JCB Article

In *Candida albicans*, the Nim1 kinases Gin4 and Hsl1 negatively regulate pseudohypha formation and Gin4 also controls septin organization

Raymond Wightman, Steven Bates, Pat Amornrrattanapan, and Peter Sudbery

Department of Molecular Biology and Biotechnology, University of Sheffield, Western Bank, Sheffield, S10 2TN UK

In the development of hyphal germ tubes of *Candida albicans*, a band of septin forms at the base of the germ tube (basal septin band). Later, a septin ring forms, which organizes the first septum within the germ tube (septin ring). We have investigated the role of the Nim1 kinases, Gin4 and Hsl1, in the formation of these septin structures. We show that during germ tube formation, Gin4 is required for the organization of the septin ring but not the basal septin band. Hsl1 is not required for the formation of either septin rings or basal bands. Unexpectedly, we found that both *gin4*Δ and *hsl1*Δ mutants form pseudohyphae constitutively, in a fashion that in the case of *gin4*Δ, is partly independent of Swe1. *Gin4*-depleted pseudohyphae are unable to form hyphae when challenged with serum, but this can be overcome by ectopic expression of Gin4 from the *MET3* promoter. Thus, Gin4 may regulate the developmental switch from pseudohyphae to hyphae.

Introduction

The human fungal pathogen *Candida albicans* can grow as yeast, pseudohyphae, or true hyphae (Odds, 1985; Gow, 1997; Brown and Gow, 1999; Berman and Sudbery, 2002). The unicellular yeast form closely resembles the budding yeast *Saccharomyces cerevisiae*. The pseudohyphal form consists of chains of cells that can display varying degrees of elongation, but retain a constriction at the septa that separate adjacent cellular compartments (Merson-Davies and Odds, 1989). The hyphal form consists of hyphae with parallel-sided walls with no constrictions at the septal junctions. It is thought that the ability to switch between the different morphological forms may be required for virulence because the different morphological forms are found in infected tissue and environmental signals that induce hyphae also coordinately induce a variety of known virulence factors such as the *SAP* family of aspartyl proteases (for reviews see Liu, 2001; Sudbery, 2001; Berman and Sudbery, 2002; Brown, 2002). Moreover, strains that are prevented from forming hyphae because of mutations in signal transduction pathways are avirulent (Lo et al., 1997).

C. albicans yeast cells can be induced to form hyphae or pseudohyphae by a variety of environmental signals such as the presence of serum, 35–37°C growth temperature, and neutral pH (Odds, 1985; Sudbery, 2001). The presence of serum at a growth temperature of 35°C, pH 6, results in a culture consisting almost entirely of the hyphal form, whereas in the absence of serum at 35°C, pH 6, cells develop as long pseudohyphae (Sudbery, 2001). Although pseudohyphae superficially resemble true hyphae, it is becoming increasingly clear that there are fundamental differences between the two forms in the organization of the cell cycle and the septin cytoskeleton (Sudbery, 2001). In the development of pseudohyphae, a septin ring forms at the junction between the mother cell and the daughter cell. The first mitosis and formation of the primary septum take place across the plane of this septin ring. Thus, the daughter cell behaves as a bud, modified by hyperpolarized growth. In hyphal development, germ tube evagination occurs before the cell cycle has initiated in the mother cell (Hazan et al., 2002). A band of longitudinal septin bars forms at the base of the germ tube, which later becomes faint and disorganized (Sudbery, 2001). A cap of septin is also present at the germ tube tip (Warenda and Konopka, 2002). A septin ring then

R. Wightman and S. Bates contributed equally to this work.

The online version of this article contains supplemental material.

Address correspondence to Peter Sudbery, Department of Molecular Biology and Biotechnology, University of Sheffield, Western Bank, Sheffield, S10 2TN UK. Tel.: 44-114-222-6186. Fax: 44-114-272-8697. email: psudbery@shef.ac.uk

Steven Bates's present address is University of Aberdeen, Department of Molecular and Cell Biology, Institute of Medical Sciences, Foresterhill, Aberdeen, AB25 2ZD UK.

Key words: basal septin band; germ tube; hypha; septin ring; *SWE1*

Abbreviations used in this paper: αCdc11, antisera to *S. cerevisiae* Cdc11; IAA, isoamyl alcohol; YEPD, yeast extract peptone dextrose.

appears within the germ tube, 10–15 μm from the mother cell, probably marking the initiation of the cell cycle. The nucleus migrates out of the mother cell and the first mitosis takes place across the plane of this septin ring. After mitosis, the septin ring organizes the formation of the primary septum (for review see Berman and Sudbery, 2002). Throughout this paper the band of septin at the base of the germ tube will be referred to as the "basal septin band" and the ring within the germ tube, which organizes the formation of the primary septum, will be referred to as the "septin ring".

In *S. cerevisiae*, septin rings at the bud neck provide a scaffold for proteins that are required for cytokinesis (for review see Longtine and Bi, 2003). The proper organization and function of the septin ring requires Gin4, a homologue of the Nim1 kinase of *Schizosaccharomyces pombe* (Russell and Nurse, 1987). In a *gin4*Δ mutant, septin is deposited in a series of longitudinal bars around the bud neck instead of a continuous ring (Longtine et al., 1998a, 2000). Gin4 localizes to the bud neck and this localization is septin dependent (Okuzaki et al., 1997; Longtine et al., 1998b; Barral et al., 1999). Therefore, the function and localization of septins and Gin4 are mutually interdependent. Mutations that disrupt septin organization cause an impairment of cytokinesis and bud elongation. This is due to the induction of the morphogenesis checkpoint that delays mitosis and prevents a switch from polarized to isometric growth of the bud (Lew and Reed, 1993, 1995a). The morphogenesis checkpoint is dependent on Swe1, the *S. cerevisiae* homologue of the *S. pombe* Wee1 kinase (Booher et al., 1993), which inhibits Cdc28 by phosphorylation of tyrosine 19 (Sia et al., 1996). A *swe1*Δ mutation abrogates the morphogenesis checkpoint in a temperature-sensitive *cdc3* mutant and consequently exacerbates its growth defect (Barral et al., 1999). The morphogenesis checkpoint operates through control of Swe1 stabilization. When first synthesized in G1, Swe1 accumulates in the nucleus. After bud formation it is targeted to the mother side of the bud neck and it is degraded in the G2/M phase (McMillan et al., 1999). Swe1 also exerts a cell size checkpoint over the onset of mitosis (Harvey and Kellogg, 2003). This observation has given rise to an alternative model to the morphogenesis checkpoint in which it is posited that abnormally prolonged bud growth occurs in cells lacking Gin4 because the signal indicating that cell size has passed the critical size threshold is not transmitted to Swe1. Apart from Gin4, the *S. cerevisiae* genome encodes two other Nim1-kinase homologues, Hsl1 and Kcc4. These also localize to the bud neck in a septin-dependent fashion (Barral et al., 1999). However, septin organization is not dependent on either kinase (Longtine et al., 2000), although Hsl1 is required for the transmission of the checkpoint signal to Swe1.

The *C. albicans* genome contains a single homologue of the *S. cerevisiae SWE1* gene and two Nim1-kinases homologous to *S. cerevisiae GIN4* and *HSL1*, respectively. In this paper, we have investigated the role of these genes in the organization of the basal septin band and the septin ring in hyphal germ tubes of *C. albicans.* We show that Gin4 is required for the organization of the septin ring but not the basal septin band. Thus, the difference in organization between these two structures may be due to the action of Gin4. Hsl1 is not required for the organization of either structure. Unexpectedly, we found that both *gin4*Δ and *hsl1*Δ mutants constitutively form pseudohyphae and that these pseudohyphae are unable to form hyphae when challenged with serum. Furthermore, as wild-type cells develop pseudohyphae, the level of Gin4 declines and they also become unable to form hyphae upon serum challenge. However, high levels of Gin4, ectopically expressed from the *MET3* promoter, overcome this block. Thus, in addition to organizing the septin ring, Gin4 appears to determine the developmental potential of pseudohyphae.

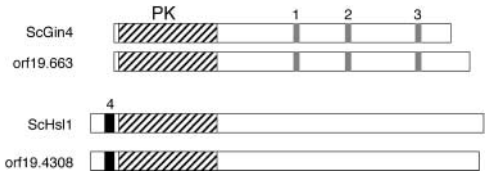

Figure 1. **Structural comparisons of the *C. albicans* Nim1 kinase homologues with *S. cerevisiae* Gin4 and Hsl1.** Unique motifs shared between homologues are shown for Gin4 (solid gray) and Hsl1 (solid black). For detailed alignments see Fig. S1. PK, protein kinase domain (black diagonals); Motif 1 (TYA); Motif 2 (SQL); Motif 3 (GSFFRK); Motif 4 (VVQSVXXATKRLS).

Results

C. albicans homologues of Nim1 kinases

We identified two homologues of Nim1 kinases in the *C. albicans* database assembly 19 (http://www-sequence.stanford.edu/group/candida/): ORF19.663 and ORF19.4308. A structural comparison of these genes with the *S. cerevisiae GIN4* and *HSL1* genes is shown diagrammatically in Fig. 1 and the full CLUSTALW alignment, including *KCC4*, is shown in Fig. S1 (available at http://www.jcb.org/cgi/content/full/jcb.200307176/DC1). Neither *C. albicans* Nim1 kinase could be easily defined as homologous to one of the three *S. cerevisiae* Nim1 kinases on the basis of the overall degree of similarity. However, the CLUSTALW alignment reveals that *C. albicans* ORF19.4308 shares an NH_2-terminal extension with *S. cerevisiae* Hsl that is not present in *C. albicans* ORF19.663, *S. cerevisiae* Gin4, or Kcc4. Moreover, *C. albicans* ORF19.663 shares three motifs that are uniquely shared with *S. cerevisiae* Gin4. Based upon these observations, ORF19.663 and ORF19.4308 will be designated as *C. albicans* homologues of *GIN4* and *HSL1*, respectively. The results in this paper are consistent with this conclusion. Furthermore, annotations in the *C. albicans* databases at Stanford (URL provided above) and Galar Fungal (http://genolist.pasteur.fr/CandidaDB/) use the same designations for these ORFs.

Loss of Gin4 results in chains of elongated cells

Both copies of the *GIN4* gene were sequentially deleted in *C. albicans* strain BWP17. The resulting homozygous deleted strain will hereafter be referred to as *gin4*Δ. In addition, a conditional *gin4* mutant was constructed in which one copy of *GIN4* was deleted and the remaining copy was placed under the control of the regulatable *MET3* promoter (Care et al., 1999). This strain will be referred to hereafter as *MET3-GIN4.*

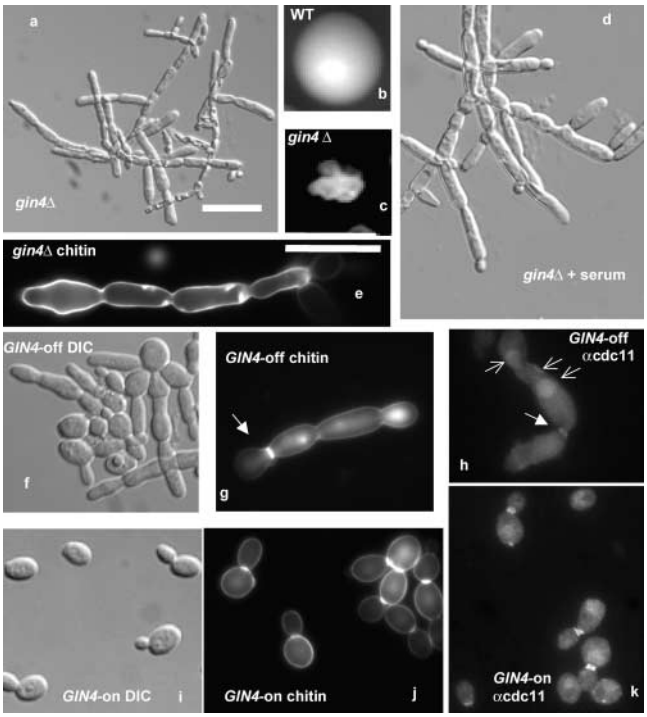

Figure 2. **The phenotype of *gin4*Δ and *MET3-GIN4* cells.** *MET3-GIN4* and *gin4*Δ strains were grown on YEPD at 30°C and the appearance of colonies and cells were recorded as indicated. Chitin was visualized with calcofluor white and septin by immunocytofluorescence using αCdc11 antiserum (Sudbery, 2001). "*MET3-GIN4* off/on" refers to whether the *MET3* promoter was repressed or derepressed. When repressed, cells were first grown in synthetic-defined medium under derepressing conditions and then grown for 6 h in derepressing conditions. Note: septin and chitin rings, indicated by arrows with solid heads in g and h, formed during the growth in derepressing conditions. The round patches of septin within the cells (h, open arrows) colocalized with nuclei as revealed by DAPI staining (not depicted). Panels a and d are shown at the same magnification. Bars, 10 μm. Panels e–k are also shown at the same magnification.

The *gin4*Δ mutant was viable, but grew in chains of elongated cells indicative of a severe defect in cytokinesis (Fig. 2 a). The failure to undergo cytokinesis resulted in a change in colony morphology from the smooth appearance of the wild-type colonies to a crenulated appearance in *gin4*Δ mutants characteristic of pseudohyphal formation in wild-type cells (Fig. 2, b and c). After the addition of serum, these cells were unable to form hyphae, which are defined by narrow parallel sides without constrictions (Fig. 2 d). Calcofluor white staining showed that the primary septum, composed of chitin, failed to form (Fig. 2 e). An identical phenotype was seen in the *MET3-GIN4* strain when the *MET3* promoter was turned off (Fig. 2, f–h). Calcofluor staining showed that primary septa fail to form (Fig. 2 g). Furthermore, immunocytofluorescence, using polyclonal antisera raised against *S. cerevisiae* Cdc11, showed that septin rings failed to form properly (Fig. 2 h). Cells were indistinguishable from wild type when the *MET3* promoter was turned on (Fig. 2, i–k), indicating that the *gin4*Δ phenotype is due to loss of Gin4.

Cell elongation in the *MET3-GIN4* strain after 6 h of growth in *MET3*-repressing medium was quantified by measuring the axial ratio (length/width). The results are presented in Table I, which also quantifies nuclear distribution. (This quantification was not performed in *gin4*Δ mutants because the strain grew in very large clumps that were difficult to separate sufficiently to count a large number of cells.) The axial ratio increased over 2.5-fold when Gin4 was turned off. The disturbance to septin ring formation and the consequent failure to form a primary septum explains the cytokinesis defect in *gin4*Δ cells. The distribution of nuclei was perturbed with a high proportion of the compartments containing either no nuclei, or more than one nucleus (Table I; Fig. 5 a). A similar phenotype is observed in *S. cerevisiae gin4*Δ mutants, but only when the *gin4*Δ mutation is combined with null alleles of *CLB1*, *3*, *4* or in other genes required for septin ring formation (Altman and Kellogg, 1997; Longtine et al., 1998a, 2000; Barral et al., 1999). Thus, Gin4 plays a more essential role in *C. albicans* than *S. cerevisiae*. Alternatively, there may be redundancy for Gin4 function in *S. cerevisiae*. This may be supplied by Kcc4, which is known to have partially overlapping functions with Gin4 (Okuzaki et al., 2003), but which has no *C. albicans* homologue.

*C. albicans gin4*Δ mutants are hyperinvasive

*C. albicans gin4*Δ cells invade the agar substratum. In contrast to wild type, *gin4*Δ colonies cannot be washed off a yeast extract peptone dextrose (YEPD; 2% glucose, 2% Dico-Bacto Peptone, 1% Dico-Bacto yeast extract) agar plate after a 3-d growth at 30°C (Fig. 3 a). Microscopic examination of a transverse section of the plate shows extensive penetration of the agar by *gin4*Δ cells, but no penetration by wild-type cells (Fig. 3 b). Agar invasion is characteristic of hyphal and pseudohyphal cells. The degree of penetration of *gin4*Δ cells growing at 30°C in the absence of serum penetrate the agar at least as much as wild-type hyphal and pseudohyphal cells (Fig. 3 b). However, there is a clear difference in the pattern of invasion. A lower proportion of wild-type hyphal and pseudohyphal cells appear to become

Table I. **Axial ratio of Gin4 and Swe1-depleted cells**

		Nuclei per compartment				
	Axial ratio (*n*)	**0**	**1**	**2**	**3**	***n***
		%	%	%	%	%
SWE1 MET3-GIN4 on	1.49 ± 0.05 (95)	0.5	99.5	0	0	188
*swe1*Δ *MET3-GIN4* on	1.24 ± 0.03 (122)	1.0	86.6	12.4	0	203
SWE1 MET3-GIN4 off	3.87 ± 0.03 (103)	17.7	35.9	24.8	21.5	181
*swe1*Δ *MET3-GIN4* off	2.36 ± 0.1 (107)	11.1	35.5	29.6	23.7	152

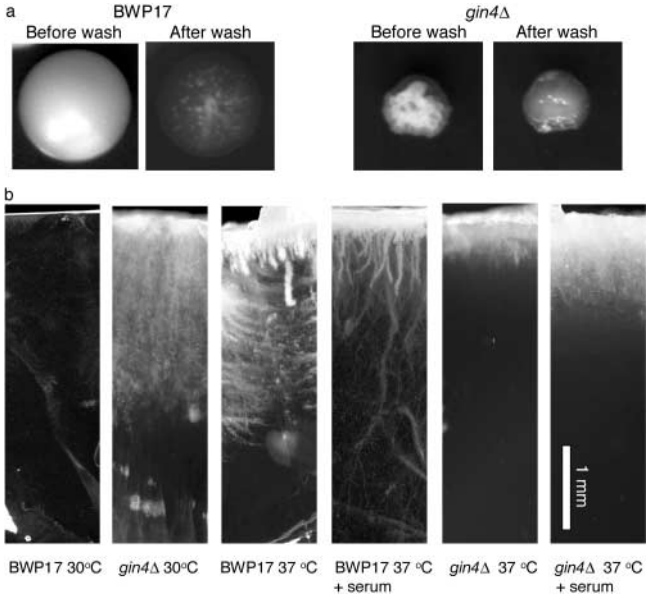

Figure 3. ***gin4*Δ cells are hyperinvasive.** (a) Wild-type (BWP17) and *gin4*Δ cells were grown on YEPD agar plates for 7 d. Colonies were photographed before and after the plates had been washed. (b) Wild-type and *gin4*Δ cells were patched over an area of a YEPD plate or a YEPD plate containing 20% serum and incubated at 30°C or 37°C, as indicated, and incubated for 7 d. After washing, transverse sections were cut from the plate and examined using a dissecting microscope.

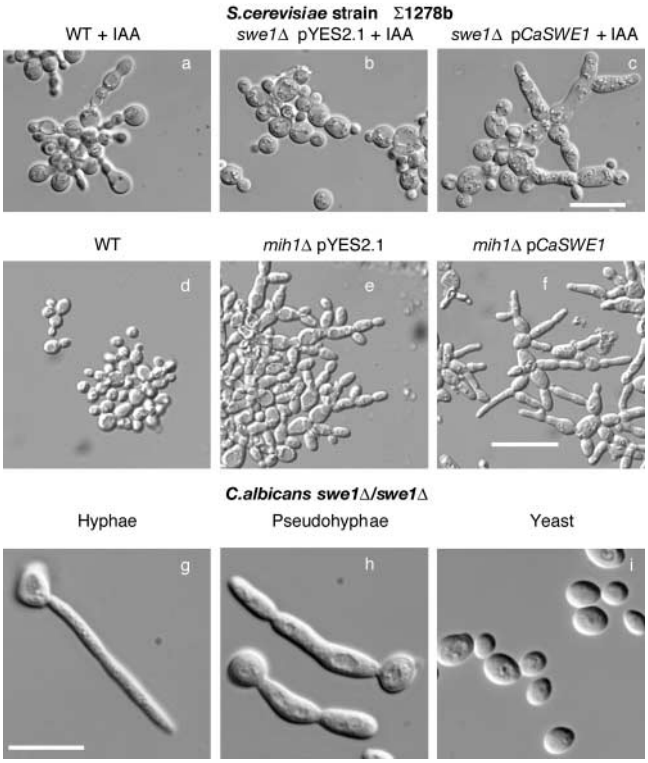

Figure 4. **The *C. albicans SWE1* gene.** (a–f) *S. cerevisiae* cells of the indicated genotype were grown for 16 h in the presence of galactose to induce the *GAL1* promoter. IAA, isoamyl alcohol. (g–i) *C. albicans swe1*Δ cells were grown to stationary phase as yeast and reinoculated for 2 h to form hyphae (g), pseudohyphae (h), or yeast (i). Bars, 10 μm.

invasive and they form structures with a feathery appearance that is not apparent in *gin4*Δ cells growing at 30°C. When grown at 37°C, *gin4*Δ cells are considerably less invasive than wild-type cells and *gin4*Δ cells growing at 30°C. Addition of serum at this temperature mildly stimulates invasion. The invasive growth defect is not due to a general temperature-sensitive growth defect because a comparison of growth of the *gin4*Δ strain on solid YEPD medium at 25°C, 30°C, 34°C, and 37°C, shows no obvious differences after a 5-d incubation. However, growth is significantly reduced in the mutant strain compared with wild type at all growth temperatures (unpublished data).

The *C. albicans* SWE1 homologue

In *S. cerevisiae*, the elongated cell-chain morphology of septin and *gin4*Δ mutants is dependent on Swe1 function. Therefore, we characterized the *C. albicans* Swe1 homologue in order to determine whether this was the case in *C. albicans*. We also wished to test the hypothesis that hypha formation is due to the operation of the morphogenesis checkpoint. We identified a single *SWE1* homologue in the *C. albicans* database (ORF19.4867; note that the corresponding sequence in assembly 6 and the annotation in the Galar Fungal database is erroneous in the 3′ region of the sequence due to incorrect contig assembly). Alignments of ORF19.4867 with Swe1 homologues of other eukaryotes are shown in Fig. S2 (available at http://www.jcb.org/cgi/content/full/jcb.200307176/DC1). To confirm that this sequence encodes the homologue of *S. cerevisiae* Swe1, we amplified it by PCR and expressed it from the *GAL1* promoter on the multicopy pYES2 vector in *S. cerevisiae* Σ1278b wild-type and *swe1*Δ strains. Σ1278b responds to 0.5% isoamyl alcohol (IAA) by growing in filaments. This response is entirely dependent on Swe1 (Martínez-Anaya et al., 2003). When expressed on galactose, the *C. albicans SWE1* completely complemented the filamentation defect of a Σ1278 *swe1*Δ mutant (Fig. 4, a–c) but had no effect in the absence of IAA (Fig. 4 d). The Mih1 tyrosine phosphatase reverses the inhibition of Cdc28 exerted by Swe1. Thus, *mih1*Δ strains are more sensitive to overexpression of *SWE1*. Overexpression of the *C. albicans SWE*1 gene in a Σ1278 *mih1*Δ background resulted in highly elongated cells, indicative of a strong G2/M cell-cycle delay (Fig. 4, d–f). Thus, ORF19.4867 encodes a functional homologue of *S. cerevisiae* Swe1.

Both copies of *SWE1* were sequentially deleted in *C. albicans,* strain BWP17. Under conditions promoting growth in the yeast form, the *swe1*Δ mutant had slightly rounder cells (compare Table I *swe1*Δ *MET3-GIN4* on with *SWE1 MET3-GIN4* on), which is consistent with it playing a role in negatively regulating the switch from apical to isotropic growth. Moreover, a small number of mother cells contained two nuclei in *swe1*Δ cells (Table I), which was never observed in wild-type cells. This observation is consistent with a loss of the morphogenesis checkpoint, which prevents mitosis when bud formation is incomplete. Recently, a survey of the *S. cerevisiae* gene deletion set identified *swe1*Δ as one of 25 genes whose deletion led to a small cell size. The *swe1*Δ mutant was reported to result in a 10% reduction in cell size compared with the wild-type parent (Zhang et al., 2002). Furthermore, Swe1 has been shown recently to provide a size

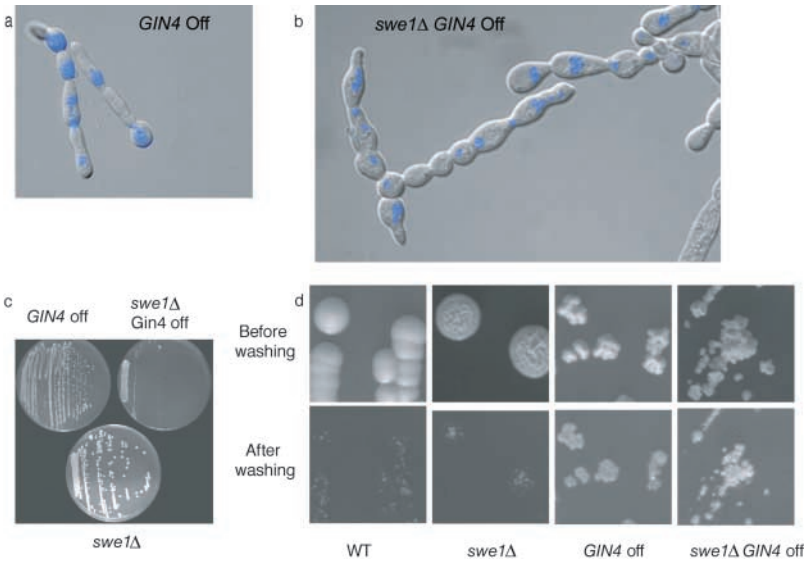

Figure 5. **A *swe1*Δ Gin4-depleted cell mutant still forms filaments.** (a and b) Cells of the indicated genotype were grown to stationary phase in derepressing conditions and reinoculated into fresh medium in repressing conditions. After 6 h, cells were stained with DAPI (blue) and images were recorded using DIC optics. (c) *MET3-GIN4* and *swe1*Δ *MET3-GIN4* strains were grown for 3 d at 30°C under *MET3*-repressing conditions as indicated. (d) The same plates were incubated for a further 2 d and examined for evidence of agar invasion by the plate washing assay described in Fig. 3.

control over the onset of mitosis, and in the absence of Swe1 daughter cells are born abnormally small (Harvey and Kellogg, 2003). Consistent with these observations we found that the *swe1*Δ allele also led to a 10% reduction in cell size in *C. albicans*: the mean volume of *swe1*Δ *GIN4*-on mother cells of the yeast form was 32.8 ± 0.66 fL (n = 122), whereas the mean volume in *SWE1 GIN4*-on mother cells was 36.7 ± 2.3 fL (n = 95). The *swe1*Δ mutant formed hyphae and pseudohyphae normally (Fig. 4, g and h). Therefore, neither hypha or pseudohypha formation is brought about by the activation of the morphogenesis checkpoint.

The pseudohyphal phenotype of *gin4*Δ cells is at least partially independent of Swe1

The pseudohyphal phenotype of the *C. albicans gin4*Δ strain could be due to the action of Swe1 acting to either exert a morphogenesis or cell size checkpoint. To address this issue, we constructed a *MET3-GIN4 swe1*Δ strain. When Gin4 was depleted in a *swe1*Δ mutant, cells were slightly less elongated than *SWE1* cells (Table I), suggesting that some of the elongation was due to the action of Swe1. Further evidence for the operation of a Swe1-dependent checkpoint was a growth defect when *Gin4* expression was turned off in *swe1*Δ *MET3-GIN4* cells, that was not evident when Gin4 expression was turned off in *SWE1* cells (Fig. 5 c). Together, these observations suggest that depletion of Gin4 triggers a Swe1-dependent checkpoint. However, *SWE1* is not entirely responsible for the pseudohyphal phenotype observed because cells remained significantly elongated when *GIN4* expression is turned off in *swe1*Δ strains. Furthermore, *swe1*Δ *GIN4*-off cells remained capable of agar invasion (Fig. 5 d). Thus, the pseudohyphal phenotype observed in *gin4*Δ cells is partly independent of a Swe1-dependent checkpoint.

Gin4 is required for the transition from pseudohyphae to hyphae

To investigate further the requirement of Gin4 for hyphal development, we made use of the conditional *MET3-GIN4* allele. Cells were grown to stationary phase with the *MET3* promoter induced. The resulting culture, consisting of over 95% unbudded yeast cells, was then reinoculated into serum-containing medium at 37°C with the *MET3* promoter either induced or repressed (*GIN4*-on and *GIN4*-off, respectively). Germ tubes evaginated at the same rate in both cultures and the resulting hyphae were identical in appearance (Fig. 6, a and b). Hyphal growth continued normally in these cultures for at least 6 h.

One interpretation of this experiment is that pseudohyphal cells, but not yeast cells, require Gin4 to form hyphae when challenged with serum. To test this hypothesis, we designed an experiment in which *MET3-GIN4* and the parental BWP17 cells were grown to stationary phase in derepressing medium. The unbudded yeast cells were then cultured as pseudohyphae for various times before being challenged to make hyphae by the addition of serum. Wild-type cells rapidly lost the ability to form hyphal germ tubes so that by 6 h <5% of elongated pseudohyphal cells formed hyphae (Fig. 6 c). In contrast, 50% of control cells, incubated in conditions that promote yeast growth, formed hyphae. This observation suggests that the pseudohyphal state is antagonistic to hypha formation. However, when the *MET3-GIN4* strain was induced during pseudohyphal growth, the proportion of elongated cells forming hyphae was only slightly lower than the wild-type control cells grown as yeast, suggesting that high levels of Gin4 can overcome the inability of pseudohyphae to form hyphae.

To confirm that levels of Gin4 are elevated when ectopically expressed from the induced *MET3* promoter, we tagged Gin4 with GFP in the *MET3-GIN4* strain (*MET3-GIN4-GFP*) and also in the wild-type strain so that *GIN4-GFP* expression was under the control of the *GIN4* promoter (*GIN4-GFP*). The capacity of these strains to form hyphae, after incubation as pseudohyphae, was similar to that observed in the previous experiment (Table S2, available at http://www.jcb.org/cgi/content/full/jcb.200307176/DC1). Cellular Gin4-GFP levels were monitored by Western blots using a mixture of two mAbs to GFP. During incubation as pseudohyphae, Gin4-GFP levels were much higher in the induced *MET3-GIN4-GFP* culture compared with the *GIN4-*

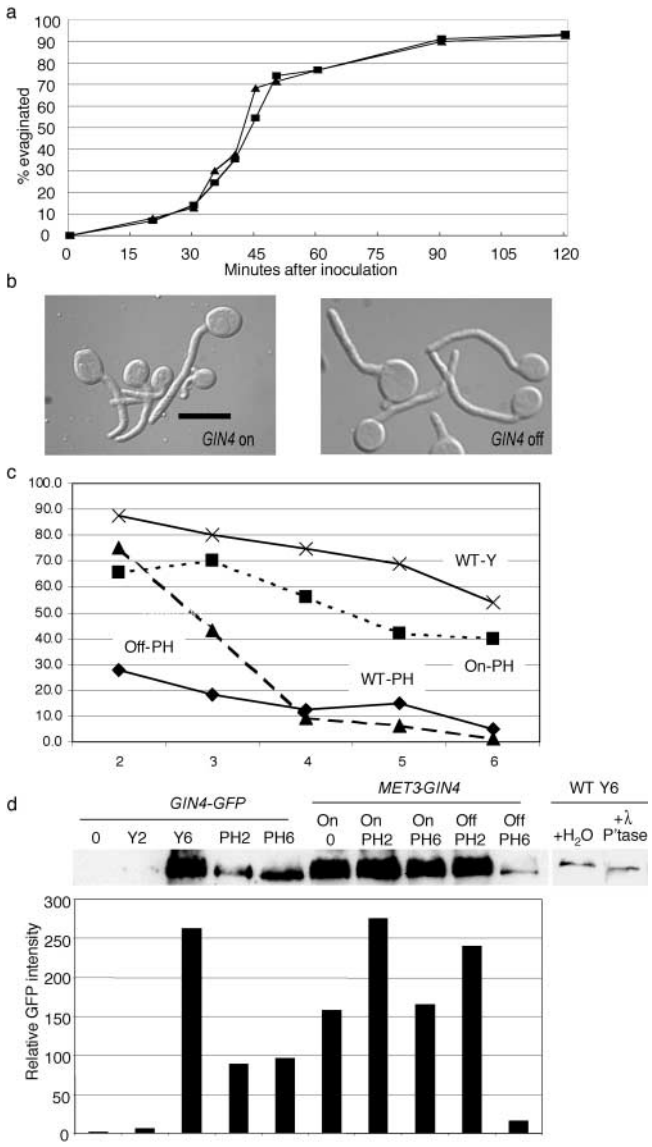

Figure 6. **Gin4-depleted cells can form hyphae but only from the yeast state.** (a and b) *MET3–GIN4* cells were grown to stationary phase under derepressing conditions. The resulting unbudded cells were reinoculated into derepressing (*GIN4* on) or repressing (*GIN4* off) medium containing 20% serum. (a) At the indicated times the percentage evagination was recorded. Solid squares, *GIN4* on; solid triangles, *GIN4* off. Each datum is the mean of at least 80 cells. (b) The appearance of hyphae recorded after 120 min using DIC optics. Bar, 10 μm. (c) *MET3-GIN4* cells were grown to stationary phase in derepressing medium. Cells were then reinoculated, at a concentration of 10^5 cells/ml^{-1}, into *MET3* derepressing ("On-PH") or repressing ("Off-PH") medium, pH 6, 36°C, which promotes the formation of pseudohyphae. At 1-h intervals, samples were withdrawn, and cells were collected by centrifugation and resuspended into the same volume of fresh, prewarmed, derepressing or repressing medium containing 20% serum. After a further 2-h incubation, the proportion of elongated cells producing hyphae was recorded. Elongated cells are defined as cells in which the length was more than twice the width. It should be noted that at early time points, some round cells persisted and that a high proportion of these produced germ tubes, which were not included in the analysis shown in Fig. 6 c. However, by the 6-h time point, <5% of the population were round cells. Parallel samples of the parental BWP17 strain were grown to stationary phase in repressing conditions and incubated either at 30°C, pH 6, which induced yeast growth (WT-Y) or pseudohyphal inducing conditions (WT-PH) before serum treatment to induce hyphae. (d) The experiment described in c was repeated with *GIN4-GFP* (*GIN4* expressed from its own promoter) grown under *MET3*-repressing conditions and *MET3-GIN4*-GFP strains grown under repressing ("Off") or derepressing conditions ("On"). Cells were grown as yeast (Y) or pseudohyphae (PH) and samples were withdrawn 2 and 6 h as indicated (Y2, Y6, PH2, PH6). Gin4 levels were determined by Western blotting. Quantification is by direct measurement of ECL. Full details, including normalization of loading, are given in supplemental material. The protein sample from wild-type cells grown as yeast for 6 h (Y6) was treated with 1,000 U λ phosphatase (New England Biolabs, Inc.) or an equivalent volume of water. The loading was reduced to show the band shift clearly. After withdrawal of the samples for Western blotting, the remaining cultures were treated with serum to induce germ tubes. Quantitation of the proportion of each culture producing germ tubes is shown in Table S2.

GFP culture (Fig. 6 d). Thus, the continued capacity to form hyphae after incubation as pseudohyphae is associated with a high level of Gin4. The repressed *MET3-GIN4-GFP* culture also showed a rise in Gin4-GFP levels after 2 h, but by 6 h the levels had declined to that seen in the *GIN4-GFP* culture. Interestingly, Fig. 6 c shows that there is delay in this culture before the ability to form hyphae is lost, providing further confirmation that there is a correlation between Gin4 levels and the ability of pseudohyphae to form hyphae. Gin4 protein levels also show variation with growth form in cells expressing the protein from the native promoter. Gin4-GFP levels were lower in *GIN4-GFP* cells grown as pseudohyphae compared with the same cells grown as yeast (Fig. 6 d). Finally, we observed that Gin4 appeared to be phosphorylated in cells grown as yeast and cells overexpressing Gin4 from the *MET3* promoter because there was a band shift in these samples, which disappeared after treatment with λ phosphatase (shown in Fig. 6 d for the yeast culture). We interpret these experiments as showing that high levels of Gin4 and/or phosphorylation are required for the transition from pseudohyphae to hyphae. Because Gin4 levels are low in pseudohyphae and Gin4 is not phosphorylated, the pseudohypha to hypha transition is normally blocked.

Gin4 is required for the formation of the septin ring but not the basal septin band

Hyphal germ tubes that formed in Gin4-depleted cells induced from the yeast state were examined to determine the pattern of septin ring formation by immunocytofluorescence using polyclonal antisera to *S. cerevisiae* Cdc11 (αCdc11; Sudbery, 2001). The appearance of basal septin bands and septin rings in representative cells is shown in Fig. 7 (a–e) and quantified in Fig. 7 (h and i). The location of nuclei is also shown in Fig. 7 (a–e). By 90 min, a septin ring had formed in 30% of germ tubes regardless of whether Gin4 was turned on or off (Fig. 7 i). In *GIN4*-off cells, the proportion declined to 10% by 180 min and all septin rings had completely disappeared by 210 min (Fig. 7 i) and primary septa failed to form (Fig. 7 f). In contrast, when *GIN4* was turned on, the proportion of cells with a ring in the germ tube had increased to nearly 90% by 180 min (Fig. 7 i) and primary septa formed normally (Fig. 7 g). The first mitosis occurred at the normal time and in the normal position (Fig. 7 c) in *GIN4*-off cells. Thus, although in wild-type cells mitosis takes place across the plane of the septin rings

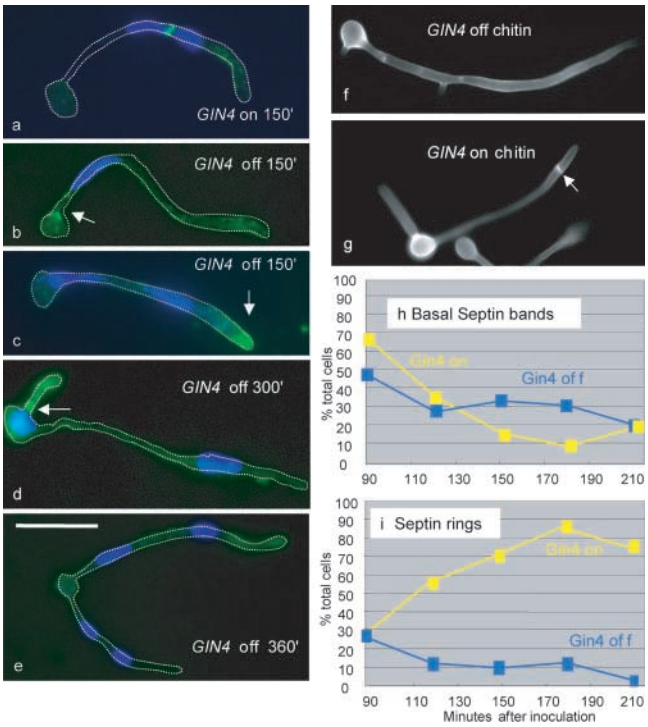

Figure 7. **Properties of *gin4*Δ hyphae.** *MET3–GIN4* cells were grown to stationary phase under derepressing conditions. The resulting unbudded cells were reinoculated into derepressing (*GIN4* on) or repressing (*GIN4* off) medium containing 20% serum and stained with αCdc11 antibody (a–e, false color green) and DAPI (a–e, false color blue) or calcofluor (f and g). Arrows in b and d indicate examples of a basal septin band. Arrow in c indicates apical staining. Arrow in g indicates primary septum. All panels are at the same magnification. Bar, 10 μm. (h and i) Quantitation of cells with tubes and septin bands. A minimum of 70 cells was counted for each time point.

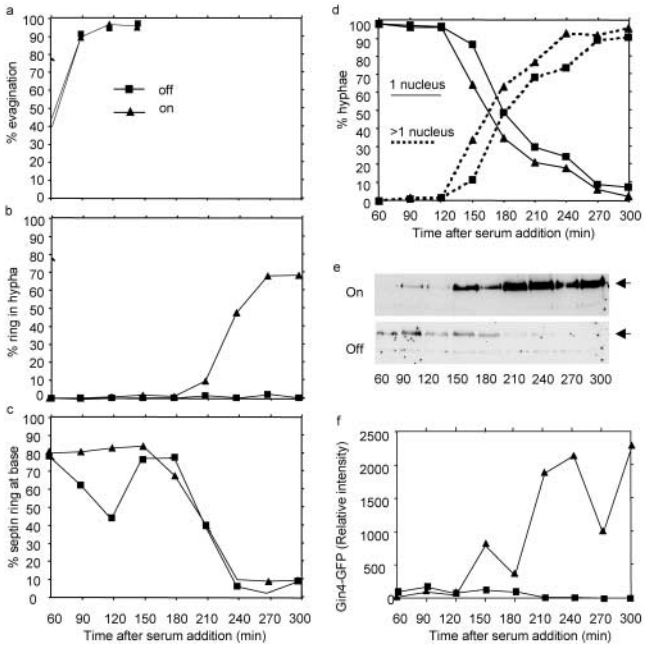

Figure 8. **Properties of hyphae dependent on the *MET3* regulated expression of *GIN4-GFP*.** *MET3-GIN4* cells were grown to stationary phase in derepressing medium, and then reinoculated into fresh medium containing serum at 37°C in repressing or derepressing conditions as described in Fig. 7. The percentage of evagination, germ tubes with septin rings, basal septin bands, and two nuclei were recorded. A minimum of 50 cells were counted for each time point. Levels of Gin4-GFP were monitored during the time course by Western blot analysis (e) and quantified (f) as described in supplemental material. Arrows indicate the Gin4-GFP band. The faint band beneath the Gin4 band is a nonspecific band present in non-GFP tagged strains. We have also used this as a loading control, with similar results to those presented.

(Fig. 7 a), the septin rings are not required for mitosis and do not determine the position at which mitosis will occur.

A basal septin band also formed at the base of the germ tube regardless of whether Gin4 was turned on or off, but appeared to persist longer in cells depleted of Gin4 (Fig. 7, b and h). Some cells depleted of Gin4 showed septin staining at the apical tip of the growing hyphae (Fig. 7 c). In wild-type cells, such staining was also observed at early times but, like the basal band, it also disappeared as the septin ring formed. The presence of septin rings in the *GIN4*-off culture at the beginning of the experiment suggests that significant quantities of Gin4 may be present initially. However, between 240 and 300 min a second germ tube emerged from many mother cells (Fig. 7 d). By this time, the absence of septin rings from the germ tube (Fig. 7 d) suggests that levels of Gin4 are below that required for function. A basal septin band was present in 10 out of 13 (77%) of these second germ tubes examined, whose length was <8 μm but in no case was a septin ring observed. The small number of cells recorded in this experiment result from the technical difficulty of recovering intact structures with two germ tubes after the staining procedure for Cdc11, which involves partial removal of the hyphal cell wall. To increase the number of observations, we constructed a Cdc10–GFP fusion in the *MET3-GIN4* strain, which allows the basal septin band to be visualized directly without the need for cell wall digestion. Nevertheless, the aggregation of cells that occurs during hyphal formation still made observations difficult. We observed a basal septin band in all 36–second germ tubes examined in the *GIN4* off culture. We also used calcofluor staining to examine cells for the presence of a chitin band at the base of the second germ tube. When *GIN4* was turned off, a chitin band was observed in 72% of second germ tubes ($n = 39$). When *GIN4* was turned on, a chitin band was observed in 83% of second germ tubes ($n = 42$). Nuclear migration and mitosis took place normally in the second germ tube in the absence of a septin ring (Fig. 7 e). Septin accumulated at the tip of 30% of second germ tubes observed in the *GIN4* off culture ($n = 30$).

To monitor the level of residual Gin4 in *MET3-GIN4*-off cells, we repeated the experiment using the *MET3-GIN4-GFP* strain and monitored Gin4-GFP levels by Western blotting as described above. Hyphal germ tubes evaginated at an identical rate regardless of whether the *MET3* promoter was turned on or off (Fig. 8 a). When the *MET3* promoter was turned on, the Gin4–GFP fusion caused a delay in the appearance of septin rings as they appeared between 240 and 270 min (compare Fig. 7 i with Fig. 8 b). Nevertheless, basal septin bands were apparent in newly evaginated germ tubes (Fig. 8 c) and persisted longer than in cells con-

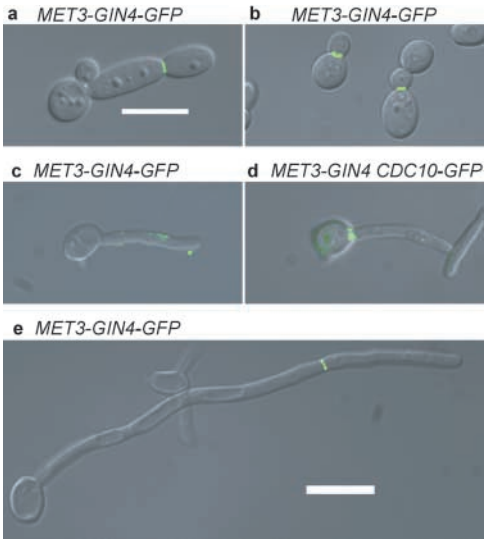

Figure 9. **Gin4-GFP does not form a ring at the base of germ tubes.** *MET3-GIN4-GFP* or *MET3-GIN4 CDC10-GFP*, as indicated, were grown in YEPD medium, pH 6.0, as follows: (a) 37°C; (b) 30°C; (c and d) 37°C plus serum (70 min); and (e) 37°C plus serum (210 min). Bars, 10 μm.

taining the untagged *MET3-GIN4* (compare Fig. 8 c with Fig. 7 h). Thus, although septin ring formation was delayed by the Gin4–GFP fusion, the appearance of basal septin bands was not affected. Again, nuclear migration and mitosis were not delayed and took place in the absence of septin rings (Fig. 8 d).

In the *GIN4*-off culture, no septin rings had appeared by 300 min (Fig. 8 b), but basal septin bands were again apparent in newly evaginated germ tubes (Fig. 8 c). Nuclear division was delayed by a small amount compared with the *GIN4*-on culture, but still took place in the absence of septin rings (Fig. 8 d). Gin4 levels were monitored by a Western blot (Fig. 8, e and f). Low levels of Gin4 were present at the start of the experiment but thereafter rapidly declined and Gin4 was undetectable by 300 min. In contrast, the level of Gin4 steadily increased when the *MET3* promoter was turned on. Thus, expression of *GIN4* from the *MET3* promoter is tightly regulated and no Gin4 was detectable at the time (300 min) when second germ tubes form, which contain basal septin bands. Furthermore, fusing Gin4 to GFP delays the formation of septin rings, but has no effect upon the formation of basal septin bands. Together, these results suggest that Gin4 is required for the formation of the septin ring but is not required for the basal septin band or the apical cap.

Gin4 localizes to the septin ring but not to the basal septin band

To further test the hypothesis that Gin4 is required for the germ tube ring, but not the basal septin band, we examined the localization of Gin4-GFP in yeast, pseudohyphae, and hyphal germ tubes. We found that it localizes to the bud neck of pseudohyphae (Fig. 9 a), yeast (Fig. 9 b), and to a ring in the germ tube consistent with the position of the septin ring (Fig. 9 e). In striking contrast, we never observed any Gin4 localization to the base of developing hyphae (Fig. 9 c) where the basal septin band forms (Fig. 9 d).

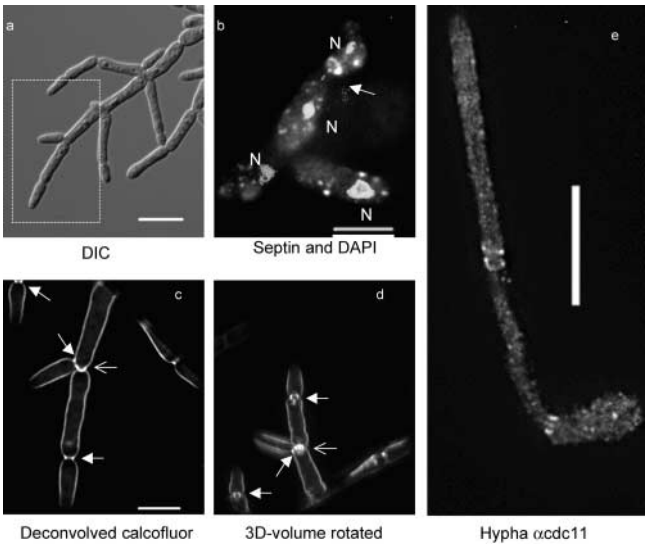

Figure 10. ***hsl1*Δ mutants form pseudohyphae constitutively despite forming normal septin rings and septa.** The *C. albicans* *hsl1*Δ strain was grown in YEPD at 30°C to an OD_{600} = 0.6. Panel and images recorded as indicated. (b) Immunocytofluorescence was performed using *S. cerevisiae* αCdc11 antisera and DAPI staining to reveal nuclei. Arrow indicates a septin ring; N = nuclei. Panels c and d were captured with a DeltaVision microscope, the fields correspond to the area within dotted lines in a. (d) A three-dimensional reconstruction of the image rotated 140° around the x axis with respect to the image in c. Arrows with solid heads in c and d indicate a ring of chitin at the base of the growing cells. The open arrow shows a fully formed primary septum. (e) *hsl1*Δ *was* grown to high cell density (OD_{600} = 0.75) to promote the formation of yeast cells. A sample was diluted into fresh YEPD plus serum medium and incubated for 2 h at 37°C. Cells were fixed and stained with anti-Cdc11 antibody as described previously (Sudbery, 2001). Bars, 5 μm.

Hsl1 also regulates pseudohypha formation, but Hsl1 is not required for septin ring organization or septum formation

To investigate the role of Hsl1, we constructed a homozygous null mutant (*hsl1*Δ*)* and a strain in which one copy of *HSL1* was deleted and the other copy expressed from the *MET3* promoter (*MET3-HSL1)*. The phenotype of the *MET3-HSL1* strain under repressed conditions was indistinguishable from the *hsl1*Δ strain, demonstrating that the *hsl1*Δ phenotype was entirely due to loss of Hsl1 (unpublished data). When grown in liquid YEPD culture at 30°C, the morphology of the *hsl1*Δ mutant was dependent on cell density. At the beginning of the growth cycle, when the cell density was low, it grew with a constitutive pseudohyphal morphology (Fig. 10 a). Compared with *gin4*Δ cells, *hsl1*Δ pseudohyphae had shapes that are more regular, clear constrictions between cellular compartments and branching filaments typical of pseudohyphae. Unlike the *gin4*Δ mutant, septin rings formed normally in the *hsl1*Δ mutant (Fig. 10 b). Chitin rings appeared at the necks of new compartments and a primary septum subsequently separated adjacent compartments (Fig. 10 c). Three-dimensional restoration microscopy indicated that this septum was intact across the entire width of the bud neck (Fig. 10 d).

At later stages in the growth cycle, when cell density was high, the *hsl1*Δ mutant reverted to the yeast morphology

characteristic of wild-type cells grown in these conditions. These yeast cells were able to respond to serum by developing germ tubes. Septin rings and the basal septin band formed normally in *hsl1*Δ hyphae (Fig. 10 e). Thus, in yeast and hyphae, Hsl1 is not required for septin ring formation. This closely parallels its function in *S. cerevisiae*, where it has been shown to act downstream of septin ring formation (Longtine et al., 2000).

Discussion

Gin4 is required for cytokinesis, but Nim1-kinases also negatively regulate pseudohyphal formation

Deletion of the *GIN4* gene in *C. albicans* disrupted the formation of septin rings, caused a severe cytokinesis defect, and gave rise to chains of elongated cells, which resemble pseudohyphae. A priori, the most conservative interpretation of this pseudohyphal appearance is that it arises from the combination of the failure to complete septum formation combined with cell elongation caused by the action of Swe1. The exact role of Swe1 in *S. cerevisiae* is currently unclear. The morphogenesis checkpoint model posits that it exerts a checkpoint in response to defects in bud formation (Lew and Reed, 1995a,b; Sia et al., 1996). The size control model posits that it delays mitosis until a critical cell size threshold is exceeded (Harvey and Kellogg, 2003). This model explains prolonged bud growth in *gin4* mutants by supposing that they form part of a network that monitors cell size. Regardless of which model is correct, the action of Swe1 in *gin4*Δ mutants could result in a phenotype that misleadingly resembles pseudohyphae, i.e., they are "pseudo pseudohyphae".

Several observations suggest that *gin4*Δ mutants may form genuine pseudohyphae. First, *gin4*Δ cells vigorously invaded the agar substratum under conditions that do not stimulate invasive growth in wild-type cells. The extent of the invasion was at least as great as that observed during hypha and pseudohypha formation in wild-type cells, although the morphology of the invading structures was different. A cytokinesis defect was reported recently to result from mutations in genes encoding septins (Warenda and Konopka, 2002). These septin mutants are not constitutively invasive, in fact, they are defective in invasive growth (Warenda and Konopka, 2002). Therefore, invasiveness is not simply a consequence of the chains of cells that result from a failure of cytokinesis. Second, chains of elongated cells still formed in a *swe1*Δ mutant depleted of Gin4, although the extent of cell elongation was reduced. This observation suggests that although a *gin4*Δ mutation induces Swe1-dependent elongation, approximately half of the cell elongation is independent of Swe1. Moreover, these cells still invaded agar, thus agar invasiveness is also not dependent on Swe1. Third, *gin4*Δ mutants were unable to form true hyphae in response to serum. Again, this contrasts with septin mutants that formed germ tubes, although there were some abnormalities in their shape. Therefore, the failure to complete cytokinesis cannot explain the inability to respond to serum. Fourth, like *gin4*Δ mutants, *hsl1*Δ mutants also have a constitutive pseudohyphal phenotype, although septin ring organization is unaffected and septum formation takes place normally. Indeed, the *hsl1*Δ mutants were more convincingly pseudohyphal than *gin4*Δ cells, possibly because there was no additional complication of a failure to properly organize the septin ring and septate. Together, these results suggest that cells depleted of either Nim1 kinase become constitutively pseudohyphal and therefore that Nim1-kinases, Gin4 and Hsl1, act as negative regulators of pseudohyphal development.

Pseudohyphae are in a developmental state that precludes the formation of hyphae

Pseudohyphae that form because of either Gin4 or Hsl1 depletion cannot form hyphae. However, using the conditional *MET3-GIN4* allele, we showed that when yeast cells are produced in the presence of Gin4, they are able to form hyphae when Gin4 is repressed during the serum challenge. One explanation of this observation is that the pseudohyphal state blocks the formation of hyphae. We tested this hypothesis using the conditional *MET3-GIN4* allele. Yeast cells formed in the presence of Gin4 were incubated in pseudohyphae-inducing conditions for various times before being challenged to make hyphae by the addition of serum. Wild-type and *MET3-GIN4* off cells lost the ability to develop into hyphae. This suggests that it is the pseudohyphal state that prevents Gin4-depleted cells from forming hyphae. However, *MET3-GIN4* cells cultured in derepressing conditions retained the ability to form germ tubes. We used a *GIN4–GFP* fusion to follow Gin4 levels during this experiment. The results of a Western blot using mAbs to GFP showed that these cells expressed high levels of Gin4 in a phosphorylated form. Thus, high levels of Gin4 and/or Gin4 phosphorylation overcomes the block that normally prevents pseudohyphae from forming germ tubes. This block may result from the low levels of Gin4 observed in wild-type pseudohyphae. The continued hyphal growth after Gin4 depletion in the *MET3-GIN4* off culture shows that Gin4 is not required for the maintenance of the hyphal state. When wild-type cells are challenged to make hyphae in the yeast state when Gin4 levels are low, germ tubes quickly emerge, suggesting that high Gin4 levels are not required for the yeast-hyphal transition.

Gin4 is required for the organization of septin rings but not basal septin bands

Multiple lines of evidence strongly suggest that Gin4 is not involved in the formation of the basal septin band. First, basal bands resemble the striated appearance of the septin ring in *S. cerevisiae gin4*Δ mutants. Second, when *GIN4* expression is turned off using the *MET3* promoter, basal septin bands persist after there is insufficient Gin4 to maintain septin rings. The slow kinetics of Gin4 depletion do not allow us to completely rule out the possibility that Gin4 is required for the organization of the basal septin band on the basis of this experiment. However, as Gin4 was depleted, septin rings disappeared but basal septin bands persisted. Moreover, by 300 min Gin4 was undetectable, but second germ tubes formed that displayed a basal septin band, but no septin rings. Third, Gin4-GFP locates to septin rings, but not basal septin bands. Fourth, a Gin4-GFP allele delays the appearance of septin rings but not basal septin bands.

Table II. **Strains used in this study**

Strain	Genotype	Source
C. albicans		This study
BWP17	*ura3Δ/ura3Δ arg4Δ/arg4Δ his1Δ/his1Δ*	Wilson et al., 1999
swe1Δ	*swe1Δ::ARG4/swe1Δ::HIS1 ura3Δ/ura3Δ arg4Δ/arg4Δ his1Δ/his1Δ*	This study
hsl1Δ	*hsl1Δ::HIS1/hsl1Δ::HIS1 ura3Δ/ura3Δ arg4Δ/arg4Δ his1Δ/his1Δ*	This study
gin4Δ	*gin4Δ::ARG4/gin4Δ::HIS1 ura3Δ/ura3Δ arg4Δ/arg4Δ his1Δ/his1Δ*	This study
MET3-GIN4	*gin4Δ::ARG4/URA3-MET3-GIN4 ura3Δ/ura3Δ arg4Δ/arg4Δ his1Δ/his1Δ*	This study
MET3-HSL1	*hsl1Δ::HIS1/URA3-MET3-HSL1 ura3Δ/ura3Δ arg4Δ/arg4Δ his1Δ/his1Δ*	This study
MET3-GIN4 swe1Δ	*gin4Δ::dpl200/URA3-MET3-GIN4 swe1Δ::ARG4/swe1ΔHIS1 ura3Δ/ura3Δ arg4Δ/arg4Δ his1Δ/his1Δ*	This study
MET3-GIN4-GFP	*gin4Δ::ARG4/URA3-MET3-GIN4-GFP-HIS1 ura3Δ/ura3Δ arg4Δ/arg4Δ his1Δ/his1Δ*	This study
BWP-17 GIN4-GFP	*GIN4-GFP-HIS1/GIN4 ura3/ura3 arg4/arg4*	This study
MET3-GIN4 CDC10-GFP	*gin4Δ::ARG4/URA3-MET3-GIN4 CDC10/CDC10-GFP-HIS ura3Δ/ura3Δ arg4Δ/arg4Δ his1Δ/his1Δ*	This study
S. cerevisiae		This study
Σ1278b	*MATa ura3-52*	Ahn et al., 1999
CMS50	*MATa swe1Δ::kanMX6 ura3-52*	Martínez-Anaya et al., 2003
SKY903	*MATα mih1Δ::LEU2*	Ahn et al., 1999

Septin rings are not required for nuclear migration and mitosis

Nuclear migration and mitosis occurred normally in *MET3-GIN4-GFP* cells and *MET3-GIN4*-off cells. In the latter case, mitosis also occurred normally in the second germ tube that formed in the mother cell when the *MET3* promoter was turned off. None of the germ tubes displayed a septin ring when mitosis occurred. Thus, the septin ring is not required for mitosis and the position where mitosis occurs cannot be determined by the position of the septin ring. Importantly, the location of the septin ring cannot be determined by the position of mitosis, because in wild-type cells, the septin ring forms in the germ tube before nuclear migration commences (Sudbery, 2001). Therefore, the location of the septin ring and the position where mitosis occurs must be specified by independent markers.

Hyphae and pseudohyphae do not result from induction of the Swe1-dependent morphogenesis checkpoint

In *S. cerevisiae*, the Swe1-dependent morphogenesis checkpoint, which delays mitosis and prevents the switch from polarized to isotropic growth. This has led to the suggestion that the operation of the morphogenesis checkpoint is the mechanism by which hyphae and/or pseudohyphae form (Kron and Gow, 1995). We have tested this hypothesis by constructing a *swe1*Δ mutant. There was evidence that Swe1 is acting to trigger a morphogenesis checkpoint in *C. albicans*, because cells are longer in a *gin4*Δ mutant compared with a *gin4*Δ *swe1*Δ mutant. Moreover, *swe1*Δ mutants accumulate a small fraction of cells with two nuclei, which are never observed in wild-type cells. However, all aspects of hyphal and pseudohyphal formation are normal in a *swe1*Δ mutant and we conclude that the morphogenesis checkpoint is not important for hyphal or pseudohyphal growth. This is consistent with the recent report that there is no change in the amount or timing of tyrosine phosphorylation of Cdc28p in developing hyphae compared with yeast (Hazan et al., 2002). Furthermore, pseudohyphae form normally in an *S. cerevisiae swe1*Δ mutant (Ahn et al., 1999).

Materials and methods

Strains and growth conditions

Strains used are listed in Table II. All *C. albicans* strains were derived from BWP17 (Wilson et al., 1999). All *S. cerevisiae* strains were derived from Σ1278b. YEPD and synthetic-defined media were prepared as described previously (Care et al., 1999; Sudbery, 2001), except that all media were supplemented with 50 mg/ml^{-1} uridine. Induction of the *MET3* promoter was achieved in minimal medium consisting of 0.67% (wt/vol) Difco yeast nitrogen base, 2% (wt/vol) glucose 50 mg/l^{-1} uridine, and any amino acids required by the strain (40 mg/l^{-1}). *MET3* expression was repressed with 0.5 mM cysteine and 2.5 mM methionine as described previously (Care et al., 1999). Conditions for hyphal and pseudohyphal cultures were prepared as described previously (Sudbery, 2001). Induction of filamentation by IAA was prepared as described previously (Martínez-Anaya et al., 2003).

Strain construction

Full details of strain construction are given in the supplemental material and oligonucleotide primers used are specified in Table S1 (available at http://www.jcb.org/cgi/content/full/jcb.200307176/DC1). In brief, disruption cassettes, consisting of either *URA3*, *ARG4*, or *HIS1* selection markers, flanked by a 60-bp sequence of the target gene, were generated by PCR. These were used to transform *C. albicans* strain BWP17 (Wilson et al., 1999). Fusion of *GIN4* to YFP was achieved by PCR cassettes as described previously (Gerami-Nejad et al., 2001). Fusions of the *MET3* promoter to the *GIN4* and *HSL1* coding sequences were generated by a promoter replacement strategy as described previously (Care et al., 1999).

Western blot analysis of Gin4-GFP

Details of protein extraction procedures and antibodies used for Western blotting are provided in the supplemental material.

Microscopy

Immunocytofluorescence using αCdc11 (Santa Cruz Biotechnology, Inc.) was performed as described previously (Sudbery, 2001). Chitin was stained with calcofluor white (Sigma-Aldrich) as described previously (Sudbery, 2001). DNA was stained with DAPI. Cells were examined either with a fluorescence microscope (model DMLB; Leica) or a microscope (model DeltaVision Linux 4; Applied Precision Instruments). Digital images were captured by a CCD camera (model RTE; Princeton Instruments) controlled by an Apple Macintosh G4 computer running Open Lab software version 2.2.5 (Improvision). Images were exported as TIFF files and edited in Adobe Photoshop version 5.5. Images from the DeltaVision microscope were captured, deconvolved, and three-dimensional images were reconstructed using the Softworx software suite supplied with the microscope. Composite figures were assembled using Microsoft PowerPoint 2000.

Axial ratio measurements

Cell images were captured using a microscope (model DMLB; Leica) using DIC optics and the 100× objective. Cell length was determined by mea-

suring the distance between the constrictions using Open Lab Version 2.2.5 software (Improvision). The width was determined at the widest part of the cell. The axial ratio is defined as length divided by width. In yeast cells, the axial ratio is reported for mother cells only as buds may still be growing in an apical fashion. In cells forming pseudohyphae in *MET3*-repressing conditions, only the penultimate cell in a chain of four or more cells was measured as these have completed their apical growth.

Cell size determination

The long and short axes of mother cells were measured as described in the previous paragraph. Cell volume was calculated according to the formula $4/3pab^2$ where a is the radius of the long axis and b is the radius of the short axis (Sudbery et al., 1980).

Online supplemental material

Supplemental material provides details of strain construction, and methods and antibodies used in Western blots. Table S1 specifies sequence of oligonucleotides. Table S2 provides quantitation of the proportion of each culture producing germ tubes in the experiment described in Fig. 6 d. Fig. S1 provides a Clustal alignment of the *C. albicans* and *S. cerevisiae* Nim1 kinases. Fig. S2 provides a Clustal alignment of the *C. albicans* and *S. cerevisiae* Swe1 kinases. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200307176/DC1.

We are grateful for strains and plasmids from the Mitchell laboratory. We are grateful to Judy Berman for invaluable discussions and critical reading of the manuscript.

This work was funded by a project grant from the Wellcome Trust.

Submitted: 28 July 2003
Accepted: 10 December 2003

References

Ahn, S.H., A. Acurio, and S.J. Kron. 1999. Regulation of G2/M progression by the STE mitogen-activated protein kinase pathway in budding yeast filamentous growth. *Mol. Biol. Cell.* 10:3301–3316.

Altman, R., and D. Kellogg. 1997. Control of mitotic events by Nap1 and the Gin4 kinase. *J. Cell Biol.* 138:119–130.

Barral, Y., M. Parra, S. Bidlingmaier, and M. Snyder. 1999. Nim1-related kinases coordinate cell cycle progression with the organization of the peripheral cytoskeleton in yeast. *Genes Dev.* 13:176–187.

Berman, J., and P.E. Sudbery. 2002. *Candida albicans*: A molecular revolution built on lessons from budding yeast. *Nat. Rev. Genet.* 3:918–930.

Booher, R.N., R.J. Deschenes, and M. Kirschner. 1993. Properties of *Saccharomyces cerevisiae wee1* and its differential regulation in response to G1 and G2 cyclins. *EMBO J.* 12:3417–3428.

Brown, A.J.P. 2002. Morphogenic signalling pathways in *Candida albicans*. *In Candida* and *Candidiasis*. R. Calderone, editor. American Society for Microbiology, Washington, DC. 95–106.

Brown, A.J.P., and N.A.R. Gow. 1999. Regulatory networks controlling *Candida albicans* morphogenesis. *Trends Microbiol.* 7:333–338.

Care, R.A., J. Trevethick, K.M. Binley, and P.E. Sudbery. 1999. The *MET3* promoter: a new tool for *Candida albicans* molecular genetics. *Mol. Microbiol.* 34:792–798.

Gerami-Nejad, M., J. Berman, and C. Gale. 2001. Cassettes for PCR-mediated construction of green, yellow and cyan fluorescent protein fusions in *Candida albicans. Yeast.* 18:859–880.

Gow, N.A.R. 1997. Germ tube growth of *Candida albicans. Curr. Top. Med. Mycol.* 8:43–55.

Harvey, S.L., and D.R. Kellogg. 2003. Conservation of mechanisms controlling entry into mitosis: budding yeast Wee1 delays entry into mitosis and is required for cell size control. *Curr. Biol.* 13:264–275.

Hazan, I., M. Sepulveda-Becerra, and H.P. Liu. 2002. Hyphal elongation is regulated independently of cell cycle in *Candida albican*s. *Mol. Biol. Cell.* 13: 134–145.

Kron, S.J., and N.A.R. Gow. 1995. Budding yeast morphogenesis: signaling, cytoskeleton and cell-cycle. *Curr. Opin. Cell Biol.* 7:845–855.

Lew, D.J., and S.I. Reed. 1993. Morphogenesis in the yeast cell cycle: Regulation by Cdc28 and cyclins. *J. Cell Biol.* 120:1305–1320.

Lew, D.J., and S.I. Reed. 1995a. A cell-cycle checkpoint monitors cell morphogenesis in budding yeast. *J. Cell Biol.* 129:739–749.

Lew, D.J., and S.I. Reed. 1995b. Cell cycle control of morphogenesis in budding yeast. *Curr. Opin. Genet. Dev.* 5:17–23.

Liu, H.P. 2001. Transcriptional control of dimorphism in *Candida albicans. Curr. Opin. Microbiol.* 4:728–735.

Lo, H.J., J.R. Kohler, B. DiDomenico, D. Loebenberg, A. Cacciapuoti, and G.R. Fink. 1997. Nonfilamentous *C. albicans* mutants are avirulent. *Cell.* 90: 939–949.

Longtine, M.S., and E.F. Bi. 2003. Regulation of septin organization and function in yeast. *Trends Cell Biol.* 13:403–409.

Longtine, M.S., H. Fares, and J.R. Pringle. 1998a. Role of the yeast Gin4p protein kinase in septin assembly and the relationship between septin assembly and septin function. *J. Cell Biol.* 143:719–736.

Longtine, M.S., A. Mckenzie, D.J. Demarini, N.G. Shah, A. Wach, A. Brachat, P. Philippsen, and J. Pringle. 1998b. Additional modules for versatile and economical PCR-based gene deletions and modification in *Sacharomyces cerevisiae. Yeast.* 14:953–961.

Longtine, M.S., C.L. Theesefield, J.N. McMillan, E. Weaver, J.R. Pringle, and D.J. Lew. 2000. Septin-dependent assembly of a cell cycle-regulatory module in *Saccharomyces cerevisiae. Mol. Cell. Biol.* 20:4049–4061.

Martínez-Anaya, C., J.R. Dickinson, and P.E. Sudbery. 2003. In yeast, the pseudohyphal phenotype induced by isoamyl alcohol results from the operation of the morphogenesis checkpoint. *J. Cell Sci.* 116:3423–3431.

McMillan, J.N., M.S. Longtine, R.A.L. Sia, C.L. Theesfeld, E.S.G. Bardes, J.R. Pringle, and D.J. Lew. 1999. The morphogenesis checkpoint in *Saccharomyces cerevisiae*: Cell cycle control of Swe1p degradation by Hsl1p and Hsl7p. *Mol. Cell. Biol.* 19:6929–6939.

Merson-Davies, L.A., and F.C. Odds. 1989. A morphology index for cell shape in *Candida albicans. J. Gen. Microbiol.* 135:3143–3152.

Odds, F.C. 1985. Morphogenesis in *Candida albicans. Crit. Rev. Microbiol.* 12: 45–93.

Okuzaki, D., S. Tanaka, H. Kanazawa, and H. Nojima. 1997. Gin4 of *S. cerevisiae* is a bud neck protein that interacts with the Cdc28 complex. *Genes Cells.* 2:753–770.

Okuzaki, D., T. Watanabe, and H. Nojima. 2003. The *Saccharomyces cerevisiae* bud neck proteins Kcc4 and Gin4 have distinct, but partially overlapping cellular functions. *Genes Genet. Syst.* 78:113–126.

Russell, P., and P. Nurse. 1987. The mitotic inducer nim1+ functions in a regulatory network of protein-kinase homologs controlling the initiation of mitosis. *Cell.* 49:569–576.

Sia, R.A.L., H.A. Herald, and D.J. Lew. 1996. Cdc28 tyrosine phosphorylation and the morphogenesis checkpoint in budding yeast. *Mol. Biol. Cell.* 7:1657–1666.

Sudbery, P.E. 2001. The germ tubes of *Candida albicans* hyphae and pseudohyphae show different patterns of septin ring localisation. *Mol. Microbiol.* 41: 19–31.

Sudbery, P.E., A.R. Goodey, and B.L.C. Carter. 1980. Genes that control cell proliferation in the yeast *Saccharomyces cerevisiae. Nature.* 288:401–404.

Warenda, A.J., and J.B. Konopka. 2002. Septin function in *Candida albicans* morphogenesis. *Mol. Biol. Cell.* 13:2732–2746.

Wilson, B., D. Davis, and A.P. Mitchell. 1999. Rapid hypothesis testing in *Candida albicans* through gene disruption with short homology regions. *J. Bacteriol.* 181:1868–1874.

Zhang, J., C. Schneider, L. Ottmers, R. Rodriguez, A. Day, J. Markwardt, and B.L. Schneider. 2002. Genomic scale mutant hunt identifies cell size homeostasis genes in *S. cerevisiae. Curr. Biol.* 12:1992–2001.