Role for the SCF^{CDC4} Ubiquitin Ligase in *Candida albicans* Morphogenesis

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The ability of *Candida albicans*, a major fungal pathogen, to switch between a yeast form, and a hyphal (mold) form is recognized as being important for the ability of the organism to invade the host and cause disease. We found that a *C. albicans* mutant deleted for *CaCDC4*, a homologue of the *Saccharomyces cerevisiae* F-box protein component of the SCF^{CDC4} ubiquitin ligase, is viable and displays constitutive filamentous, mostly hyphal, growth. The phenotype of the *Cacdc4*–/– mutant suggests that ubiquitin-mediated protein degradation is involved in the regulation of the dimorphic switch of *C. albicans* and that one or more regulators of the yeast-to-mold switch are among the substrates of SCF^{CaCDC4}. Epistasis analysis indicates that the *Cacdc4*–/– phenotype is largely independent of the filamentation-inducing transcription factors Efg1 and Cph1. We identify *C. albicans* Far1 and Sol1, homologues of the *S. cerevisiae* SCF^{CDC4} substrates Far1 and Sic1, and show that Sol1 is a substrate of *C. albicans* Cdc4. Neither protein is essential for the hyphal phenotype of the *Cacdc4*–/– mutant. However, ectopic expression and deletion of *SOL1* indicate a role for this gene in *C. albicans* morphogenesis.

INTRODUCTION

Candida albicans is a commensal organism of the gastrointestinal tract that can cause life-threatening, systemic infection among immunocompromised or debilitated patients (Richardson and Warnock, 1997). One of the central and defining characteristics of *C. albicans* is its ability to switch between at least three distinct growth forms: a yeast form, a hyphal form—characterized by parallel cell walls without constrictions at the sites of septation, and a pseudohyphal form—characterized by unseparated chains of elongated cells (Sudbery *et al.*, 2004). It is thought that the hyphal form of growth promotes the ability of the fungus to penetrate the mucous membranes and gain access to the underlying tissues, eventually reaching the bloodstream and causing life-threatening candidemia. In support of this view, *C. albicans* mutants unable to switch from the yeast form to the hyphal form showed strongly reduced virulence in a mouse model of systemic infection (Lo *et al.*, 1997; Zheng and Wang, 2004).

A variety of environmental stimuli are known to promote the switch to hyphal growth in *C. albicans*: neutral or alkaline pH, carbon starvation, nitrogen starvation, cell density, oxygen concentration, and elevated temperature (>35°C) (reviewed in Ernst, 2000). Incubation in serum at 37°C is a common and potent way to induce hyphal formation, possibly because serum imposes several types of signals. Several transcription factors were identified that regulate filamentous growth: Cph1 (Liu *et al.*, 1994), Efg1 (Lo *et al.*, 1997; Stoldt *et al.*, 1997), Cph2 (Lane *et al.*, 2001), CaTec1 (Schweizer *et al.*, 2000), CaTup1 (Braun and Johnson, 1997),

CaNrg1 (Braun *et al.*, 2001), CaMcm1 (Rottmann *et al.*, 2003), and CaFkh2 (Bensen *et al.*, 2002). Deletion of *CaTUP1*, *CaNRG1*, or *CaFKH2* cause constitutive pseudohyphal growth. Deletion of either *CPH1* or *EFG1* causes a reduction in the ability to form hyphae, with the *efg1* mutant having the stronger defect, and the double mutant being virtually unable to form hyphae under most conditions (Lo *et al.*, 1997).

Cell morphogenesis is closely associated with regulation of the cell cycle in many eukaryotes, particularly in budding yeasts (Lew and Reed, 1995; Rua *et al.*, 2001). The morphogenetic switch in *C. albicans* may therefore be expected to involve regulation at the level of the cell cycle regulatory machinery. Indeed, repression of the Polo-like kinase CaCdc5, a mitotic regulator, was found to induce hyphal growth (Bachewich *et al.*, 2003). Furthermore, CaCln1/Ccn1, a protein with homology to the *Saccharomyces cerevisiae* G₁ cyclins, was found to be required for maintenance of hyphal growth (Loeb *et al.*, 1999), and Hgc1, a *C. albicans* cyclin related to the *S. cerevisiae* G₁ cyclins Cln1 and Cln2 was shown to be necessary, but not sufficient, for induction of hyphal growth (Zheng and Wang, 2004). Recently, depletion of the *C. albicans* Cln3 homologue, an essential protein, was shown to induce hyphal or pseudohyphal growth, depending on the conditions (Bachewich and Whiteway, 2005; Chapa y Lazo *et al.*, 2005).

Ubiquitin-mediated protein degradation (Hershko and Ciechanover, 1998; Kornitzer and Ciechanover, 2000) plays a central role in cell cycle control. Skp1-Cullin-1/Cdc53-F-box protein (SCF)^{CDC4}, an SCF ubiquitin ligase complex that includes Cdc4 as substrate recognition component, is one of the ubiquitin ligases implicated in cell cycle entry and progression in yeast and mammalian cells. The mammalian homologue of Cdc4 is involved in degradation of Cyclin E (Koepp *et al.*, 2001; Moberg *et al.*, 2001; Strohmaier *et al.*, 2001) and of the proto-oncogene c-Myc (Welcker *et al.*, 2004; Yada *et al.*, 2004) and was shown to function as a tumor

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Table 1. *C. albicans* strains

Name	Genotype	Source
CAI4	<i>Caura3Δ::imm434/Caura3Δ::imm434</i>	Fonzi and Irwin (1993)
THE1	<i>Caura3Δ::imm434/Caura3Δ::imm434 ade2::hisG/ade2::hisG</i> <i>ENO1/eno1::ENO1-tetR-ScHAP4AD-3xHA-ADE2</i>	Nakayama <i>et al.</i> (2000)
HLC69	<i>CAI4 cph1::hisG/cph1::hisG efg1::hisG efg1::hisG</i>	Lo <i>et al.</i> (1997)
KC16	<i>CAI4 crp1Δ::hisG/crp1Δ::hisG</i>	Weissman <i>et al.</i> (2000)
KC135	<i>CAI4 CaCDC4/Cacdc4Δ::hisG-URA3-hisG</i>	This work
KC136	<i>CAI4 CaCDC4/Cacdc4Δ::hisG</i>	This work
KY137,138	<i>CAI4 Cacdc4Δ::hisG/Cacdc4Δ::hisG-URA3-hisG</i>	This work
KC163	<i>CAI4 CaFAR1/Cafar1Δ::hisG-URA3-hisG</i>	This work
KC165	<i>CAI4 Cafar1Δ::hisG/Cafar1Δ::hisG-URA3-hisG</i>	This work
KC166	<i>CAI4 Cafar1Δ::hisG/Cafar1Δ::hisG</i>	This work
KC178	<i>HLC69 CaCDC4/Cacdc4Δ::hisG-URA3-hisG</i>	This work
KC180	<i>HLC69 Cacdc4Δ::hisG/Cacdc4Δ::hisG-URA3-hisG</i>	This work
KC181	<i>KC166 CaCDC4/Cacdc4Δ::hisG-URA3-hisG</i>	This work
KC183	<i>KC166 Cacdc4Δ::hisG/Cacdc4Δ::hisG-URA3-hisG</i>	This work
KC184	<i>CAI4 SOL1/sol1Δ::hisG-URA3-hisG</i>	This work
KC185	<i>CAI4 SOL1/sol1Δ::hisG</i>	This work
KC186	<i>CAI4 sol1Δ::hisG/sol1Δ::hisG-URA3-hisG</i>	This work
KC187	<i>CAI4 sol1Δ::hisG/sol1Δ::hisG</i>	This work
KC194	<i>KC187 CaCDC4/Cacdc4Δ::hisG-URA3-hisG</i>	This work
KC196	<i>KC187 Cacdc4Δ::hisG/Cacdc4Δ::hisG-URA3-hisG</i>	This work
KC197	<i>THE1 Cacdc4Δ::hisG-URA3-hisG/CaCDC4</i>	This work
KC198	<i>THE1 Cacdc4Δ::hisG/CaCDC4</i>	This work
KC200	<i>THE1 Cacdc4Δ::hisG/CaCDC4::TETp-SAT-1</i>	This work
KC239	<i>CAI4 sol1Δ::hisG/sol1Δ::hisG Caade2::CaURA3 SOL1</i>	This work

suppressor gene (Spruck *et al.*, 2002; Mao *et al.*, 2004; Rajagopalan *et al.*, 2004). In *S. cerevisiae*, in contrast, *CDC4* is essential for cell proliferation, and temperature-sensitive mutants arrest in G₁ with elongated, multiple buds (Hereford and Hartwell, 1974). The critical substrate of yeast SCF^{CDC4} for the G₁-to-S transition is the cyclin-dependent kinase inhibitor Sic1 (Schwob *et al.*, 1994; Bai *et al.*, 1996; Feldman *et al.*, 1997; Skowrya *et al.*, 1997; Verma *et al.*, 1997b). Other substrates of SCF^{CDC4} include another cyclin-dependent kinase (CDK) inhibitor, Far1 (Henchoz *et al.*, 1997), and a transcription factor, Gcn4 (Meimoun *et al.*, 2000; Chi *et al.*, 2001).

Here, we describe the identification and characterization of the functional *C. albicans* homologue of Cdc4 and of a *C. albicans* Sol1, a homologue of the *S. cerevisiae* Cdc4 substrate Sic1. As opposed to ScCDC4, CaCDC4 is not essential. Deletion of CaCDC4 leads to constitutive filamentous growth, suggesting a role for ubiquitin-mediated protein degradation in the dimorphic switch of *C. albicans*.

MATERIALS AND METHODS

Media and Strains

Media included either YPD, or synthetic complete medium, both described in Sherman *et al.* (1986). Lee's serum was prepared as described previously (Lee *et al.*, 1975). Selection for uridine prototrophy was performed on synthetic complete plates lacking uridine and uracil. Uridine auxotrophy was performed on synthetic complete plates containing 50 μg/ml uridine and 1 mg/ml 5-fluoroorotic acid. Nourseothricin selection was performed by first plating the cells on YPD plates, incubating for 2 d at 30°C, and then replica-plating on YNB plates containing 400 μg/ml nourseothricin. Copper induction was carried out by adding 0.1 mM CuSO₄ to synthetic complete medium. The *S. cerevisiae* *dbf2-3* strain (Donovan *et al.*, 1994) was kindly supplied by Lee Johnston (MRC, London, United Kingdom). The *C. albicans* strains were derived either from CAI4 (Fonzi and Irwin, 1993) or from THE1 (Nakayama *et al.*, 2000). *C. albicans* strains are listed in Table 1.

Plasmids

CaCDC4. The CaCDC4 genomic region was PCR amplified using primers at positions -1067 and +2564 relative to the CaCDC4 open reading frame (2307

nucleotides [nt] in length) and was cloned between the *Hind*III and *Xho*I sites of plasmid B2205, a 2μ vector based on pRS306 (Sikorski and Hieter, 1989), to generate KB1083. In a second step, CaCDC4 was transferred as a *Sac*I-*Xho*I fragment from KB1083 to pRS314 (Sikorski and Hieter, 1989), to generate KB1261. For deleting CaCDC4, in a first step, a genomic region extending between a *Sac*I site located at position -1228 relative to the CaCDC4 start site and a *Kpn*I site located at position +2727 (421 nt beyond the CaCDC4 stop codon), was PCR amplified and cloned in pBSII-SK+ (Stratagene, La Jolla, CA) digested with the same enzymes, to generate KB1342. In a second step, the hisG-CaURA3-hisG *Bam*HI-*Bgl*III "blaster" fragment (Fonzi and Irwin, 1993) was cloned into KB1342 digested with *Bcl*I, to generate KB1344-1 and KB1344-3 (two orientations). *Bcl*I cuts the CaCDC4 region at three positions: -218, +239, and +1574 relative to the CaCDC4 start site. Thus, this deletion construct removes 218 nt of the promoter region and the majority of the coding sequence of CaCDC4, including the F-box domain and some of the WD40 repeats. For deleting CaCDC4, KB1344 was digested with *Sac*I and *Kpn*I before transformation.

TET-CaCDC4. Strain THE1 (Nakayama *et al.*, 2000) was deleted for one allele of CaCDC4 by using plasmid KB1344. The second allele was placed under the regulation of the TET promoter by transforming the strain with a fragment containing SAT-1 (nourseothricin resistance) and the TET promoter (Roemer *et al.*, 2003), PCR amplified with primers TET-CaCDC4up (5'-CAAAGTATCT-GTGTGAATTTTGGATACAAAAGTTTCTGTGATTTTCAGTTTGTCACAAAC-AAAACCCGGGATCGATAGAGCT) and TET-CaCDC4dn (5'-CTGTACT-CAAAATTAGCCGTCTCTCGCTCAAAGGATATTGAATAGCTTTGATTTTCTATCCATATCCGGTAATTTAGTGTG). For epitope tagging of CaCdc4, in a first stage a plasmid was built containing the 13 × Myc cassette followed by the CaURA3 marker: the CaURA3 *Bam*HI-PmeI fragment of pFA-URA3-MAL2p (Gola *et al.*, 2003) was cloned into pFA6a-13Myc-TRP1 (Longtine *et al.*, 1998) digested with *Bgl*III and *Pme*I, to generate pFA6a-13Myc-CaURA3 (KB1541). In a second stage, the 13 × Myc-CaURA3 was PCR amplified using primers containing 65 nt preceding the stop codon of CaCDC4 followed by sequence F2 (Longtine *et al.*, 1998), and the 65 nt after the stop codon followed by sequence S2 (Gola *et al.*, 2003). This fragment was used for transformation, and transformants were screened by Western blotting.

CaFAR1. The CaFAR1 (Orf19.7105; 2307 nt) deletion plasmid was constructed by sequentially introducing the 3' region of the gene (coordinates +2313 to +3093) as an *Spe*I-*Sac*I fragment, and the 5' region (coordinates -850 to +50) as an *Xho*I-*Hind*III fragment, into KB985 and KB986, to generate KB1387 and KB1388, respectively. KB985 and KB986 contain the hisG-CaURA3-hisG *Bam*HI-*Bgl*III blaster fragment (Fonzi and Irwin, 1993) cloned into the *Bam*HI site of pBSII-SK+ in two opposite orientations. For deleting CaFAR1, the plasmids were digested with *Xho*I and *Sac*I before transformation.

SOL1. The *SOL1* (Orf19.6930; 717 nt)-carrying plasmid KB1606 was isolated as a clone of the 2 μ *URA3* *C. albicans* genomic library (Liu *et al.*, 1994). The library insert extended from –317 to +1506 relative to the *SOL1* transcription start site; *SOL1* is the only complete open reading frame (ORF) in this insert. To express *SOL1* in *S. cerevisiae*, the coding sequence was PCR amplified with primers that added *Hind*III and *Kpn*I sites at positions –5 and +872, and the resulting PCR fragment was cloned in p416-GAL1 (Mumberg *et al.*, 1994) digested with the same enzymes, to generate KB1599. To express an N-terminally truncated *SOL1* a 5' primer adding a *Hind*III site and an ATG start codon at position +319 was used with the same 3' primer, to generate KB1600. To express epitope-tagged *Sol1* in *C. albicans* under the *CaMAL2* promoter, a *Clal*-*Hind*III PCR fragment carrying the whole *SOL1* open reading frame was cloned instead of the *Clal*-*Hind*III fragment of plasmid KB1575 (Gildor *et al.*, 2005), to generate KB1578. For transformation, KB1578 was linearized with *Asc*I. For deleting *SOL1*, the 5' (–785 to +3; *Kpn*I-*Pst*I) and 3' (+719 to +1558; *Spe*I-*Sac*I) regions of the gene were sequentially introduced into KB985, KB986 to generate KB1476-1 and -2, respectively. The plasmids were digested with *Kpn*I and *Sac*I before transformation. For reintegration of *SOL1*, a *Pst*I-*Kpn*I PCR fragment extending from –667 to +865 relative to the *SOL1* initiation codon was cloned into BES116 (Feng *et al.*, 1999) to generate KB1622.

CaCUP1p-SOL1, -SIC1. The *CaCUP1p* expression vector was constructed by introducing the *CaCUP1* promoter fragment as a *Not*I-*Spe*I PCR fragment extending from –518 to –1 relative to the *CaCUP1* open reading frame (Weissman *et al.*, 2000) into BES116 (Feng *et al.*, 1999), digested with the same enzymes, to generate KB1317. In a second stage, an ATG start codon followed by a single Myc tag sequence was introduced as a *Spe*I-ATG-Myc-*Bgl*II-*Pst*I synthetic oligonucleotide (5'-ACTAGTCCATGGAACAAAAGTTGATTCTCTGAAGAAGATTGAGATCTGCAG) between the *Spe*I and *Pst*I sites of KB1317 to generate KB1321. The following fragments were then cloned into KB1321: *SIC1* as a *Pst*I-*Hind*III fragment (–2 to +1080) to generate KB1386, *SIC1* Δ N as a *Bgl*II-*Hind*III fragment (+408 to +1080) to generate KB1389, *SOL1* as a *Pst*I-*Hind*III fragment (–2 to +900) to generate KB1472, and *SOL1* Δ N as a *Bgl*II-*Hind*III fragment (+253 to +900) to generate KB1473. All plasmids were linearized with *Asc*I for transformation.

Protein Analysis

Protein levels were assayed by Western blotting analysis by using the monoclonal antibodies 9E10 to detect the Myc epitope or 12CA5 to detect the hemagglutinin epitope. Proteins were extracted by the quantitative NaOH/2-mercaptoethanol method, as described previously (Weissman and Kornitzer, 2004). 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.

Microscopy

Cells were fixed in 70% ethanol and visualized with a Zeiss Axioskop 2 epifluorescence microscope equipped with differential interference contrast optics, by using a 63 or 100 $\times$ objective. For cell wall staining, fixed cells were incubated 15–30 min in 1 μ g/ml calcofluor white, washed in phosphate-buffered saline, and observed by epifluorescence with a 4,6-diamidino-2-phenylindole (DAPI) filter. *Cacdc4* $^{-/-}$ mycelia were homogenized with a motorized Kontes pellet pestle in 1.5-ml microfuge tubes before microscopic observation. Colonies were visualized with a Zeiss Stemi 2000C binocular microscope. Small colonies were visualized with a Zeiss Axiolab microscope by using a 10 $\times$ long working distance objective. Time-lapse microscopy was performed on an $\sim$ 0.5-ml agar pad on a microscope slide, on which the cells (1 μ l) were laid down and covered with a coverslip. We found that at room temperature, cells actively divided for at least 8 h under these conditions.

Flow Cytometry

Cells were prepared according to Haase and Reed (2002), with some modifications. Cells (10^7 – 10^8) were fixed in 70% ethanol for 1 h to overnight, washed with 0.2 M Tris, pH 7.5, and then incubated overnight in a shaker at 37°C in 0.2 M Tris, pH 7.5, 10 mM EDTA with 1 mg/ml RNaseA. The cells were then spun down, resuspended in 50 mM HCl with 5 mg/ml pepsin, incubated a further 2 h at 37°C, and washed with 0.2 M Tris, pH 7.5. Approximately 5×10^6 cells were then incubated at least 15 min at 30°C while shaking in 0.3 ml of 0.2 M Tris, pH 7.5, with 2 μ M SYTOX (Molecular Probes, Eugene, OR). The cells were diluted 1:10 in 0.2 M Tris, pH 7.5, just before injection into the flow cytometer (BD Biosciences FACScalibur). Ungated events (20,000) were recorded for each run. In cases where the 2n versus 4n peak assignment was in doubt, the culture was spiked with a stationary phase *G*₁-arrested culture, and the increased peak was assigned as 2n.

Sequence Analysis

Sequences of *C. albicans* proteins were obtained from <http://www-sequences.stanford.edu/group/candida>; sequences of *Saccharomyces* spp. and *A. gossypii* proteins were obtained from <http://www.yeastgenome.org/>. The se-

Figure 1. *CaCDC4* is able to suppress the lethality of a deletion of *CDC4*. (A) Colonies of a *cdc4* Δ strain carrying either *S. cerevisiae* or *C. albicans* *CDC4* on plasmid KB1083 and grown for 2 d on YPD plates at 30 or 37°C, as indicated. (B) Cell morphology of the same strains grown at 30°C in YPD. Bar, 10 μ m.

quences were aligned by ClustalW, implemented with the MEGALIGN software (DNASar, Madison, WI), which also was used to generate the homology trees.

RESULTS

Identification of *CaCDC4*

In the course of studying the degradation of *CaGcn4*, the *C. albicans* homologue of the *S. cerevisiae* *Cdc4* substrate *Gcn4* (Tripathi *et al.*, 2002; Gildor *et al.*, 2005), we identified a *Cdc4* homologue in the *C. albicans* genome by sequence homology search (our unpublished data). This 768 codon-long open reading frame (*C. albicans* Orf19.2559; GenBank EAK99394), encodes a predicted F-box domain (residues 295–344) and a WD40 repeat domain (residues 394–763) that, when aligned with *S. cerevisiae* *Cdc4*, is found to carry all the essential and conserved residues defined by Orlicky *et al.* (2003). *CaCDC4* was exchanged with an *ScCDC4* carrying plasmid in a *cdc4* Δ background strain (MTY1260) (Nash *et al.*, 2001) by plasmid shuffle. The *CaCDC4* gene was able to rescue the deletion of the essential *CDC4* gene of *S. cerevisiae* (Figure 1A), although the enlarged and elongated bud morphology of the *CaCDC4*-substituted cells suggested a partial defect in cell cycle progression (Figure 1B). These results support the assignment of *CaCdc4* as the functional homolog of *S. cerevisiae* *Cdc4*.

Phenotype of the *Cacdc4* $^{-/-}$ Mutant

To investigate the function of *CaCdc4* in *C. albicans*, the two alleles of *CaCDC4* gene were deleted sequentially from the *C. albicans* genome (Figure 2A). The homozygous *Cacdc4* deletant displayed a striking phenotype: the colonies contained large number of aerial hair-like protrusions (Figure 2B, b and d) as well as filaments that penetrated deeply into the agar (Figure 2B, e); the protrusions, when observed under the microscope, seemed to consist of bundles of hyphae (Figure 2C, a and b). The consistency of the colonies was solid, rather than soft like a typical yeast colony. When *Cacdc4* $^{-/-}$ cells were scraped off the mutant colony and grown in liquid, the cells grew in one or several mycelial balls. To determine whether the filamentous *Cacdc4* $^{-/-}$

Figure 2. Deletion of *C. albicans* *CDC4*. (A) Southern blot analysis of the *CaCDC4* region in the following strains: lane 1, KC135 (*CaCDC4/Cacdc4Δ::hisG-URA3-hisG*); lane 2, KC136 (*CaCDC4/Cacdc4Δ::hisG*); lane 3, CAI4 (*CaCDC4/CaCDC4*); and lanes 4–6, KC137, 138, 139 (CAI4 *Cacdc4Δ::hisG/Cacdc4Δ::hisG-URA3-hisG*). The genomic DNA was digested with *Xho*I and *Xba*I, and probed with the *Xho*I–*Spe*I fragment of plasmid KB1344-1, which corresponds to the 3' region of *CaCDC4*. (B) *Cacdc4*^{−/−} colony phenotype of cells grown for 2 d at 30°C on YPD. Wild-type *C. albicans* (CAI4) (a); *Cacdc4*^{−/−} mutant (KC138) on a YPD plate; backlit exposure of wild-type and mutant colonies (c and d), to show the structure of the colony; and the halo around the mutant colony (KC138) (e) represents hyphae penetrating the agar. (C) Micrographs of the protuberances displayed by the *Cacdc4*^{−/−} mutant colonies, at 100× (a) and 200× (b) magnifications; calcofluor staining of cells from the *Cacdc4*^{−/−} colony, grown in liquid YPD overnight (d), or diluted in liquid YPD from an overnight culture and grown for 5 h (e). For c and d, mycelia were broken mechanically before visualization; for e, the edge of a small mycelium is shown. Bar, 10 μm.

cells are hyphal or pseudohyphal, part of a colony or a ball of liquid-grown cells were separated with a homogenizer and stained with calcofluor to visualize the cell walls and the septa or the sites of cell separation. Hyphae usually exhibit straight walls with no constriction at the site of the septum, whereas pseudohyphal cells are typically wider in the middle and show constrictions at the sites of cytokinesis (Sudbery *et al.*, 2004). According to these criteria, cells from the *Cacdc4*^{−/−} colony showed a majority of hyphal cells and some pseudohyphal cells (Figure 2C, c). For the liquid cultures, both stationary and exponentially growing, cell mor-

phology similarly seemed to be a mixture of hyphal and pseudohyphal cells, with a higher proportion of pseudohyphal cells (Figure 2C, d and e).

ECE1 and *HWP1* are two genes that are normally expressed only under hyphal induction conditions such as serum or Lee's medium (Birse *et al.*, 1993; Sharkey *et al.*, 1999). We tested expression of these genes in wild-type versus *Cacdc4*^{−/−} mutant cells, under normal or hyphal-inducing conditions. The *Cacdc4*^{−/−} mutant cells showed constitutive high expression of both *ECE1* and *HWP1* regardless of the growth conditions (Figure 3).

Figure 3. Northern blot of HWP1 and ECE1 in wild-type and *Cacdc4*^{-/-} cells. Cultures were grown in YPD (Y) or in two types of inducing conditions: YPD + 20% serum for 2 h at 37°C (S) or Lee's medium for 4 h at 37°C (L). RNA (20 μ g) was loaded in each lane and detected with PCR products corresponding to the whole open reading frame of *ECE1* or to nucleotides +72 to +1120 of *HWP1*.

Figure 4. Effect of CaCDC4 shutoff on *C. albicans* morphology. (A) Western blot of CaCdc4-13 $\times$ Myc in a wild-type background strain or in strain KC200 after addition of 100 mg/ml tetracycline. C indicates a nontagged control strain. (B) Colony phenotype of strain KC200 (TET-CaCDC4) after 18 or 48 h on YPD agar with or without 100 mg/ml tetracycline. (C) Cell morphology of strain KC200 in liquid YPD at the indicated times after addition of 100 mg/ml tetracycline. The culture was diluted several times over the time course to keep it in logarithmic phase. Cells were stained with calcofluor and visualized by epifluorescence with a DAPI filter. Bar, 10 μ m. (D) Cell cycle distribution upon depletion of CaCdc4. KC200 cells were treated as for C, and aliquots, removed at the indicated time points after tetracycline addition, were subjected to FACS analysis. The cultures were diluted throughout the experiment in order to keep them in logarithmic phase. The starting culture was in late log phase, and therefore in the control culture, the 0-h time point contained a somewhat larger proportion of 2n cells than the subsequent time points.

To better manipulate CaCdc4 activity, a tetracycline-regulatable promoter (Roemer *et al.*, 2003) was integrated upstream of the remaining CaCDC4 allele of a *Cacdc4*^{+/-} heterozygote, in a strain background expressing the TetR-SchHap4AD protein (Nakayama *et al.*, 2000). The TET-regulated CaCDC4 allele was epitope-tagged to monitor the disappearance of the CaCdc4 protein upon tetracycline addition; within 90 min of tetracycline addition, most of the CaCdc4-13 $\times$ Myc protein had disappeared from the cells (Figure 4A). Growth on tetracycline plates yielded the same characteristic *Cacdc4*^{-/-} colonies (Figure 4B). In liquid culture, growth in tetracycline-containing medium did induce a switch to pseudohyphal growth after 8–14 h; after 24 h of logarithmic growth, true hyphae could be detected, but a

majority of cells still showed pseudohyphal morphology, with constrictions at the sites of cytokinesis (Figure 4C).

In *S. cerevisiae*, *cdc4* temperature-sensitive mutants arrest in G₁ (Hereford and Hartwell, 1974). To monitor the effect of CaCdc4 depletion on the cell cycle distribution of *C. albicans*, fluorescence-activated cell sorting (FACS) analysis was performed on the TET-CaCDC4. At 5.5 h after tetracycline addition, a significant shift toward the 4n peak was observed; at 7.5 h, the majority of cells were in the 4n peak, and an 8n peak occurred, presumably representing pseudohyphal chains of cells (Figure 4D). Beyond 7.5 h, the distribution shifted to a broad peak of high ploidy (our unpublished data), as expected from the exclusive occurrence of pseudohyphal chains at later time points (Figure 4C).

Epistasis Analysis of *Cacdc4*^{-/-} with *efg1*^{-/-} *cph1*^{-/-} and *Cafar1*^{-/-}

If the filamentation phenotype of the *Cacdc4*^{-/-} mutant is due to stabilization of a positive regulator of filamentous growth, then a mutant of this gene should be epistatic to *Cacdc4*^{-/-} and should prevent filamentation in the double mutant. *Efg1* and *Cph1* are two transcription factors that are required for filamentation; the double *efg1*^{-/-} *cph1*^{-/-} mutant fails to form hyphae under almost all hyphae-inducing conditions (Lo *et al.*, 1997). Thus, one possibility was that Cdc4 is required for the turnover of one of these factors, or both. Deleting CaCDC4 in the *efg1*^{-/-} *cph1*^{-/-} background still yielded a strain that was highly filamentous on plates, although with a larger proportion of pseudohyphae versus hyphae (Figure 5). Growth in liquid yielded a flocculent but uniform culture that consisted of pseudohyphal cells (Figure 5). The fact that this mutant was still filamentous indicated that an additional factor is responsible for the phenotype of the *Cacdc4*^{-/-} mutant.

Another potential SCF^{CDC4} substrate that may play a role in the filamentation phenotype of the *Cacdc4*^{-/-} mutant is Far1, an inhibitor of G₁ cyclin-kinase complexes. Far1 is involved in the cell cycle arrest and formation of mating projections upon exposure of *S. cerevisiae* cells to the mating pheromone (Chang and Herskowitz, 1990; Valtz *et al.*, 1995). Far1 degradation in *S. cerevisiae* depends on Cdc4 (Henchoz *et al.*, 1997). Orf19.7105 (<http://www-sequence.stanford.edu/group/candida>) is the closest bidirectional *C. albicans* homologue of FAR1. Deletion of both alleles of CaFAR1 did not lead to any discernible phenotypes under normal growth conditions or to any defects in filamentation under serum induction conditions (data not shown). In addition, deletion of CaFAR1 in the *Cacdc4*^{-/-} mutant background did not alter the colony morphology or the hyphal cell morphology of this mutant (Figure 5), arguing against a role for this gene in the filamentation phenotype of the *Cacdc4*^{-/-} mutant.

Hyphal Induction by Serum in *Cacdc4*^{-/-} Cells

Serum is the most potent inducer of hyphae in *C. albicans*. The *Cacdc4*^{-/-} mutant is constitutively filamentous, mainly hyphal, raising the question whether CaCdc4 is part of the signaling pathway that is involved in hyphal induction by serum. If it were, then adding serum to the *Cacdc4*^{-/-} strain should not cause any further induction of filamentation. However, given the constitutively filamentous phenotype of the *Cacdc4*^{-/-} mutant, further induction of filamentation by serum would be hard to measure on a whole cell population. Therefore, to monitor the response of this mutant to serum, we followed individual *Cacdc4*^{-/-} pseudohyphal cells by time-lapse micrography. The *Cacdc4*^{-/-} pseudohyphal cells were isolated from the mycelial cells based on their slower sedimentation. Wild-type cells and triple *Cacdc4*^{-/-}

Figure 5. Epistasis of *Cacdc4*^{-/-} over *efg1*^{-/-} *cph1*^{-/-} and *Cafar1*^{-/-}. Strains KC138 (*Cacdc4*^{-/-}), KC180 (*efg1*^{-/-} *cph1*^{-/-} *Cacdc4*^{-/-}) and KC183 (*Cafar1*^{-/-} *Cacdc4*^{-/-}) were grown on YPD plates or in liquid YPD medium. Top row, colony morphology. Cell morphology visualized by calcofluor staining of cells from the colonies (middle row) or from an overnight liquid culture (bottom row). Bar, 20 μ m.

efg1^{-/-} *cph1*^{-/-} mutant cells also were followed by time-lapse micrography. As shown in Figure 6, the *Cacdc4*^{-/-} mutant cells did form germ tubes in response to serum and did so more rapidly and vigorously than the wild-type cells: at 40 min, several germ tubes are visible in the mutant, whereas germ tubes only start to emerge at 60 min in the wild-type. This suggests that CaCdc4 is not itself part of the serum response signal transduction pathway but that it functions as a negative regulator of this pathway. In contrast, the *Cacdc4*^{-/-} *efg1*^{-/-} *cph1*^{-/-} cells did not respond to serum and continued to grow as pseudohyphal cells. Thus, the nonresponsiveness of the *efg1*^{-/-} *cph1*^{-/-} mutant to serum induction (Lo *et al.*, 1997) is epistatic to *Cacdc4*^{-/-}, indicating that no alternative serum response pathway is induced in the absence of CaCdc4.

Effect of *S. cerevisiae* Sic1 Expression in *C. albicans*

The best studied substrate of SCF^{CDC4} is the cyclin-dependent kinase inhibitor Sic1, stabilization of which is the cause for the G₁ arrest of mutants of SCF^{CDC4} components (Schwob *et al.*, 1994; Feldman *et al.*, 1997; Nash *et al.*, 2001). If

Figure 6. Response to serum. Cells from an overnight culture of CAI4 (WT), KC138 (*Cacdc4*^{-/-}), and KC180 (*efg1*^{-/-} *cph1*^{-/-} *Cacdc4*^{-/-}) were set down on a 50% fetal calf serum (FCS), 2% agar pad on a microscope slide, and an individual field of cells was followed over several hours at room temperature. To obtain an enrichment in *Cacdc4*^{-/-} pseudohyphal cells, the rapidly sedimenting part of a KC138 overnight culture was discarded, and the slowly sedimenting supernatant, which consists almost exclusively of pseudohyphal cells, was concentrated by centrifugation.

the reason for the hyphal growth phenotype of the *Cacdc4*^{-/-} mutant is that the *C. albicans* homologue of Sic1 is stabilized, then overexpressing a stabilized version of that protein might by itself lead to hyphal growth. To test this hypothesis, in a first stage we expressed in *Candida* a truncated version of the *S. cerevisiae* SIC1 gene, which is expected to yield an active, constitutively stable protein (Verma *et al.*, 1997b). Expression of SIC1 Δ N was achieved by cloning it under the copper-inducible CaCUP1 promoter. The construct was transformed into the *crp1*^{-/-} mutant, which defective in the copper extrusion pump Crp1 and is therefore relatively sensitive to the addition of copper to the medium (Weissman *et al.*, 2000). Expression of this construct induced the cells to form highly elongated buds (Figure 7), indicating that *C. albicans* is sensitive to this CDK inhibitor.

Identification of a *C. albicans* Sic1 Homologue

Because *S. cerevisiae* Sic1 expression did induce morphological changes in *C. albicans*, suggesting that stabilization of a Sic1 homologue might be responsible for the *C. albicans* *cdc4*^{-/-} phenotype, we next attempted to identify such a homologue. A sequence homology search (using the BLAST algorithm) failed to uncover any obvious Sic1 homologues

Figure 7. Effect of *S. cerevisiae* Sic1 on *C. albicans* cell morphology. N-terminally truncated Sic1 was expressed in strain KC16 from the CaCUP1 promoter of plasmid KB1389, by adding 100 μ M CuSO₄ to the cultures. Micrographs were taken after 6 h of copper induction. Bar, 20 μ m.

in the nearly complete *C. albicans* genome database. We therefore attempted to isolate this homologue functionally, based on the suppression phenotype of Sic1 overexpression on mitotic exit mutants, such as *dbf2*^{ts} (Donovan *et al.*, 1994). A *C. albicans* genomic library (Liu *et al.*, 1994) was introduced in the *dbf2*-3 mutant, and transformants able to grow at 37°C were selected. The single type of suppressor plasmid contained a 238 codon-long open reading frame, Orf19.6930 (<http://www-sequence.stanford.edu/group/candida>) with no homology to Dbf2 but with limited homology to Sic1. Because functional analysis showed that this protein, although related, seems to be functionally distinct from Sic1 (see below), we called it Sic one-like (Sol1) (Figure 8A). Comparing this ORF to the *S. cerevisiae* proteome by using the BLAST algorithm did not reveal any significant homologies. The putative transcription factor Hcm1 was obtained as closest homologue. However, when multiple alignment was performed with several ascomycete Sic1 homologues, together with the corresponding Hcm1 homologues, Sol1 clearly clustered with the Sic1 group (Figure 8B). Figure 8C shows the Sol1 sequence, marked at the sites of homology to the consensus of four Saccharomycetaceae Sic1 sequences. Like ScSic1, Sol1 contains a significant number of potential CDK sites (S/T-P), concentrated in the amino-terminal half of the protein. The region of highest conservation, around Thr140, corresponds to a potential CDK/MAPK site that serves in *S. cerevisiae* Sic1 as a target for Hog1 (Escote *et al.*, 2004).

To further analyze Sol1, the full-length protein and an amino-terminal truncation of the protein were cloned under the control of the *GAL1* promoter and expressed in *S. cerevisiae*. The amino-terminal half of Sol1 contains the majority of its 14 potential CDK target sites and was, by analogy with the Sic1 protein, expected to carry its degradation signal, but not its CDKI function (Verma *et al.*, 1997b; Hodge and Mendenhall, 1999). Whereas the full-length Sol1 protein showed little effect on growth of the *S. cerevisiae* cells under inducing conditions, the truncated Sol1 protein strongly inhibited growth (Figure 9A). Furthermore, expression of full-length Sol1 caused the appearance of enlarged, elongated buds, and this phenotype was exacerbated in the cells expressing the truncated protein (Figure 9B). However, unlike stable Sic1, expression of which causes cell cycle arrest in G₁ (Schwob *et al.*, 1994; Verma *et al.*, 1997a; see below), the truncated Sol1, although able to inhibit growth of *S. cerevisiae*, did not lead to G₁ arrest in *S. cerevisiae* (Figure 9C).

Dependence of Sol1 Degradation on CaCdc4

To test whether, by analogy with Sic1 and ScCdc4, Sol1 is a substrate of degradation by CaCdc4, we cloned a Myc

Figure 8. Isolation of *SOL1*. (A) Suppression of the temperature sensitivity of *dbf2-3* by *SOL1*. The *dbf2-3* mutant transformed with a vector plasmid or with KB1606 (*SOL1*), and a wild-type S288C strain, were incubated for 3 d at 37°C on a YPD plate. (B) Multiple alignment of the *Sol1* with four ascomycetes Sic1 homologues, as well as the corresponding Hcm1 homologues, by using the ClustalW program with PAM250 matrix. Sc, *S. cerevisiae*; Sca, *S. castellii*; Sk, *S. kluyveri*; Ag, *Ashbya gossypii*; and Ca, *C. albicans*. (C) Sequence of *Sol1* marked with the homologies to the four ascomycete Sic1 homologues. Identities to three of the four Sic1 sequences are marked by boldface and to four of four, by a star. Potential CDK sites are underlined. The bracket marks the position homologous to the start of the C-terminal domain of ScSic1 shown to be essential for its CDKI activity (Hodge and Mendenhall, 1999).

epitope-tagged version of *SOL1* under the regulation of the *CaMAL2* promoter. This construct was transformed into the TET-*CaCDC4* strain, and *Sol1* levels were followed in the presence or absence of tetracycline. In the presence of tetracycline, i.e., in *CaCdc4*-depleted cells, there was an accumu-

lation of *Sol1* (Figure 10A, top, time 0 lanes). In addition, shut-off of the *CaMAL2* promoter by the addition of glucose showed that, whereas in the control cells, *Sol1* is rapidly degraded, in the *CaCdc4*-depleted cells, the protein is stable, suggesting that *Sol1* is indeed an SCF^{CaCDC4} substrate (Fig-

Figure 9. Expression of *Sol1* in *S. cerevisiae*. (A) Growth of cells expressing *SOL1* or *SOL1ΔN* from the *GAL1* promoter of plasmids KB1599 and KB1600, respectively. Increasing dilutions of W303 cells transformed with the indicated plasmids were plated on SC + 2% galactose or SC + 2% glucose, as indicated. The plates were incubated for 2 d at 30°C. (B and C) The same strains as in A were grown overnight in raffinose and then induced for 7 h with 2% galactose and photographed (B) or subjected to FACS analysis (C). The relative percentage of cells under the 1n and 2n peaks is indicated.

Figure 10. Degradation of Sol1 in CaCdc4-depleted cells. Myc-tagged Sol1 was expressed from the *MAL2* promoter of plasmid KB1578 in strain KC200 (TETp-CaCDC4). The cells were grown overnight in SC medium + maltose and then diluted in the same medium with or without 100 μ g/ml tetracycline and grown for another 8 h. Glucose was then added to the cultures to shut off the *MAL2* promoter, and the protein levels were detected by Western blotting against the Myc epitope. Hydroxyurea (70 mM) was added to a portion of the cultures 2 h before glucose addition. (A) Sol1 degradation in the presence or absence of tetracycline (TET), in the absence (top) or the presence (bottom) of HU. Lane C is a non-epitope-tagged control cell extract. (B) The “+TET” samples shown in A were run adjacent to each other and submitted to a shorter exposure, to visualize the accumulation of a lower mobility species of Sol1 in the presence of hydroxyurea.

ure 10A, top). However, it was possible that the stabilization of Sol1 is a secondary effect of cell cycle changes induced by the disappearance of CaCdc4 from the cells, affecting the Sol1 degradation pathway upstream of the ubiquitination step, e.g., at the level of Sol1 phosphorylation (Feldman *et al.*, 1997; Nash *et al.*, 2001). Therefore, we also tested Sol1 degradation in cells arrested in S phase with hydroxyurea (HU). In preliminary results, we found that addition of 70 mM HU to *C. albicans* induced an S-phase arrest within 2 h (our unpublished data). In the HU-treated control culture, Sol1 was barely detectable, suggesting that it is even more rapidly degraded under these conditions. In the CaCdc4-depleted, HU-treated cells, in contrast, Sol1 was still strongly stabilized (Figure 10A, bottom). Furthermore, when the

CaCdc4-depleted samples were run adjacent to each other and subjected to a shorter exposure, Sol1 was found to migrate as two electrophoretic species, with the slower species accumulating in the HU-treated cells (Figure 10B). The slower migrating species presumably represents a hyperphosphorylated form of the protein. In spite of this hyperphosphorylation, Sol1 is still stabilized in the CaCdc4-depleted cells. These results support the possibility that Sol1 is a substrate of CaCdc4 and that stabilization in CaCdc4-depleted cells is a direct effect of the loss of the SCF^{CaCDC4} activity.

Phenotypes of the *SOL1* overexpression in *C. albicans*

Overexpression of *SOL1* in *C. albicans* was achieved by cloning it under the *CaCUP1* promoter and expressing it in the *crp1*^{−/−} mutant, as described above for *SIC1*. To directly compare the activities of Sol1 and Sic1, both proteins were expressed from the same promoter, in full-length form or as N-terminal truncations. Whereas ectopic expression of the full-length proteins showed little effect on cell growth, expression of the N-terminally truncated proteins caused inhibition of cell proliferation (Figure 11A). At the level of cellular morphology, expression of full-length Sol1 had no discernible effect (Figure 11B, d), whereas full-length Sic1 showed some extent of bud elongation (Figure 11B, b). The truncated proteins both induced the formation of elongated buds, but buds induced by Sol1 Δ N (Figure 11B, e and f) were markedly longer than those induced by Sic1 Δ N (Figures 11B, c and 7). FACS analysis of the cultures also revealed significant differences between the two proteins: whereas expression of Sic1 in its native form caused an accumulation of cells in G₁, and expression of the truncated Sic1 caused cell cycle arrest in G₁, overexpression of the full-length Sol1 did not affect the cell cycle distribution of the culture, and the truncated Sol1 only moderately increased the G₁ cell population (Figure 11C). Because all four proteins were expressed as fusions to an identical N-terminal Myc epitope sequence, we compared the protein levels by Western blotting with an anti-Myc monoclonal antibody (mAb) (Figure 11D). The protein levels of the truncated proteins were markedly higher than their full-length counterparts. This was expected for Sic1, because the deleted region contains the residues required for its degradation (Verma *et al.*, 1997b), and it suggests that truncation of Sol1 likewise caused its stabilization. Importantly, levels of the two native proteins were comparable, and the level of Sol1 Δ N seemed, if anything, higher than that of Sic1 Δ N. Thus, lower protein levels cannot be invoked to explain the weaker effect of Sol1 on G₁ accumulation.

Phenotype of the *C. albicans* *Sol1*^{−/−} Mutant

The strain deleted for both alleles of the *SOL1* gene displayed a moderate growth defect, and yielded heterogeneously-sized colonies when streaked on agar plates. The cellular morphology of overnight cultures (OD₆₀₀ = 18) looked mostly like wild-type cells, with a minority of highly enlarged and elongated cells (Figure 12B). Outgrowth of such a stationary culture into log phase by diluting 100 $\times$ into fresh medium, however, showed after 3 h a large proportion of cells displaying a highly elongated single bud (Figure 12B). As the culture matured, more normal-sized cells became visible: by the time it had reached OD₆₀₀ = 5, the majority of cells were normal sized (our unpublished data). The *SOL1*^{+/−} heterozygote, as well as the *sol*^{−/−} <*SOL1*> reintegrant showed a small proportion of mildly elongated cells, suggesting a gene dosage effect of *SOL1* (Figure 12B). We also tested whether the *sol1*^{−/−} strain was

Figure 11. Effect of the overexpression of Sic1 and Sol1 in *C. albicans*. (A) Cellular growth is inhibited upon overexpression of truncated Sic1 and Sol1. Plasmids KB1321 (vector), KB1386 (*CUP1p-SIC1*), KB1389 (*CUP1p-SIC1ΔN*), KB1472 (*CUP1p-SOL1*), and KB1473 (*CUP1p-SOL1ΔN*) were transformed into strain KC16. Plates were photographed after 2 d at 30°C. (B) Morphology of cells overexpressing Sic1 and Sol1. The same strains as in A were photographed after 6 h in SC + 100 μM CuSO₄. Vector (a), *CUP1p-SIC1* (b), *CUP1p-SIC1ΔN* (c), *CUP1p-SOL1* (d), and *CUP1p-SOL1ΔN* (e and f). Bar, 20 μm. (C) FACS analysis of the same strains after 6 h of growth in SC medium + 100 μM CuSO₄. The numbers above the peaks represent the relative percentages of the 2n and 4n cell populations (averages of three independent experiments ± standard deviations). (D) Western blot analysis of a protein extract from the same strains. The Myc-tagged proteins were visualized with the 9E10 mAb. The * indicates an unrelated cross-reacting band.

defective in germ tube induction by serum. However, no difference was seen between the wild-type and mutant strain (Figure 12C).

Phenotype of the *sol1*^{-/-} *Cacdc4*^{-/-} Double Mutant

To test whether stabilization of Sol1 can account for the filamentation phenotype of the *Cacdc4*^{-/-} mutant, we created the double mutant by deleting both alleles of *CaCDC4* in the *sol1*^{-/-} background. The resulting double mutant showed a constitutive hyphal morphology similar to the single *Cacdc4*^{-/-} mutant, suggesting that stabilization of Sol1 does not play an essential role in the filamentation phenotype of *Cacdc4*^{-/-} (Figure 12D).

DISCUSSION

Viability of the *Cacdc4*^{-/-} Mutant

We have found that Cdc4, the substrate recognition components of the SCF^{CDC4} ubiquitin ligase (Orlicky *et al.*, 2003), profoundly affects morphogenesis of the dimorphic fungus *C. albicans*. The *C. albicans cdc4*^{-/-} mutant grows exclusively in a filamentous form under all conditions tested, largely as true hyphae. The first surprising observation was that the *CaCDC4* gene, unlike its *S. cerevisiae* counterpart, is not essential. No additional *CDC4* homologue is detectable

in the *C. albicans* genomic sequence, the closest homologue being Orf19.3301, which is the ortholog of another *S. cerevisiae* F-box protein, Met30 (our unpublished data). *S. cerevisiae cdc4-1* mutants arrest at the G₁-to-S transition due to the inability to degrade Sic1, an inhibitor of the S phase and mitotic kinases (Schwob *et al.*, 1994; Feldman *et al.*, 1997). In *C. albicans*, in contrast, we could not even detect a transient G₁ accumulation upon depletion of *CaCdc4* from the cells (Figure 4D). One possibility was that the *C. albicans* S-phase kinase is not sensitive to inhibition by Sic1. We excluded this possibility by showing that expression of *S. cerevisiae* Sic1 in *C. albicans* caused morphological changes and G₁ arrest, similar to the effect of Sic1 in *S. cerevisiae*.

C. albicans Sol1 Is a Functional Homologue of *S. cerevisiae* Sic1

A second possibility for the viability of the *C. albicans cdc4*^{-/-} mutant was that *C. albicans* does not possess a Sic1 homologue. Sic1 plays important roles in cell cycle progression in *S. cerevisiae*, both in coordinating bud emergence with the initiation of DNA replication and in promoting mitotic exit (Donovan *et al.*, 1994; Nugroho and Mendenhall, 1994; Schwob *et al.*, 1994). However, it was possible that *C. albicans* uses alternative mechanisms for these functions. Indeed, database searches failed to uncover any candidates

Figure 12. Phenotypes of the *sol1*^{-/-} deletion. (A) Southern blotting of the *SOL1* region of the following strains: lane 1, *SOL1/SOL1* (CAI4); lane 2, *SOL1/sol1Δ::hisG-URA3-hisG* (KC184); lane 3, *SOL1/sol1Δ::hisG* (KC185); and lane 4, *sol1Δ::hisG/sol1Δ::hisG-URA3-hisG* (KC186). Genomic DNA was digested with *Xba*I and probed with a 757-base pair *Bam*HI-*Xba*I fragment from plasmid KB1606, corresponding to positions +719 to +1476 relative to the *SOL1* start codon. (B) Cellular morphology of *sol1*^{-/-} in a logarithmic and stationary culture. The CAI4, KC184, KC186, and KC239 strains were diluted 1:100 from an overnight culture and grown for 3 h at 30°C (LOG). Also shown is the KC186 overnight culture (o.n.). Bar, 20 μm. (C) Response of the *sol1*^{-/-} strain to serum induction of filamentation. CAI4 (*SOL1*^{+/+}) and KC186 (*sol1*^{-/-}) overnight cultures were diluted in FCS and incubated for 2 h at 37°C. Bar, 20 μm. (D) Colony and cellular morphology of the *sol1*^{-/-} (KC138) versus the *Cacdc4*^{-/-} *sol1*^{-/-} (KC196) strains. Colonies were grown for 2 d on YPD plates at 30°C (top row). Cells from these colonies (middle row) or from an overnight liquid culture (bottom row) were stained with calcofluor and visualized by epifluorescence. Bar, 20 μm.

based on sequence homology. By applying a functional screen based on the ability of Sic1 to suppress mitotic exit mutants, we were nonetheless able to isolate Sol1, a homologue of Sic1 from *C. albicans*. Mitotic exit in *S. cerevisiae* requires down-regulation of Clb2/Cdc28, which occurs via activation in telophase of APC/C-Cdh1, a Clb2-specific

ubiquitin ligase (Visintin *et al.*, 1998). Inhibition of Clb2/Cdc28 activity by the CDK inhibitor Sic1 represents an alternative pathway to promote mitotic exit. Thus, the ability of Sol1 overexpression to likewise suppress the mitotic exit mutant *dbf2-3* supports its function as an inhibitor of the mitotic kinase. In spite of the low sequence conservation,

multiple alignment of Sol1 with several presumed ascomycete Sic1 orthologues further supports its assignment as a Sic1 homologue.

Sol1 and Sic1 Activities Overlap Only Partially

However, the overexpression phenotype of Sol1 in both *S. cerevisiae* and *C. albicans* does not correspond to the phenotype of Sic1 overexpression. This is most clearly seen when Sic1 and Sol1 overexpression was directly compared in *C. albicans*: whereas full-length Sic1 lead to a G₁ delay, and truncated Sic1 caused G₁ arrest, the full-length and truncated Sol1 caused no visible effect, and at most a G₁ delay, respectively (Figure 11). This is not due to differential accumulation of the Sic1 and Sol1 proteins, because Western blotting analysis indicates that the full-length proteins are equivalently expressed, and the truncated Sol1 is, if anything, more abundant than the truncated Sic1. The morphology of cells overexpressing Sic1 and Sol1 is also different: *C. albicans* cells overexpressing truncated Sic1 exhibit elongated buds, but cells overexpressing truncated Sol1 exhibit even longer buds that are reminiscent of germ tubes. Thus, we are led to the conclusion that Sic1 and Sol1 are functionally distinct. We speculate that the distinction derives from different interaction with specific cyclin-CDK complexes: because the G₁ arrest elicited by Sic1 is due to its inhibition of the S-phase kinase Clb5, Clb6/Cdc28 (Schwob *et al.*, 1994), it is possible that Sol1 has lower affinity for these cyclin-CDK complexes. The effect of Sol1 on cellular morphology may derive from its inhibitory effect on Clb1,2/Cdc28. This kinase is responsible for the switch from apical to isotropic growth after bud emergence (Lew and Reed, 1993). Inhibition of this switch would lead to highly elongated buds, as observed upon Sol1 overexpression. Conversely, in the *sol1*^{-/-} mutant, one might expect an acceleration in the switch to isotropic growth, which might explain the peculiar thick-budded morphology of this mutant (Figure 12B). The elongation of the *sol1*^{-/-} buds could be a secondary effect of a block, e.g., in mitotic progression (Bachewich *et al.*, 2003). The elongated *sol1*^{-/-} buds usually show a single elongated DNA mass in the middle of the bud, consistent with a block in telophase (our unpublished data); however, we were unable to measure the cell cycle distribution of this mutant by flow cytometry.

Does C. albicans Contain Another Sic1 Homologue?

If Sol1 is not a "true" Sic1 homologue, i.e., one that inhibits initiation of replication, does such a homologue exist at all in *C. albicans*? We cannot exclude that a bona fide Sic1 homologue still hides in the *C. albicans* genome. However, we note that in *S. cerevisiae*, Sic1 is required for the coordination between budding and S-phase initiation. This results from the inhibitory function of Sic1 on the S-phase kinase Clb5,6/Cdc28, together with the fact that Cln1, 2/Cdc28, the same kinase that causes bud emergence (Lew and Reed, 1993), phosphorylates Sic1, which leads to its degradation and to the initiation of S phase (Schneider *et al.*, 1996; Tyers, 1996). In *C. albicans*, initiation of hyphal bud evagination is not necessarily coordinated with a specific cell cycle phase (Hazan *et al.*, 2002), i.e., a mechanism must exist that allows the initiation of polarized growth independently of the regulation of the S-phase kinase. In addition, during hyphal growth, DNA replication is regulated independently of the hyphal tip elongation, which is continuous. Thus, it is possible that the Sic1 S-phase kinase inhibitory function, which imposes an obligatory coordination between polarized growth and the initiation of S phase, is absent in *C. albicans*.

Alternatively, such a function could exist, but it may be restricted to the yeast mode of growth.

Role of CaCdc4 in the Signaling Pathway of Filamentation

Analysis of the dynamics of filament formation subsequent to the loss of CaCdc4 protein and of SCF^{CaCDC4} function indicates that within 90 min of tetracycline addition, most of the CaCdc4 protein has disappeared from the cell (Figure 4). SCF^{CaCDC4} function, as measured by the accumulation of Sol1, is impeded already 4 h after tetracycline addition, but high accumulation of Sol1 only occurs some 5.5 h after tetracycline addition (our unpublished data). The discrepancy might indicate that CaCdc4 is present in excess under normal conditions, and significant levels of SCF^{CaCDC4} function are maintained even when most of CaCdc4 has disappeared from the cell; alternatively, it is possible that the tagged CaCdc4 protein is less stable than the untagged protein. Importantly, however, 10 h after tetracycline addition, when promoter shut-off analysis indicated that Sol1 is completely stabilized, only pseudohyphal cells were visible in the culture. Only 24 h after tetracycline addition could some true hyphal cells be detected in the culture. The lag between the loss of SCF^{CaCDC4} activity and the appearance of hyphae suggests that SCF^{CaCDC4} knockdown by itself may be sufficient to induce pseudohyphal growth but not hyphal growth. The rapid response of the *CaCdc4*^{-/-} mutant to serum (Figure 6) suggests that this strain may be hypersensitized to hyphal induction signals but that a signal is still required. The lag between disappearance of CaCdc4 activity and appearance of hyphae in the TET-*CaCDC4* strain may reflect the requirement of an additional signal such as, e.g., starvation, or growth on a semisolid agar medium. Thus, SCF^{CaCDC4}-mediated degradation seems to modulate the cellular response to morphogenetic signals rather than to initiate these signals.

Substrates of SCF^{CaCDC4}

Even though Sol1 carries morphogenetic functions and is a substrate of SCF^{CaCDC4}, epistasis analysis indicated that stabilization of Sol1 alone does not explain the hyperfilamentation phenotype of the *CaCdc4*^{-/-} mutant. We also identified and excluded the *C. albicans* Far1 homologue, another SCF^{CaCDC4} substrate in *S. cerevisiae* that plays a role in cell morphogenesis. Thus, the critical substrate of SCF^{CaCDC4} for the induction of filamentation remains elusive. Notably, until this substrate is identified, we cannot reject the formal possibility that the phenotype of the *CaCdc4*^{-/-} mutant reflects a function of CaCdc4 that is independent of the SCF complex. Additional potential SCF^{CaCDC4} candidate substrates include any one of the large number of transcription factors that have been shown to promote hyphal growth. Of these transcription factors, CaTec1 (Schweizer *et al.*, 2000) stands out because its yeast homologue was recently shown to be targeted for degradation by SCF^{CaCDC4} (Chou *et al.*, 2004). However, the Cdc4 consensus targeting sequence identified in ScTec1 is not conserved in CaTec1, and in preliminary experiments, we found that CaTec1 is degraded equally rapidly in wild-type and CaCdc4-depleted cells (our unpublished data). The critical SCF^{CaCDC4} substrate might alternatively be found among cell cycle regulators that promote filamentous growth. G₁ cyclins, in particular, are often targeted for degradation by SCF complexes in many organisms (Willems *et al.*, 2004). Of the three G₁ cyclins described in *C. albicans*, CaCln1/Ccn1 (Loeb *et al.*, 1999), Hgc1 (Zheng and Wang, 2004), and CaCln3 (Bachewich and Whiteway, 2005; Chapa y Lazo *et al.*, 2005), the former two play a

positive role in filamentous growth. CaCln3, in contrast, negatively regulates filamentous growth, and depletion of CaCln3 induces a pseudohyphal or hyphal phenotype somewhat reminiscent of the phenotype obtained upon CaCdc4 depletion (Bachewich and Whiteway, 2005; Chapa y Lazo *et al.*, 2005). Thus, it is possible that CaCln3, rather than being a substrate of CaCdc4, operates in the same pathway, one possibility being that CaCln3 phosphorylates a target(s) that is subsequently recognized by SCF^{CaCdc4}.

The signal transduction pathways that mediate the filamentous response of *C. albicans* to different growth conditions are incompletely understood. The finding that a protein degradation system heavily influences the dimorphic switch adds an additional potential layer of regulation to the yeast-to-hyphae transition. The targets of SCF^{CaCdc4} that cause its filamentation phenotype, and the mode of regulation of their turnover, will likely represent central regulators of morphogenesis. Future studies will aim to identify these SCF^{CaCdc4} targets.

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