressed (Fig. 4C, top panel).

The *C. albicans* homolog of this gene, CaCLN3 (32, 33), was similarly able to partly suppress CaUme6 toxicity in *S. cerevisiae* (Fig. 4C, bottom panel).

**CaCln3 suppresses CaUme6-induced filamentation in *C. albicans***. Ectopic overexpression of CaUME6 can induce hyphal growth, even in rich medium (20, 21). To test whether the suppression of CaUme6 by CaCln3 can be recapitulated in *C. albicans*, we assayed the effect of ectopic overexpression of CaCln3 on the CaUme6-induced hyphal growth. While CaUME6 expression under the control of the MAL2 promoter indeed induced robust hyphal growth, co-overexpression of CaCLN3 suppressed this effect in a large measure and left the cells for the most part in the yeast morphology (Fig. 5A). This microscopic morphology was mirrored in the sedimentation of the culture: while elongated cells typically sediment faster than yeast cells, the cells in a culture overex-
pressing CaUME6 formed extended hyphae to such an extent that a never-sedimenting mycelium was formed in the test tube (Fig. 5B). In contrast, cells co-overexpressing CaCLN3 with CaUME6 sedimented normally. A corresponding effect was also seen at the level of colony morphology: the crenellated morphology, characteristic of filamentous growth, induced by CaUme6 was largely suppressed by co-overexpression of CaCln3 (Fig. 5C). Finally, we concomitantly tested expression of the hypha-induced genes HWP1, ECE1, and HGC1 by Northern blotting in cells overexpressing CaUME6, alone or together with CaCLN3. All three genes were strongly induced upon CaUME6 induction alone, but this induction was strongly suppressed by co-overexpression of CaCLN3 (Fig. 5D). CaUME6 expression was barely affected by CaCLN3 co-overexpression, confirming that the effect of CaCLN3 on CaUme6 is posttranscriptional.

**Role of Cdc28 cyclins in CaUme6 degradation.** Following the suppression of CaUme6 activity by CaCln3, we tested whether CaCln3 overexpression induces CaUme6 degradation. Unexpectedly in view of the previous results, rather than causing CaUme6 degradation, co-overexpression of CaCLN3 caused stabilization of CaUme6 (Fig. 6A). This occurred in spite of the fact that in the same cells, co-overexpression of CaCLN3— but not of other G1 cyclins— with CaUME6 caused suppression of the hyphal induction (see Fig. S2 in the supplemental material). Since Cdc28/Cdk1 had been found to be involved in CaUme6 degradation in *S. cerevisiae*, we screened additional *Candida albicans* Cdc28 cyclins for effects on CaUme6 degradation. Among the cyclins tested, only Hgc1 overexpression induced an acceleration of CaUme6 degradation (Fig. 6A). Conversely, a mutant lacking *HGC1* showed almost complete stabilization of CaUme6, placing the Cdc28 cyclin Hgc1 within the degradation pathway of CaUme6 (Fig. 6B) (a mutant lacking CaCLN3 was not tested since that mutant was inviable). Surprisingly, alongside stabilization of CaUme6, the *hgc1−/−* mutant also exhibited markedly reduced steady-state levels of CaUme6. We tested whether this was due to reduced mRNA expression in the *hgc1−/−* strain. However, levels of CaUme6 mRNA expressed under the control of the MAL2 promoter were very similar in the wild-type and *hgc1−/−* backgrounds.
We next tested whether this was an idiosyncrasy of our specific mutant strain by constructing a new mutant strain by clustered regularly interspaced short palindromic repeat (CRISPR) mutagenesis (47). In this new strain set, CaUme6 was still stabilized but showed lower expression levels. In order to test whether these lower levels were due to lower translation levels, cells were subjected to pulse-labeling with \([35S]\)methionine (Fig. 6C). In three independent experiments, levels of CaUme6 were 65% lower in the mutant than in the wild-type strain, suggesting that, in addition to promoting CaUme6 degradation, Hgc1 promotes CaUme6 translation.

The stabilization of CaUme6 observed in C. albicans hgc1 mutant strain prompted us to revisit degradation of CaUme6 in S. cerevisiae. By sequence alignment, the closest homologs in S. cerevisiae to C. albicans Hgc1 are the G1 cyclins Cln1 and Cln2 (48, 49). We had found that Cln2 overexpression was unable to suppress CaUme6 toxicity in S. cerevisiae (Fig. 4C). Indeed, in S. cerevisiae, neither overexpression of Hgc1 nor overexpression of...
Cln2 induced any acceleration of CaUme6 degradation (Fig. S4). However, in the reciprocal experiment, deleting both the CLN1 and CLN2 HGC1 homologs from the *S. cerevisiae* genome resulted in almost complete stabilization of CaUme6 (Fig. 6D). This suggests that the kinase responsible for SCFCDC4-mediated CaUme6 degradation in *S. cerevisiae* is Cdc28-Cln1/2, the closest homolog of *C. albicans* Cdc28-Hgc1.

**DISCUSSION**

The observation that a mutant of CaCDC4, one of the substrate recognition factors of the SCF ubiquitin ligase, is locked in the hyphal morphology demonstrated the involvement of ubiquitin-mediated protein degradation in *C. albicans* morphogenesis (34). Here, we identify a key hyphal morphogenesis transcription factor, CaUme6, as the critical SCFCDC4 substrate responsible for the hyphal phenotype of the Cacdc4−/− mutant. Like all known SCFCDC4 substrates, CaUme6 was expected to require phosphorylation in order to be recognized by its ubiquitin ligase. We identify here the Cdc28 cyclin Hgc1 and, in *S. cerevisiae*, the Cdc28 kinase, together with the G1 cyclins Cln1 and Cln2, as kinases that are required for CaUme6 degradation.

The *C. albicans* G1 cyclin Hgc1 was shown to be absolutely necessary for hyphal growth (49). Specific substrates phosphorylated by Hgc1 under hyphal growth conditions include the septin Cdc11 (50), the Cdc42 GTPase-activating protein (GAP) Rga2 (51), and the transcription factor Efg1 (52). HGC1 is an essential transcriptional target of CaUme6 in the hyphal induction pathway (53). Thus, the Hgc1-mediated degradation of CaUme6 leads to a negative-feedback loop that keeps cellular CaUme6 levels in check (Fig. 7), similarly to the negative-feedback loop between the transcription factor Gcn4 and the cyclin Pcl5 in both *C. albicans* and *S. cerevisiae* (45, 46). Complicating the picture, however, we also found a stimulatory effect of Hgc1 on CaUme6 translation, via an unknown mechanism. The translational effect shown here does not depend on the extended 5′ untranscribed region (5′ UTR) of the native CaUME6 transcript (54), since this region is absent from our MAL2 promoter-driven CaUME6 expression construct. Thus, Hgc1 exerts both positive and negative effects on CaUme6 levels. Further elucidation of the mechanism of CaUme6 translational regulation by Hgc1 will be required to understand how Hgc1 affects net CaUme6 levels under different conditions.

CaUme6 degradation was previously found to be regulated by external conditions such as high CO2 and low O2 concentrations, in part via binding of the C terminus of Ofd1 (55). However, the negative-feedback regulation described here is operative in rich media under nonfilamentous growth conditions and appears thus to be distinct from this previously described regulation of CaUme6 degradation by CO2 and O2.

In the course of our search for the Cdc28 cyclin involved in CaUme6 degradation, we identified Cln3 as a cyclin that antagonizes CaUme6 activity. In the heterologous
S. cerevisiae system, the toxicity of CaUme6 was suppressed by Cln3/CaCln3 overexpression. In C. albicans, in the presence of elevated CaCln3 levels, the ectopic induction of hyphal growth by CaUme6 overexpression was abolished. It is worth mentioning that under standard physiological hyphal induction conditions (37°C, 10% serum), overexpression of CaCLN3 also suppressed hyphal morphogenesis (T. Gildor and D. Kornitzer, unpublished data). The suppression of the CaUme6 phenotypes by CaCln3 in C. albicans was accompanied by a dramatic reduction in the CaUme6-induced expression of its target genes. The reduction in CaUme6 activity was also accompanied by a strong stabilization of CaUme6, which was initially surprising. However, the identification of the CaUme6-Hgc1 negative-feedback loop immediately suggests a solution to this apparent paradox: if CaCln3 affects CaUme6 transcriptional activity, then it would also disrupt the CaUme6-Hgc1 feedback loop by interfering with expression of HGC1, causing stabilization of CaUme6 (Fig. 7).

If CaCln3 suppresses CaUme6 transcriptional activity, then deletion of CaCLN3 should lead to increased CaUme6 activity. CaCLN3 is an essential gene and cannot be deleted; however, when CaCLN3 was placed under the control of a repressible promoter, it was found that yeast cells depleted of CaCln3 arrested in G1, grew in size, and eventually formed hypha- or pseudohypha-like extensions (32, 33). Interestingly, CaCln3-depleted cells were found to exhibit increased HGC1 expression, which is consistent with an increase in CaUme6 activity (33).

The simplest explanation for the effect of HGC1 and CaCLN3 on CaUme6 activity and stability is that Cdc28-Hgc1 and Cdc28-CaCln3 directly phosphorylate CaUme6. We were, however, unable to produce sufficient recombinant full-length CaUme6 to reconstitute these reactions in vitro. Instead, we were able to reconstitute in S. cerevisiae both the Cdc28-Cln1/2 dependence of CaUme6 degradation and the suppression of CaUme6 activity by Cdc28-Cln3/CaCln3. Reconstitution of these effects in a heterologous organism, in the absence of additional C. albicans factors, reinforces the assumption of direct phosphorylation of CaUme6 by these kinases in S. cerevisiae and, consequently, by their homologs Cdc28-Hgc1 and Cdc28-CaCln3 in C. albicans. Nonetheless, the possibility of an indirect effect of these kinases on CaUme6 via a third factor present in both organisms cannot be excluded.

The transcription factor CaUme6 lies at the heart of hyphal regulation in C. albicans as a necessary and sufficient factor for hyphal growth (3, 20) and as a key transcriptional target for the various signal transduction pathways that mediate hyphal induction stimuli (21). Here, we show that in addition to its transcriptional regulation, CaUme6 is also regulated posttranslationally by CaCln3. Whereas the transcriptional regulation of CaUME6 mediates the response to extracellular signals that induce hyphal growth, the CaCln3-mediated regulation may represent the input of cellular physiology in the dimorphic switch decision. The notion that cellular physiology affects responsiveness to hyphal induction is not new: for example, it was shown that stationary cells released in fresh hyphal induction medium exhibit more robust hyphal morphogenesis than mid-log-phase cells (24). This phenomenon could be explained in part by release from the inhibition mediated by the quorum-sensing molecule farnesol but likely involves cell-autonomous effects as well. However, the mechanism for this link between cell physiology and hyphal induction was unknown.

Our observation that CaCln3 suppresses CaUme6 activity provides a plausible mechanistic explanation for the antagonistic relationship between yeast proliferation and hyphal morphogenesis. Cell proliferation in all organisms is regulated by nutrient availability. The S. cerevisiae ortholog of CaCln3, Cln3, is the most upstream regulator at the start of the cell cycle (56, 57) and is subject to several transcriptional and posttranscriptional regulations linking its levels to the nutritional state of the cell (58–60). The regulation of CaCLN3 has not been investigated, but as an essential regulator of cell proliferation, it is likely to be similarly regulated by the physiological state of the cell. Thus, by responding inversely to CaCln3 levels, CaUme6 activity would be lower under optimal growth conditions and higher under nutrient-limiting conditions.

The proliferation-differentiation antagonism is well established in animal cells (61).
In particular, cyclin D1, the functional homolog of fungal Cln3, has been widely shown not only to promote proliferation but also to inhibit epithelial differentiation (62) as well as myogenesis and neurogenesis (63). To the extent that the switch between proliferation as yeast cells and differentiation into hyphal cells prefaces differentiation pathways in higher organisms, the role of Cln3 in hyphal morphogenesis may mirror the antagonistic role of the cell cycle apparatus, and of cyclin D in particular, in multicellular differentiation systems.

MATERIALS AND METHODS

Plasmids and strains. (i) Plasmids. The CaUME6 deletion plasmids KB2022 and KB2023 were generated by cloning the 5' region (position −640 to position +1; SacI-SpeI) and 3' region (position +2537 to position +3315; HindIII-KpnI) into KB985 and KB986 (34), respectively. KB2028 is CaUME6 (position +1 to position +3315; HindIII-KpnI) cloned into p416-Gall (64). KB2073 contains the CaUME6 region from position −1500 to position +2900 (NotI-KpnI) cloned into BES116 (65). KB2117 was constructed by first introducing the single Myc epitope sequence of KB1321 (34) into KB1321; the CaUME6 sequence was then fused downstream of the epo1 tag sequence of KB1319. KB1994 is CaUME6 cloned at BamHI-HindIII and fused to the Myc epitope of KB1321. KB2147 was constructed by first cloning the CaUME6 open reading frame (ORF) (position +1 to position +2529) at EcoRv-HindIII under the control of the MAL2 promoter of BES119 (65), followed by introduction of the 6xMyc-ScCYC1 terminator sequence of KB1578 (34) at HindIII-KpnI downstream of CaUME6. For KB2270, first, a Tet-on vector plasmid (KB1868) was constructed by introducing an Xmal-EcoRI-NotI multicloning site between Sall and BgIII of pNIM6 (66), followed by cloning of CaUME6-6xMyc-SCYC1 amplified from KB2147 between Sall and NotI. KB1615, KB1697, and KB1698 were previously described (40). KB1828 is CaCEN3 cloned at ClaI-ApaI under the control of the MAL2 promoter of KB1817 (67). KB991 is a URA3 2µ GAL1-CEN3 plasmid obtained from Gerry Fink. KB1826 contains the CEN3 open reading frame cloned at EcoRv-XbaI between the galactose (GAL) promoter of plasmid p415Gall (64) and the 3xMyc tag of plasmid KB989 (41). KB2144 contains the CaCEN3 sequence (position +1 to position +2083), PstI-Xhol, first cloned into p424-Gall (64) and then transferred as a Gal1-CaCEN3 fragment, at SacI-Xhol, to p416Gall (CEN URA3). KB2139 contains the HGC1 open reading frame cloned at Xbal-EcoRI under the control of the GAL1 promoter of plasmid p416-Gall (64).

(ii) Strains. The C. albicans strains are listed in Table 1. C. albicans deletion of CaUME6 was achieved by sequential deletion of both alleles using plasmids KB2022 and KB2023 to generate KC445. CaCENDC4 was deleted in KC445 using plasmid KB1344 (34) to generate KC449. To generate KC462, CaUME6 was deleted in KC446 using plasmid KC196 obtained by 5-fluoroorotic acid (5-FOA) selection. KC533 is a CaUME6 reintegrant strain obtained by transformation of KC464 (the ura3- derivative of KC462 obtained by 5-FOA selection) using plasmid KB2073 digested with Smal. Smal targets the plasmid to the promoter region of the deleted CaUME6 allele and reconstitutes the full gene. We found that targeting the KB2073 plasmid to the ADE2 locus instead does not complement CaUME6, suggesting that the sequences extending 1,500 nucleotides (nt) upstream of the translation start site are not sufficient to support full expression. This is consistent with analyses indicating that CaUME6 possesses exceptionally long 5' UTR and promoter sequences (54, 68). KC651 contains the MAL2 promoter of plasmid pFA-URA3-MAL2 (69) integrated upstream of the CaUME6 open reading frame by PCR-targeted recombination. KC965 was constructed by transforming KC2 with the nourseothricin-resistant (Nat') CRISPR-associated gene 9 (Cas9) pV1025 plasmid (47) following by removal of the Nat' marker as described in reference 47. To generate KC1014, HGC1 was mutated by CRISPR (47) using a guide RNA corresponding to positions 125 to 144 on the antisense strand and a mutagenic oligonucleotide introducing an Xhol site and a frameshift at position 124. The S. cerevisiae strains (Table 2) were all in the W303 background. KY879 was generated.

**TABLE 1** List of *C. albicans* strains

| Name | Genotype | Reference or source |
|------|----------|---------------------|
| KC2 = CAI4 | ura3Δ::imm434/ura3Δ::imm434 | 73 |
| KC138 | ura3Δ/ura3Δ cdc4Δ::hisG-URA3-hisG/cdc4Δ::hisG | 34 |
| KC196 | ura3Δ/ura3Δ sol1Δ/sol1Δ cdc4Δ::hisG-URA3-hisG/cdc4Δ::hisG | 34 |
| KC200 | ura3Δ/ura3Δ ENO1/eno1::ENO1-tetrR-SchAP4AD-3*HA-ADE2 cdc4Δ::hisG/Tc-CDC4-SAT1 | 34 |
| KC271 = SN78 | ura3Δ/ura3Δ leu2Δ/leu2Δ | 74 |
| KC274 = SN148 | ura3Δ/ura3Δ his1Δ/his1Δ leu2Δ/leu2Δ arg4Δ/arg4Δ | 74 |
| KC363 | KC271 cdc53Δ::E2U2/cdc53-1 | 40 |
| KC445 | ura3Δ/ura3Δ ura3Δ::hisG/urna6Δ::hisG | This work |
| KC449 | KC445 cdc4Δ::hisG-URA3-hisG/cdc4Δ::hisG | This work |
| KC462 | ura3Δ/ura3Δ sol1Δ/sol1Δ cdc4Δ::cdc4A::urna6Δ::hisG-URA3-hisG/urna6Δ::hisG | This work |
| KC532 | ura3Δ/ura3Δ his1Δ/his1Δ leu2Δ/leu2Δ arg4Δ/arg4Δ hgc1Δ::His1/hgc1Δ::E2U2 | Yue Wang |
| KC533 | ura3Δ/ura3Δ sol1Δ/sol1Δ cdc4Δ::cdc4A::urna6Δ::urna6Δ::ADE2/ade2::<urna3 UME6> | This work |
| KC651 | KC271 URA3 MAL2p::UME6 UME6 | This work |
| KC965 | KC2 ENO1/eno1::CaCEN9 | This work |
| KC1014 | KC965 his1Δ::/-- | This work |
TABLE 2 List of S. cerevisiae strains

| Name     | Genotype                          | Reference or source |
|----------|-----------------------------------|---------------------|
| KY337    | W303-1A                           | R. Rothstein        |
| KY440    | MTY668                            | M. Tyers            |
| KY442    | MTY740                            |                     |
| KY387    | W303 cdc4-1                       | 75                  |
| KY414    | W303 ura3-1 leu2-3,112 trp1-1 cdc28-1N | A. Amon             |
| KY879    | W303 ura3-1 his3-11, 15 trp1-1cdc4::HIS3 <CEN TRP1 CaCDC4> | This work          |

by shuffling the CaCDC4 gene on plasmid KB1261 (34) into MTY1260 (W303 ura3-1 his3-11 15 trp1-1cdc4::HIS3 <CEN URA3 CDC4::>; M. Tyers).

mRNA analysis. For RNA analysis by Northern blotting, a 10-ml volume of yeast culture was collected for each sample, harvested, and frozen in liquid nitrogen. The RNA was extracted according to the “hot phenol” method (70). A total of 3 μg of RNA was loaded in each lane. Radioactive DNA probes were synthesized using a NEBlot kit (New England Biolabs). The radioactive signals were quantitated with a phosphorimager. All specific gene signals were normalized to the 18S rRNA signal of the same gel lane.

Protein analysis. Protein levels were assayed by Western blotting using monoclonal antibody 9E10 to detect the Myc epitope. Proteins were extracted by the quantitative NaOH/2-mercaptoethanol method, as described previously (71). To compare steady-state protein levels, equal protein amounts were loaded; to monitor protein disappearance after promoter shutoff, equal culture volume equivalents were loaded. Loading and transfer were monitored by Ponceau staining of the membrane and by actin enhanced chemiluminescence (ECL) signals with a Bio-Rad Chemidoc apparatus, or using Li-COR infrared fluorescence IRDye secondary antibodies, followed by detection performed with an Odyssey imaging phosphorimager. All specific gene signals were normalized to the 18S rRNA signal of the same gel lane.

Microscopy. Cells were fixed in 70% ethanol and visualized with a Zeiss AxioImager M1 microscope equipped with differential interference contrast (DIC) optics, using a 40× or 100× objective. Colonies were visualized with a Zeiss Stemi 2000C binocular microscope.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSphere.00248-16.

FIG S1, JPG file, 1.9 MB.
FIG S2, JPG file, 1.3 MB.
FIG S3, JPG file, 0.4 MB.
FIG S4, JPG file, 0.6 MB.

ACKNOWLEDGMENTS

We thank Y. Wang (A*STAR Singapore), M. Tyers (U. of Montreal), A. Amon (MIT), and E. Elion (Harvard Medical School) for yeast mutant strains; Nadine Sela for constructing plasmid KB1826; and Sara Selig for critical reading of the manuscript.

This work was supported by Israel Science Foundation grant no. 1574/11 to D.K.

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