The *Candida albicans pescadillo* homolog is required for normal hypha-to-yeast morphogenesis and yeast proliferation

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A single species, *Candida albicans*, causes half of all invasive fungal infections in humans. Unlike other fungal pathogens, this organism switches between growth as budding yeast and as pseudohyphal and hyphal filaments in host organs and *in vitro*. Both cell types play a role in invasive disease: while hyphal and pseudohyphal filaments penetrate host cells and tissues, yeast cells are likely to facilitate dissemination through the bloodstream and establishment of distant foci of infection. Many regulators of the yeast-to-hypha switch have emerged from intensive investigations of this morphogenetic step, but the hypha-to-yeast switch remains poorly understood. Using a forward genetic approach, a novel putative regulator involved in the hypha-to-yeast switch was identified, the *C. albicans pescadillo* homolog, *PES1*. In eukaryotes from yeast to human, *pescadillo* homologs are involved in cell cycle control and ribosome biogenesis, and are essential. We find a *pescadillo* homolog to act in fungal morphogenesis, specifically in lateral yeast growth on filamentous cells. We also find essentiality of *PES1* in *C. albicans* to be dependent on cell type, because hyphal cells, but not yeast cells, tolerate its loss. *PES1* is therefore critical for completion of the *C. albicans* life cycle, in which the fungus switches between filamentous and yeast growth. Consistent with these *in vitro* findings, *PES1* is required for *C. albicans* virulence in an *in vivo* insect model of infection.

filament | virulence | essential gene | cell cycle

In humans, *Candida albicans* causes more invasive disease than any other fungal species. Mortality from *C. albicans* bloodstream infections is ≈49% (1). In addition to its role as a pathogen, *C. albicans* colonizes the mucous membranes of 30–60% of humans (2). How has *C. albicans* evolved as the most successful human fungal commensal and pathogen? This fungus's dramatic and frequent switch between growth as ovoid yeast and as filamentous pseudohyphae and hyphae has long been implicated in its success as a pathogen (3). Work from many laboratories has elucidated regulatory mechanisms for the switch from yeast to filamentous growth, and the role of hyphal growth in virulence (3). Little is known about the reverse switch, from filamentous to yeast growth, although mutants defective in the hypha-to-yeast switch show reduced virulence in each case in which this trait was examined (4–10).

The switch from yeast to hyphal growth is induced by specific conditions, such as low cell density, alkaline pH, high temperature, a poor carbon source, and serum. In yeast-inducing conditions, yeast do not produce hyphal cells. In contrast, hyphal and pseudohyphal filaments constitutively produce yeast cells on their subapical segments, while apical segments continue to produce filamentous cells. We call these cells lateral yeast (11). Lateral yeast growth presumably occurs *in vivo* and *in vitro*, because filamentous forms are practically never seen without yeast forms in deep organs of individuals with invasive candidiasis.

We propose that the filament-to-yeast switch is a critical part of the life cycle of *C. albicans*, because yeast cells fulfill specific roles during colonization and infection. Yeast possess suitable dimensions and physical properties to provide the fungus facile access to the host's bloodstream. While cultured endothelial cells from human umbilical cord veins phagocytose hyphae (12), other types of endothelial cells phagocytose yeast. Endocytosis of yeast by endothelial cells was observed in cells of the human blood brain barrier (13), endothelium of porcine whole blood vessel strips (14), and bovine aortic endothelial cells (15). In addition to their function in bloodborne dispersal during invasive disease, *C. albicans* yeast cells have been found to play distinct roles from hyphal cells in the interaction with the host immune system. Exposure of human monocytes to *C. albicans* yeast blocks their development into dendritic cells, a critical class of antigen-presenting cells (16). The ability to manipulate specific features of the host immune system by its different cell types is likely to contribute to the success of *C. albicans* in maintaining its commensal status and in invasive disease.

So far, two genes are known to affect lateral yeast growth: mutants in *PDE2* have decreased, and those in *CAP1* have increased lateral yeast growth (9, 17). Given the dearth of studies of the hypha-to-yeast switch, our goal was to identify novel regulators of this switch. This search identified a hyperfilamentous mutant with a transposon insertion in the promoter of the *C. albicans pescadillo* homolog, *PES1*.

Pescadillo was originally identified in a screen for genes required for development of zebrafish embryos (18). In organisms from yeast to human, the highly conserved homologs of this gene participate in cell cycle control, DNA replication, and ribosome biogenesis (19, 20). In all organisms studied to date, *pescadillo* homologs are essential (19, 20). However, the biochemical activity of *pescadillo* homologs in proliferation, cell cycle control, and ribosome biogenesis is unknown.

Here, we report that the *C. albicans pescadillo* homolog is required for normal growth of cells induced as yeast, and for normal production of lateral yeast on filaments. While a heterozygote retaining an intact allele of the *C. albicans pescadillo*

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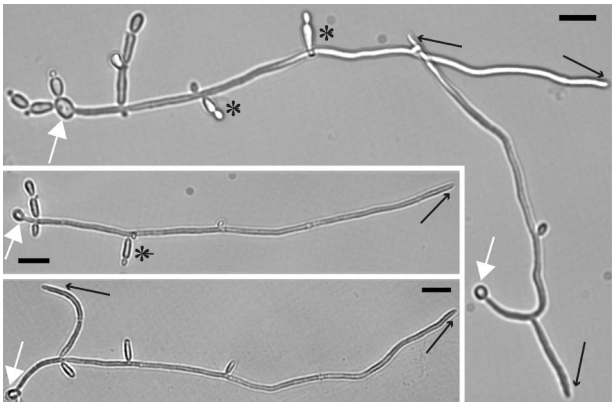

Fig. 1. Lateral yeast are produced under conditions that induce hyphal tip growth and hyphal branching. Yeast cells of SC5314 (*PES1*/*PES1*) were inoculated into Spider medium, permitted to settle on a fibronectin-coated coverslip, and incubated at 37 °C for 6.5 h. Cells of the same culture on the same coverslip were photographed. White arrow, original yeast from which hyphal outgrowth occurred. Black arrow, hyphal branch or tip. Black star, lateral yeast. (Scale bar, 10 μm.)

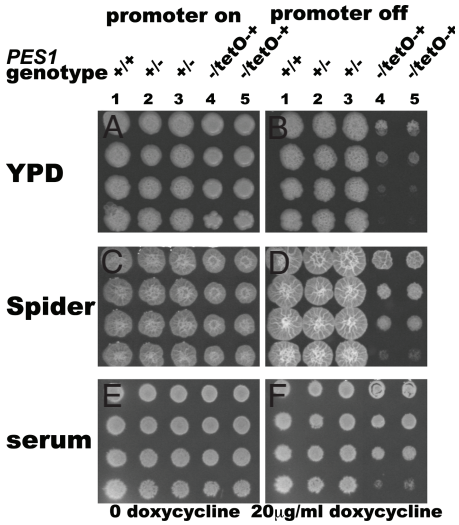

Fig. 2. Hyphae-inducing conditions permit growth when expression of *PES1* is repressed from *tetO*. Strains were grown overnight in YPD without doxycycline at 30 °C and diluted to an initial density of OD_{600} 0.2. Fivefold dilutions were spotted onto YPD (*A*), YPD with 20 μg/ml doxycycline (*B*), Spider (*C*), Spider with 20 μg/ml doxycycline (*D*), 20% calf serum 2% glucose (*E*), and 20% calf serum 2% glucose with 20 μg/ml doxycycline (*F*). Plates were incubated at 37 °C for 4 days. (1) JKC915 (*HIS1*/*his1*::*FRT-tetR PES1*/*PES1*). (2) JKC956 (*HIS1*/*his1*::*FRT-tetR PES1*/*pes1*::*FRT*). (3) JKC962 (*HIS1*/*his1*::*FRT-tetR PES1*/*pes1*::*FRT*). (4) JKC1137 (*HIS1*/*his1*::*FRT-tetR pes1*::*FRT*/*FRT-tetO-PES1*). (5) JKC1143 (*HIS1*/*his1*::*FRT-tetR pes1*::*FRT*/*FRT-tetO-PES1*).

homolog is viable (21), a previous large-scale study found this gene to be essential in *C. albicans* (22). In contrast, we find that *C. albicans* cells tolerate loss of the *pescadillo* homolog under hyphae-inducing, but not under yeast-inducing conditions. Delineating the pathways in which *PES1* functions in the hypha-to-yeast switch will begin to elucidate this step of the *C. albicans* life cycle.

Results

Lateral Yeast Are Produced in Conditions That Induce Hyphal Tip Growth and Hyphal Branching. In all media tested, lateral yeast were produced from subapical cells of both pseudohyphal and hyphal filaments, whose apical cells were producing new filamentous cells. The same cultures contained filaments producing hyphal branches (Fig. 1 and data not shown). Because in liquid cultures, microenvironments of apical and subapical filamentous cells are expected to be similar, we conclude that lateral yeast growth occurs in hyphae-inducing conditions.

A Hyperfilamentous Transposon Mutant in the *C. albicans pescadillo* Homolog, *PES1*. We sought to identify novel regulators of lateral yeast growth. A previous forward genetic approach to filamentous growth successfully used haploinsufficiency phenotypes of *C. albicans* heterozygous mutants to identify regulators of this process (23). From a new heterozygous *mariner* transposon mutant collection, we isolated a hyperfilamentous mutant with a transposon insertion 47 nt upstream of the putative translational start site of ORF 19.4093. This ORF encodes a homolog of a protein conserved from yeast to human, originally called *pescadillo* when it was cloned in zebrafish (18), and *YPH1* (**y**east ***p***escadillo **h**omolog) or *NOP7* in *Saccharomyces cerevisiae* (24, 25). We named this gene *CaPES1* and abbreviate it as *PES1* here. We created *de novo* mutations in *PES1* in the prototrophic wild type, SC5314 (26), by deleting *PES1* on one chromosome from the putative start to the putative stop codons, and replacing the second allele with a conditional allele as described below. The strains carrying targeted mutations in *PES1* did not recapitulate the phenotype of the transposon mutant, which grew exclusively as pseudohyphal and hyphal filaments under all conditions tested. Instead, they showed specific defects in yeast growth when *PES1* was depleted, as described below.

***PES1* Is an Ortholog of the *S. cerevisiae pescadillo* Homolog *YPH1*.** The Pes1 predicted protein sequence is highly homologous to a large number of eukaryotic *pescadillo* orthologs, including *S. cerevisiae YPH1*. To examine whether the *C. albicans pescadillo* homolog can functionally substitute for loss of the essential gene *YPH1*, its ability to rescue growth of *S. cerevisiae yph1* mutants was tested in two experiments. First, in a *S. cerevisae yph1* deletion mutant in the Σ1278b genetic background, *PES1* was able to substitute for *YPH1* when expressed in either low- or high-copy number. Supporting information (*SI*) Fig. S1, published on the PNAS Web site, shows complementation of *yph1*Δ by *PES1* in low-copy number. Second, expression of *PES1* from a high-copy plasmid permitted growth of *S. cerevisiae yph1*Δ temperature-sensitive (ts) mutants in the W303 genetic background (19) at the restrictive temperature (not shown). *C. albicans PES1* can complement loss of *S. cerevisiae YPH1* in two genetic backgrounds, establishing that it is an ortholog of *YPH1*.

***PES1* Is Essential in *C. albicans* in Yeast-Inducing Conditions.** The effect of loss of Pes1 function was examined by constructing three conditional alleles of *PES1*. Given the essentiality of *pescadillo* homologs in other organisms, we did not attempt to construct a homozygous *PES1* deletion strain. Two different repressible promoters were used to control expression of *PES1* in heterozygotes that contain a deletion of one allele and a replacement of the native *PES1* promoter of the remaining allele with the repressible *MAL2* or *tetO* promoters (*pMAL2* and *tetO*) (27, 28). Transcription from *pMAL2* is repressed by glucose in the growth medium, partially repressed by mannitol, and induced by maltose. In our system, transcription from *tetO* is repressed by doxycycline or tetracycline and induced in the absence of these drugs. When *PES1* transcription was repressed from either promoter on rich yeast-inducing medium, YPD, there was minimal growth of *pes1*Δ/*pMAL2-PES1* or *pes1*Δ/*tetO-PES1* strains (Fig. 2 and Fig. S2).

To test the effect of an abrupt perturbation of Pes1 function, we constructed heterozygotes in which the only allele of *PES1* encodes a ts protein $Pes1^{W416R}$ (Fig. S3). A homologous amino

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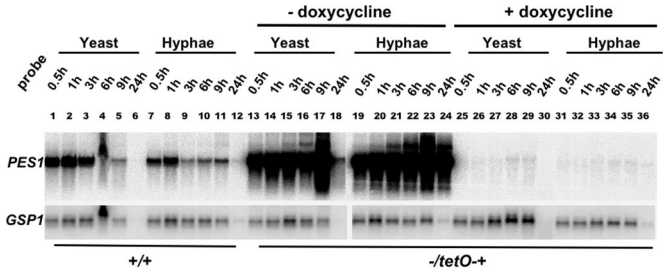

Fig. 3. *PES1* is expressed in both yeast and hyphal cells, and control of *PES1* expression from the *tetO* promoter is independent of yeast- or hyphae-inducing conditions. Strains were grown overnight in YPD at 30 °C, diluted to an initial density of OD_{600} 0.2, and incubated in four conditions: YPD at 30 °C (samples *1-6* and *13-18*), YPD with 10% calf serum at 37 °C (samples *7-12* and *19-24*), YPD with 100 μg/ml doxycycline at 30 °C (samples *25-30*), and YPD with 10% calf serum and 100 μg/ml doxycycline at 37 °C (samples *31-36*). Cells were harvested at the indicated time points, and RNA preparations were blotted and probed with *PES1*. *GSP1* was used as a loading control probe. (*1-12*) SC5314 (*PES1/PES1*). (*13-36*) JKC1137 (*HIS1/his1::FRT-tetR pes1::FRT/FRT-tetO-PES1*).

acid substitution confers temperature sensitivity on *S. cerevisiae* mutants in *YPH1* (25). Mutants with the genotype *pes1*Δ/*PES1*^{W416R} had a growth defect on YPD at 24 °C and did not grow at 37 °C (Fig. S3). On rich yeast-inducing medium, functional Pes1 is required for growth.

Loss of Pes1 Is Not Lethal in Hyphae. The effect of loss of Pes1 function on survival and growth of yeast or hyphal cells was examined in mutants containing three different conditional *PES1* alleles. On yeast-inducing medium ts Pes1^{W416R} mutants were not viable after 3 days at the restrictive temperature, 37 °C. In contrast, on hyphae-inducing media, these mutants were able to survive for 3 days at the restrictive temperature and then resume growth at the semipermissive temperature, 24 °C (Fig. S3). Like the Pes1^{W416R} ts mutants, mutants expressing the repressible promoter alleles of *PES1* showed a differential requirement for *PES1* in yeast- vs. hyphae-inducing conditions. Cells in which *PES1* expression was repressed from either *pMAL2* or *tetO* grew under hyphae-inducing conditions (Fig. 2 and Fig. S2), indicating that in hyphal cells, loss of *PES1* is not lethal.

To confirm that *PES1* message is depleted during promoter repression of *pes1*Δ/*tetO-PES1* strains in both yeast- and hyphae-inducing conditions, levels of *PES1* message were examined by Northern blot. Cells were grown under yeast- and hyphae-inducing conditions, in medium with and without doxycycline to repress transcription from *tetO*. *pes1*Δ/*tetO-PES1* cells grown in medium without doxycycline strongly overexpressed *PES1* compared to wild-type cells, irrespective of morphogenetic conditions. Conversely, *PES1* expression was strongly repressed by doxycycline in both conditions, although a minimal amount of *PES1* message was still detectable (Fig. 3). *PES1* expression in these strains is therefore controlled effectively from the *tetO* promoter, independently of morphogenesis-inducing conditions.

***PES1* Is Expressed in Yeast and Hyphal Cells.** We examined whether *PES1* is differentially expressed in yeast- vs. hyphae-inducing conditions. We used conditions previously described in large-scale microarrray analyses of the yeast-to-hypha switch (29). The wild type was found to express *PES1* at comparable levels in both yeast- and hyphae-inducing conditions (Fig. 3).

Loss of *PES1* Perturbs Lateral Yeast Growth. The effect of loss of Pes1 function on morphogenesis was examined. *pes1*Δ/*pMAL2-PES1* strains growing on media containing mannitol, which partially represses *PES1* expression, had considerably reduced

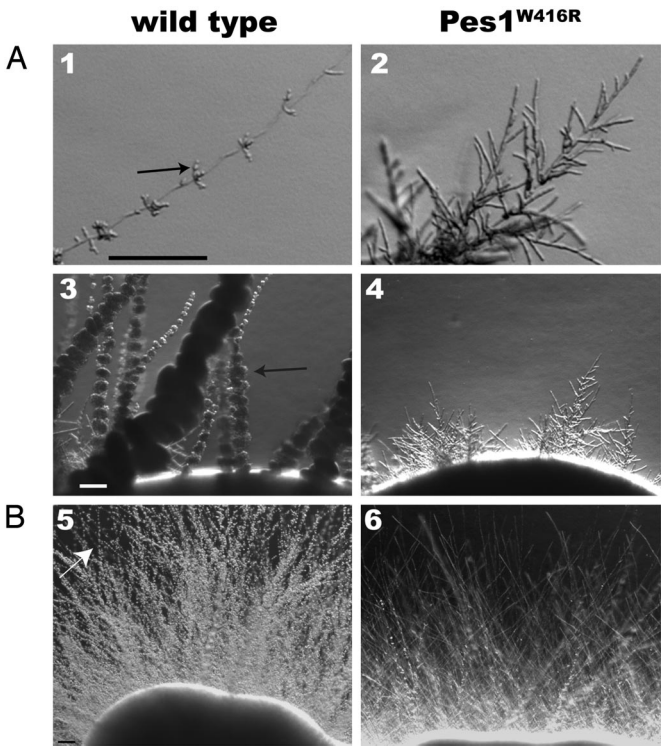

Fig. 4. Lateral yeast growth is defective during Pes1 inactivation. Wild-type and *pes1*Δ/*PES1*^{W416R} strains were grown for 9 days at 24 °C on YP 2% galactose (*A*), and on RPMI1640, buffered to pH 7.5 with 165 mM Mops (*B*). (*1*, *3*, and *5*) SC5314 (*PES1/PES1*). (*2* and *4*) JKC1155 (*pes1::FRT/FRT-PES1*^{W416R}). (*6*) JKC1160 (*pes1::FRT/FRT-PES1*^{W416R}). (*1* and *2*) Tips of young filaments. (*3–6*) Filaments emanating from colony rim. Arrows, clusters of lateral yeast. (Scale bar, 100 μm.)

lateral yeast growth (Fig. S4). Pes1^{W416R} ts mutants were also defective in lateral yeast growth at the semipermissive temperature (Fig. 4). Loss of Pes1 function therefore results in defective lateral yeast growth.

Loss of *PES1* Does Not Lead to Constitutive Polarized Growth. Formation of the ovoid shape of a budding yeast cell requires that the polarized growth direction of an emerging bud switches to isotropic expansion around the entire cell circumference as the growing bud prepares for mitosis (30). We tested whether defective lateral yeast growth of *PES1* mutants is the result of an inability to appropriately switch to isotropic growth, i.e., whether these mutants remain trapped in a polarized growth mode. Terminal phenotypes of Pes1^{W416R} ts mutants were examined in hyphae- and yeast-inducing conditions. Mutant yeast cells inoculated into hyphae-inducing conditions at the restrictive temperature produced germ tubes indistinguishable from those of wild type, which arrested as short hyphae (Fig. S5). When Pes1^{W416R} ts yeast cells were inoculated into yeast-inducing conditions at the restrictive temperature, they arrested in the yeast form (Fig. S5).

The terminal phenotype of *pes1*Δ/*pMAL2-PES1* yeast during promoter repression was followed for 3 days. Mother cells divided several times with each daughter receiving a nucleus, eventually forming clusters of attached large yeast. After 3 days of *PES1* depletion, the nuclei appeared fragmented (Fig. S5). Thus, both abrupt inactivation of Pes1 and depletion of *PES1* from a repressible promoter did not interfere with appropriate switching of yeast cells from polarized to isotropic growth. Mutants in *PES1* are not trapped in a polarized growth state.

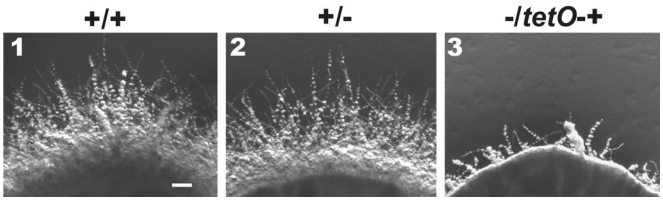

Fig. 5. *PES1* overexpression leads to decreased hyphal growth and increased lateral yeast growth. Strains were streaked on Spider medium without doxycycline and incubated at 37 °C for 4 days. (1) JKC915 (*HIS1/his1*::*FRT-tetR PES1/PES1*). (2) JKC962 (*HIS1/his1*::*FRT-tetR PES1/pes1*::*FRT*). (3) JKC1143 (*HIS1/his1*::*FRT-tetR pes1*::*FRT/FRT-tetO-PES1*). (Scale bar, 100 μm.)

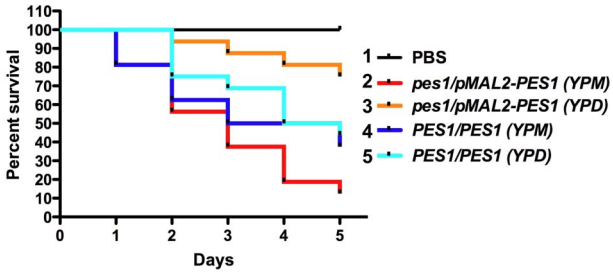

Fig. 6. *PES1* depletion leads to loss of virulence in a *Galleria mellonella* model. *C. albicans pes1*Δ*/pMAL2-PES1* mutant and wild-type strains were grown overnight at 30 °C in YPD to repress *PES1* expression in the mutant, or in YP 2% maltose (YPM) to induce *PES1* expression in the mutant, and resuspended in PBS to an inoculum of 10^6 cells. Sixteen larvae per condition were injected and kept at 37 °C. (*1*) Larvae injected with PBS only. (*2*) Larvae injected with JKC681 (*pes1*::*FRT/FRT-pMAL2-PES1*) pregrown in YPM. (*3*) Larvae injected with JKC681 (*pes1*::*FRT/FRT-pMAL2-PES1*) pregrown in YPD. (*4*) Larvae injected with SC5314 (*PES1/PES1*) pregrown in YPM. (*5*) Larvae injected with SC5314 (*PES1/PES1*) pregrown in YPD. The *P* value for difference in survival curves between larvae injected with JKC681 pregrown in YPD and with JKC681 pregrown in YPM is 0.0001 (Mantel-Cox test). The *P* value for difference in survival curves between larvae injected with SC5314 pregrown in YPD and with SC5314 pregrown in YPM is 0.547.

Quorum Sensing Is Not Perturbed by Pes1 Inactivation. To test whether *PES1* mutants' defect in lateral yeast growth is the result of an inability to respond to quorum-sensing molecules that stimulate a switch from hyphal to yeast growth and could be secreted by subapical filamentous cells, we examined the response of $Pes1^{W416R}$ ts mutants to two compounds that activate the *C. albicans* quorum-sensing response: farnesol and dodecanol (31). At the restrictive temperature for $Pes1^{W416R}$ ts cells, 37 °C, wild-type yeast switched to filamentous growth at a significant rate when diluted into rich medium without quorum-signaling compounds (29). Filamentous growth was suppressed by addition of dodecanol or farnesol to the medium, with no observable difference between $Pes1^{W416R}$ ts cells and the wild type (Fig. S5). Defective lateral yeast growth in *PES1* mutants is not the result of an inability to respond to quorum-signaling molecules.

***PES1* Overexpression Results in Decreased Filamentous Growth and Increased Lateral Yeast Growth.** To investigate whether *PES1* overexpression has the opposite effect from *PES1* depletion, *pes1*Δ*/tetO-PES1* mutants were grown on medium without doxycycline. In these conditions, *PES1* is strongly overexpressed (Fig. 3). On all solid media tested without doxycycline, these strains showed decreased hyphal growth, and the hyphae that were formed showed overabundant lateral yeast (Fig. 5). In liquid rich medium, strains overexpressing *PES1* did not have a significantly altered growth rate compared to the wild type (Fig. S2). Overexpression of *PES1* increases, while its depletion decreases lateral yeast growth.

***PES1* Is Required for Virulence in the *Galleria mellonella* Larva Model.** An insect model was used to examine the effect of Pes1 perturbation on *C. albicans* virulence (32). When injected at inocula between 5×10^5 and 10^6 cells, wild-type *C. albicans* SC5314 rapidly kill *G. mellonella* larvae held at 37 °C. *pes1*Δ*/pMAL2-PES1* cells, preincubated in glucose to deplete *PES1*, were significantly reduced in virulence in this system (Fig. 6). Consistent results were obtained with the *pes1*Δ*/tetO-PES1* and *pes1*Δ*/*$PES1^{W416R}$ mutants (data not shown). *PES1* is therefore required for *C. albicans* virulence in this insect model.

Discussion

In *C. albicans*, the *pescadillo* homolog *PES1* is essential in yeast, whereas hyphal cells tolerate *PES1* depletion (Fig. 2 and Fig. S2). The *pes1* yeast growth defect has implications for host–pathogen interactions because *C. albicans* uses its yeast form during its commensal state and during dissemination and deep organ proliferation in invasive disease. Wild-type *C. albicans* constitutively produce lateral yeast on filaments in conditions that induce hyphal tip growth (Fig. 1), and this process is disturbed when functional Pes1 is lacking (Fig. 4 and Fig. S4). In an insect model, virulence of cells deficient in Pes1 was attenuated. A caveat here is that attenuation of *pes1* mutant cells is likely to be potentiated by any growth defect. *PES1*-depleted cells have the least growth defect when they are exposed to a combination of strong hyphae-inducing signals (Fig. 2 and Fig. S2). Larvae were held at the hyphae-inducing temperature of 37 °C, but we do not know whether other hyphae-inducing signals prevail in the larva. Virulence attenuation during loss of *PES1* is nevertheless consistent with a pathogenic role of Pes1-dependent yeast growth.

Pescadillo homologs of yeast, mouse, and human act in proliferation control and ribosome biogenesis (19, 20). It is not clear whether these distinct functions are based in distinct biochemical activities. *PES1* is cotranscribed with genes involved in rRNA processing and ribosome biogenesis (33) (Candida Genome Database), suggesting that it may participate in these functions in *C. albicans* as well. One possibility is that growth arrest during *PES1* depletion is caused by disruption of rRNA processing, leading to activation of a "nucleolar stress" cell cycle checkpoint (34), or to depletion of ribosomes and slowing of translation. If this is the case, tolerance of hyphae for *PES1* depletion might indicate that hyphal cells need less ribosomes to grow than yeast cells. Alternatively, Pes1 may have separate roles in proliferation control and rRNA processing.

Cell cycle control in *S. cerevisiae* is linked to determination of cell shape (30). To form an ovoid yeast cell, new material is secreted toward the tip of a young bud from S phase until the G2/M transition, in a polarized or apical direction. At that point, growth is redirected to distribute new material isotropically around the entire cell circumference. Delay of mitosis, by prolonging the duration of polarized growth, leads to the elongated cells that form a pseudohyphal filament, and this effect is enhanced by mutations in cell cycle regulators required for progression through S and for the G2/M transition (30). The link between cell cycle control and morphogenesis is more complex in *C. albicans* because perturbation of many *C. albicans* cell cycle regulators, involved at all phases of the cell cycle, results in constitutive polarized growth (35). In *C. albicans*, the gene encoding G1 cyclin Cln3 is specifically required for growth in yeast-inducing conditions (36, 37), similarly to *PES1*. But unlike *PES1* depletion, depletion of *CLN3* in yeast-inducing conditions leads to growth arrest and then outgrowth of a hyphal or pseudohyphal filament (36, 37). In contrast, the terminal phenotype of *pes1* mutants consists of growth arrest of yeast cells (Fig. S5). Consequently, inability to switch from polarized to

isotropic growth cannot be the reason for the growth defect of *PES1*-depleted yeast cells. In *S. cerevisiae*, the *pescadillo* homolog Yph1 is required for entry into the cell cycle from G0, for progression from G1 to S, and for progression from G2 to M (25). It remains to be determined whether Pes1 is required in one or in multiple phases of the *C. albicans* cell cycle and how this activity differs between yeast and hyphal cells.

In *S. cerevisiae*, cell cycle entry and ribosome biogenesis are tightly coordinated at the transcriptional level in response to nutrient availability (38, 39). Du and Stillman found evidence that in *S. cerevisiae*, the *pescadillo* homolog Yph1 participates in nutritional signaling (25). Pes1 could participate in a signaling network that relays information about the cell's nutritional status to both the cell cycle machinery and the ribosomal biogenesis machinery simultaneously to coordinate these two processes. High levels of *PES1* expression in fresh rich media, and decrease of *PES1* levels over time (Fig. 3), may be consistent with this idea. The finding that overexpression of *PES1* has the opposite morphogenetic effect as its depletion (Fig. 5) may support a regulatory role for Pes1. If Pes1 is linked to a nutritional signaling network, the cAMP-dependent protein kinase (PKA) pathway is a candidate for functional interactions with Pes1. The only previously described *C. albicans* mutants with altered lateral yeast growth, in *PDE2* and *CAP1*, have abnormal intracellular cAMP levels (9, 17). In addition, a phosphatase linked to the PKA pathway, Yvh1, interacts physically with the *pescadillo* homolog in *S. cerevisiae* and in *Plasmodium falciparum* (40, 41). Signaling through the PKA pathway is required for ribosome biogenesis (38), consistent with a model whereby Pes1, in interaction with the PKA pathway, responds to availability of nutrients to promote rRNA processing and cell proliferation. Whether *C. albicans* hyphal cells require less *PES1* because hyphal growth is less dependent on such a nutritional signal remains to be determined.

In summary, Pes1 is a putative regulator implicated both in the filament-to-yeast switch and in proliferation of yeast, two processes that are critical for the vegetative life cycle of this principal fungal pathogen of humans.

Materials and Methods

Strains and Culture Conditions. The *C. albicans* and *S. cerevisiae* strains used in this study are described in Table S1. All *C. albicans* strains were generated in the SC5314 genetic background using the *CaNAT1* selectable marker, as described in ref. 42. Each of the three conditional alleles of *PES1* was generated from two independent heterozygotes. *PES1* deletion and replacement of the *PES1* promoter with *pMAL2* and *tetO*, and introduction of the Pes1^{W416R} ts-encoding mutation were confirmed by Southern blot. The genotype of *S. cerevisiae* strains was confirmed by PCR spanning the upstream and downstream homologous recombination junctions of transforming constructs. For routine growth of yeast strains, YPD (1% yeast extract/2% peptone/2% glucose) was used. Spider medium was made as in ref. 43. Solid serum medium was made with 10% or 20% calf serum (Gibco/Invitrogen cat. no.16170–078), water, and 2% agar; 2% glucose or maltose were added as indicated.

The heterozygous transposon mutant collection will be described elsewhere in detail. Briefly, a *C. albicans* genomic library, consisting of 4.9×10^4 independent clones (44), was mutagenized by transposon insertion *in vitro*. A hyperactive C9 *mariner* transposase was purified as a fusion with maltose binding protein, and transposition conditions were optimized according to considerations described in ref. 45. The transposon was adapted to contain the *CaNAT1* marker, which confers resistance to the aminoglycoside nourseothricin on *C. albicans* and on *Escherichia coli* (42). A transposon-mutagenized genomic *C. albicans* plasmid library consisting of 1.26×10^5 clones was generated. In our system, each clone represents an independent insertion event because *in vitro* mutagenesis was followed by electroporation of products of transposition into *E. coli* and selection of *E. coli* transformants on nourseothricin (clonNAT, Werner BioAgents).

C. albicans wild-type strain SC5314 was transposon mutagenized by transformation with inserts excised from the *in vitro* mutagenized genomic library and selection on rich medium (YPD) containing nourseothricin. Mutagenesis occurred by integration of a fragment of genomic DNA carrying a transposon, into the *C. albicans* chromosome by homologous recombination, thereby integrating the transposon into the transformant's chromosome. A total of 17,000 mutants was arrayed in 96-well dishes; of these, 14,000 were viable in standard conditions. The *PES1*/*pes1*::*tn* transposon mutant was isolated as a hyperfilamentous clone directly from the transformation plate (YPD + nourseothricin).

Plasmid Construction. Plasmids constructed for this study are described in *SI Text* on the PNAS Web site. Previously published plasmids used in this study are shown in Table S1, and primers are shown in Table S2, both of which are published as *SI Text*.

Microscopy of Lateral Yeast Growth in Liquid Medium. Wild-type strain SC5314 was grown in YPD to saturation overnight at 24 °C, diluted to an OD_{600} of 0.005 in Spider medium, and inoculated into wells of 6 well plates containing fibronectin-coated coverslips. After 6.5 h incubation at 37 °C, cultures were fixed with formaldehyde and examined by brightfield microscopy.

Growth Assays. For cell dilutions spotted onto solid media, cells were grown overnight in YPD at 24 °C for experiments using Pes1^{W416R}-containing mutants, and at 30 °C for all other experiments. Cells were diluted to an OD_{600} of 0.2 in PBS and further diluted in fivefold steps in PBS. Cell suspensions were spotted onto appropriate media using a replicator with prongs calibrated to deliver 1.5 μl (V&P Scientific, VP407). For growth curves in liquid media, all strains were grown to saturation overnight in YPD with 0.01 μg/ml of doxycycline at 24 °C. They were washed once in the cognate growth medium (YPD, Spider, or 20% serum and 2% glucose in water) without or with 20 μg/ml of doxycycline and diluted to an OD_{600} of 0.05. One hundred microliters of each cell suspension was inoculated into wells of flat bottom 96-well dishes, and cultures were incubated at the cognate temperature without shaking in a Spectramax250 plate reader. OD_{600} readings were obtained every 30 min and imported into Excel at the end of the experiment. Means and standard deviations were calculated and graphed in Excel.

Northern Blot. Cells of the wild-type strain SC5314, and a *pes1*/*tetO-PES1* strain, JKC1137, were grown to saturation (OD_{600} >12) in YPD at 30 °C overnight. They were diluted to an initial density of OD_{600} 0.2 into 12 ml medium per sample. The *pes1*/*tetO-PES1* strain was incubated at 200 rpm in four conditions: YPD at 30 °C, YPD with 10% calf serum at 37 °C , YPD with 100 mg/ml doxycycline at 30 °C and YPD with 10% calf serum and 100 mg/ml doxycycline at 37 °C . The wild type was incubated in the first two conditions. Cells were harvested at six time points: 0.5, 1, 3, 6, 9, and 24 h. Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. Total RNA was blotted to a nylon membrane (Hybond XL) and probed with the insert of pJK945, which was labeled with [^{32}]ATP by random priming. As the loading control probe, a portion of the *GSP1* ORF was amplified with primers 871 and 998. *GSP1* encodes a small G protein whose expression level is not affected during morphogenesis (D. Kadosh, personal communication).

Quorum-Sensing Response Assay. Strains were grown in YPD overnight at 24 °C, and diluted to an OD_{600} of 0.025 into 250 μl of each medium: YPD with 10% calf serum, YPD with 0.5% DMSO, and YPD with 200 μM dodecanol or farnesol, and incubated in 24-well dishes at 37 °C without shaking.

***G. mellonella* Killing Assay.** Injection of *C. albicans* in the *G. mellonella* killing assay was performed essentially as described (46). In brief, larvae in the final instar larval stage were obtained from Canadian Feeders. Inocula of *C. albicans* for the injections were prepared by growing 50 ml YPD cultures overnight at 30 °C. Cells were pelleted by centrifugation at 3,000 rpm for 10 min followed by three washes in PBS. Cells were resuspended in 1 ml of PBS and cell densities were determined by hemacytometer count and additionally confirmed by OD_{600} comparison for *PES1*-depleted cultures in which yeast cells formed aggregates of unseparated yeast. Final dilutions for injection of 10^6 cells were prepared in PBS. Sixteen larvae (330 $\pm$ 25 mg) were used per group and 5-μl injections were performed via the last left proleg. Larvae were incubated in Petri dishes at 37 °C in the dark and the number of dead larvae was scored daily. Kill curves were plotted and estimation of differences in survival by log-rank (Mantel-Cox) test was analyzed by the Kaplan-Meier method using GraphPad Prism statistical software.

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