The Yak1 Kinase Is Involved in the Initiation and Maintenance of Hyphal Growth in *Candida albicans*

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Members of the dual-specificity tyrosine-phosphorylated and regulated kinase (DYRK) family perform a variety of functions in eukaryotes. We used gene disruption, targeted pharmacologic inhibition, and genome-wide transcriptional profiling to dissect the function of the Yak1 DYRK in the human fungal pathogen *Candida albicans*. *C. albicans* strains with mutant *yak1* alleles showed defects in the yeast-to-hypha transition and in maintaining hyphal growth. They also could not form biofilms. Despite their in vitro filamentation defect, *C. albicans yak1Δ/yak1Δ* mutants remained virulent in animal models of systemic and oropharyngeal candidiasis. Transcriptional profiling showed that Yak1 was necessary for the up-regulation of only a subset of hypha-induced genes. Although downstream targets of the Tec1 and Bcr1 transcription factors were down-regulated in the *yak1Δ/yak1Δ* mutant, *TEC1* and *BCR1* were not. Furthermore, 63% of Yak1-dependent, hypha-specific genes have been reported to be negatively regulated by the transcriptional repressor Tup1 and inactivation of *TUP1* in the *yak1Δ/yak1Δ* mutant restored filamentation, suggesting that Yak1 may function upstream of Tup1 in governing hyphal emergence and maintenance.

INTRODUCTION

Candida albicans is an opportunistic fungal pathogen in humans, causing various forms of candidiasis ranging from superficial mucosal infections to life-threatening systemic diseases, predominantly in patients with a compromised immune system. In addition, *C. albicans*, like many pathogens, adheres to and subsequently form biofilms on implanted devices, especially on indwelling intravascular catheters. Organisms growing in biofilms have decreased susceptibility to antifungals (Donlan and Costerton, 2002; Douglas, 2003; Nobile and Mitchell, 2006). *C. albicans* is a polymorphic organism that undergoes reversible morphogenetic transitions between budding yeast, pseudohyphal and hyphal growth forms. The ability to transition from the yeast form to the hyphal form has been considered a major requirement for

virulence (Saville *et al.*, 2003), but it is also critical for biofilm formation (Ramage *et al.*, 2002; Richard *et al.*, 2005).

A variety of factors, including temperature, amino acids, pH changes, and serum can induce the yeast-to-hypha transition in *C. albicans* (Ernst, 2000; Eckert *et al.*, 2007). The contribution of a range of signaling pathways to morphogenesis has been investigated, suggesting that the yeast-to-hypha transition is the subject of both positive and negative regulation (Eckert *et al.*, 2007; Whiteway and Bachewich, 2007). Central to the positive regulation of filamentation is the transcription factor Efg1 that acts downstream of the cAMP signaling pathway. Indeed, a *C. albicans efg1Δ/efg1Δ* mutant is unable to form hyphae under a variety of hypha-inducing conditions (Lo *et al.*, 1997; Stoldt *et al.*, 1997), indicating that Efg1 plays a critical role at the onset of filamentation. Nevertheless, it has also been reported that *efg1Δ/efg1Δ* mutants display increased filamentation during growth under anaerobic or microaerophilic (embedded) conditions, suggesting that Efg1 acts as a possible repressor of filamentation under certain conditions (Giusani *et al.*, 2002; Doedt *et al.*, 2004). Negative regulation of filamentation is achieved by the general repressor Tup1 in association with the DNA-binding proteins Nrg1 and Rfg1. These negative regulators of filamentation act independently of Efg1 and other positive regulators of filamentation (Braun and Johnson, 1997; Braun *et al.*, 2001; Kadosh and Johnson, 2001; Murad *et al.*, 2001b). The interplay between positive and negative regulation of the yeast-to-hypha switch is illus-

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Abbreviations used: 1-NMPP1, 4-amino-1-(tert-butyl)-3-(1'-naphthylmethyl)pyrazolo-(3,4-*d*)-pyrimidine; DYRK, dual-specificity tyrosine-phosphorylated and regulated kinase; PKA, cAMP-dependent protein kinase.

trated by the complex structure of the promoter regions of hypha-specific genes such as *ALS3* and *HWP1*, which integrate inputs from multiple activators and repressors and are under the control of chromatin remodeling proteins (Argimon *et al.*, 2007; Kim *et al.*, 2007). To date, the exact relationships, interconnections, and feedback regulations of the signaling pathways and transcription factors that positively and negatively regulate filamentation remain largely uncharacterized.

Candida glabrata is another pathogenic yeast that represents the second leading cause of candidiasis (Pfalter and Diekema, 2007). *C. glabrata* is relatively distantly related to *C. albicans*, and it has only been reported to form pseudohyphae (Csank and Haynes, 2000). However, it is able to form biofilms, and we have previously taken advantage of this property to identify *C. glabrata* genes that are involved in biofilm formation (Iraqi *et al.*, 2005). A genetic screen for *C. glabrata* insertional mutants altered in their ability to form biofilms enabled us to identify four genes involved in biofilm formation: *YAK1*, encoding a protein kinase; *RIF1* and *SIR4*, encoding two components of the subtelomeric silencing machinery; and *EPA6*, encoding a member of the *C. glabrata* family of Epa adhesins (Iraqi *et al.*, 2005). Experimental evidence suggested a model whereby the *C. glabrata* Yak1 kinase is required to repress the subtelomeric silencing machinery and in turn allow expression of the *EPA6* and *EPA7* adhesin genes that contribute to cell-to-cell adhesion within biofilms (Iraqi *et al.*, 2005). In *C. albicans*, adhesins such as *Als3* and *Als1* are structurally related to the *C. glabrata* Epa adhesins, and they have been implicated in biofilm formation (Chandra *et al.*, 2001; Garcia-Sanchez *et al.*, 2004; Nobile *et al.*, 2006a; Zhao *et al.*, 2006). However, *C. albicans* *ALS* genes are not located at subtelomeres (van het Hoog *et al.*, 2007), suggesting that mechanisms other than silencing are involved in the regulation of these genes during biofilm formation.

In the present study, we investigated the *C. albicans* Yak1 kinase, a member of the family of dual-specificity tyrosine-phosphorylated and regulated kinases (DYRKs; Becker and Joost, 1999). The founding member of the DYRK family, the *Saccharomyces cerevisiae* Yak1 kinase, was originally identified as a suppressor of the lethality associated with the loss of either *RAS* function or the *Tpk1*, *Tpk2*, and *Tpk3* catalytic subunits of the cAMP-dependent protein kinase A (PKA) (Garrett and Broach, 1989). The different phenotypes associated with deletion or overexpression of the *S. cerevisiae* *YAK1* gene suggest that Yak1 acts as a negative regulator of growth and modulates PKA-regulated processes such as heat sensitivity and pseudohyphal growth (Garrett and Broach, 1989; Hartley *et al.*, 1994; Ward and Garrett, 1994; Zhang *et al.*, 2001). The function of Yak1 may be directly regulated by PKA, because it is phosphorylated *in vitro* and *in vivo* by PKA (Garrett *et al.*, 1991; Zappacosta *et al.*, 2002; Ptacek *et al.*, 2005). *S. cerevisiae* Yak1 and other DYRKs are Ser/Thr protein kinases whose activity is modulated by autophosphorylation at a conserved tyrosine residue (Garrett *et al.*, 1991; Kassis *et al.*, 2000). Known targets of *S. cerevisiae* Yak1 include Bcy1, the regulatory subunit of PKA; Crf1, a corepressor of the forkhead-like transcription factor Fhl1 involved in the repression of ribosomal protein genes; and Pop2, which is required for expression of glucose-repressed genes (Griffioen *et al.*, 2001; Moriya *et al.*, 2001; Martin *et al.*, 2004). Phosphorylation by Yak1 modulates the nucleocytoplasmic distribution of these proteins and their contribution to growth-related processes. Recent results indicate that *S. cerevisiae* Yak1 may influence the nucleocytoplasmic distribution of the Msi1 (Cac3) protein and its asso-

ciation in the chromatin assembly factor-I (CAF-I), suggesting a potential role of Yak1 in regulating chromatin structure (Pratt *et al.*, 2007).

In this study, we investigated the role of *C. albicans* Yak1 in biofilm formation, the yeast-to-hypha transition, and hyphal maintenance by using null and conditional alleles of the *C. albicans* *YAK1* gene. We identified genes whose up-regulation at the yeast-to-hypha switch is dependent upon a functional Yak1, and we showed that Yak1 is not required for the proper activation of the cAMP/PKA/Efg1 pathway and transcription factors necessary for the up-regulation of Yak1-dependent hypha-specific genes. Moreover, we demonstrated that deletion of *TUP1* could bypass the Yak1 requirement for filamentation. Together, our results suggest that Yak1 may function in the Tup1 pathway to govern hyphal emergence and maintenance.

MATERIALS AND METHODS

C. albicans Strains and Media

C. albicans strains used in this study are listed in Table 1. These strains were routinely grown at 30°C in YPD (1% yeast extract, 2% peptone, and 2% glucose) or in YNB (0.67% yeast nitrogen base without amino acids; Difco, Detroit, MI) with 0.4 or 2% glucose, supplemented with 20 mg/l histidine, 20 mg/l arginine, and 40 mg/l uridine when necessary. Different protocols were used to induce hyphal formation on solid medium or in liquid culture. Filamentation on plate was assessed on YPD FCS 20% (YPD, 20% fetal calf serum [Sigma-Aldrich, St. Louis, MO]), and 1.5% Bacto-agar) and on RPMI medium (1× RPMI [Invitrogen, Carlsbad, CA] buffered with 50 mM HEPES, pH 7.5, and 1.5% Bacto-agar). Cells (5×10^5) in 5 μ l of water were spotted on plates that were incubated at 37°C, under atmospheric or 5% CO₂ concentration for 2–10 d. Agar invasion was tested by washing the cells at the surface of YPD FCS 20% or RPMI plates and observing whether cells remained attached to the agar. Filamentation in embedded condition was done by embedding ≈ 100 yeast cells between two layers of YPS medium (1% yeast extract, 2% peptone, 2% saccharose, and 1% agar), and then incubating plates at 22°C for 7 d (Brown *et al.*, 1999). Colonies on solid agar were observed with an SZX9 stereomicroscope at 16–57× and photographed using a DP-12 digital system (Olympus, Melville, NY). Filamentation in liquid media was assessed by inoculating $2\text{--}5 \times 10^6$ cells ml⁻¹ of an overnight culture grown in YPD at 30°C in Lee's medium (Lee *et al.*, 1975) or YPD FCS 10% (Sigma-Aldrich) and incubation with shaking at 37°C. Filamentation was assessed at various time points. 4-Amino-1-(tert-butyl)-3-(1'-naphthylmethyl)pyrazolo-(3,4-d)-pyrimidine (1-NMPP1; Toronto Research Chemicals, North York, ON, Canada) was dissolved in dimethyl sulfoxide at 30 mM and used at the final concentrations of 1.5–5 μ M. cAMP was added to solid or liquid media at the final concentration of 5 mM. Cells were stained with 5 μ g/ml Calcofluor white for 5 min, and then they were examined on a Nikon eclipse E 600 microscope with a 40× objective under fluorescence conditions, and photographed.

C. albicans Mutant Construction

All strains constructed for this study are derivatives of BWP17 (Wilson *et al.*, 1999). The lithium-acetate procedure was used to transform *C. albicans* as described previously (Walther and Wendland, 2003). Replacement of the complete open reading frames (ORFs) of both alleles of *C. albicans* *YAK1* by polymerase chain reaction (PCR)-generated *ARG4* and *HIS1* disruption cassettes flanked by 100 base pairs of target homology region was performed through sequential transformation as described by Gola *et al.* (2003). Primers used for amplification of the *ARG4* and *HIS1* cassettes and confirmation of the deletions are listed in Table 2. Independent transformants were produced and the disruptions were verified by PCR on whole yeast cells as described previously (Gola *et al.*, 2003) and by Southern analysis using a 834-base pair PCR product (primers YakF1 and YakR1) encompassing 109 base pairs of the 5'-noncoding region and the first 725 coding nucleotides (nt) of the *YAK1* gene as a probe on EcoRI-digested genomic DNA prepared with the Yeast DNA Purification kit (Epicenter Technologies, Madison, WI). Before phenotypic analysis, if necessary, the strains were sequentially converted to histidine prototrophy or arginine prototrophy by using the *HIS1* or *ARG4* genes that had been amplified from the pFA-HIS1 or pFA-ARG4 plasmids, respectively (Gola *et al.*, 2003), and to uracil prototrophy using the *StuI*-linearized Clp10 plasmid that carries the *URA3* gene and integrates at the *RPS10* locus (Murad *et al.*, 2000).

The *C. albicans* *YAK1* gene along with 885 base pairs of the 5'-noncoding and 629 base pairs of the 3'-noncoding regions was amplified with primers YakSalI and YakEcoRV (Table 2). After digestion with SalI and EcoRV, the PCR product was inserted into XhoI, EcoRV-digested Clp10 (Murad *et al.*, 2000), yielding pCIP-YAK1. Site-directed mutagenesis of the *YAK1* gene was

Table 1. Strains used in this study

Strain	Genotype	Parent	Reference
SC5314	<i>Candida albicans</i> wild type		Gillum <i>et al.</i> (1984)
BWP17	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG</i>		Wilson <i>et al.</i> (1999)
BWP17AHU	<i>ura3::λimm434/ura3::λimm434 arg4::hisG/ARG4 his1::hisG/HIS1 RPS10/RPS10::Clp10-URA3</i>	BWP17	This study
BCA2-10	<i>ura3::λimm434/ura3::λimm434 tup1Δ::hisG/tup1Δ::hisG</i>	CAI4	Braun and Johnson (1997)
CEC356	<i>YAK1/yak1Δ::ARG4 ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG RPS10/RPS10::Clp10-URA3</i>	BWP17	This study
CEC360	<i>YAK1/yak1Δ::ARG4 ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG</i>	CEC356	This study
CEC364	<i>YAK1/yak1Δ::ARG4 ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/HIS1 RPS10/RPS10::Clp10-URA3</i>	CEC360	This study
CEC354	<i>YAK1/yak1::HIS1 ura3::λimm434/ura3::λimm434 arg4::hisG his1::hisG/his1::hisG</i>	BWP17	This study
CEC361	<i>YAK1/yak1::HIS1 ura3::λimm434/ura3::λimm434 arg4::hisG his1::hisG/his1::hisG RPS10/RPS10::Clp10-URA3</i>	CEC354	This study
CEC365	<i>YAK1/yak1::HIS1 ura3::λimm434/ura3::λimm434 arg4::hisG/ARG4 his1::hisG/his1::hisG RPS10/RPS10::Clp10-URA3</i>	CEC361	This study
CEC359	<i>yak1::HIS1/yak1::ARG4 ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG</i>	CEC356	This study
CEC358	<i>yak1::ARG4/yak1::HIS1 ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG</i>	CEC354	This study
CEC362	<i>yak1::HIS1/yak1::ARG4 ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG RPS10/RPS10::Clp10-URA3</i>	CEC359	This study
CEC363	<i>yak1::ARG4/yak1::HIS1 ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG RPS10/RPS10::Clp10-URA3</i>	CEC358	This study
CEC366	<i>yak1::HIS1/yak1::ARG4 ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG RPS10/RPS10::Clp10-URA3-YAK1</i>	CEC359	This study
CEC382	<i>yak1::HIS1/yak1::ARG4 ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG RPS10/RPS10::Clp10-URA3-yak1^{F507A}</i>	CEC359	This study
CEC399	<i>yak1::HIS1/yak1::ARG4 ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG/PCK1p::TPK1</i>	CEC359	This study
CEC396	<i>yak1::HIS1/yak1::ARG4 ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG/PCK1p::EFG1</i>	CEC359	This study
CEC989	<i>yak1::HIS1/yak1::ARG4 ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG TUP1/tup1Δ::hisG</i>	CEC359	This study
CEC990	<i>yak1::HIS1/yak1::ARG4 ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG TUP1/tup1Δ::hisG</i>	CEC989	This study
CEC991	<i>yak1::HIS1/yak1::ARG4 ura3::λimm434/ura3::λimm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG tup1Δ::hisG/tup1Δ::hisG</i>	CEC990	This study

performed using pCIP-YAK1 as a template for amplification and oligonucleotides YakA1 and YakA2 (Table 2) carrying mutations that direct the change of Phe507 into Ala in the Yak1 protein and introduce an AluI restriction site in the YAK1 ORF. After amplification, DpnI digestion was used to counterselect parental DNA. The occurrence of the mutation in derivatives of pCIP-YAK1 obtained upon *Escherichia coli* K-12 transformation was verified first by testing the presence of the AluI site and then by sequence analysis. This resulted in the identification of plasmid pCIP-YAK1^{F507A} that differs from pCIP-YAK1 by the expected nucleotide changes only. StuI-linearized pCIP-YAK1 and pCIP-YAK1^{F507A} were used to transform *C. albicans* strain CEC359, yielding strains CEC366 and CEC382, respectively (Table 1).

Plasmids pSKM28 with *TPK1* under the control of the *PCK1* promoter (Bockmuhl *et al.*, 2001) and pRC2312P-H with *EFG1* under the control of the *PCK1* promoter (Stoldt *et al.*, 1997) were kindly provided by J. Ernst (Düsseldorf Universität, Germany), and they were used to transform *C. albicans* strain CEC359 yielding strains CEC399 and CEC396, respectively. SphI-digested plasmid p383c was used to disrupt *TUP1* in the *C. albicans* *yak1Δ/yak1Δ* strain, CEC359, as described previously (Braun and Johnson, 1997). Candidate heterozygous *TUP1/tup1Δ::hisG-URA3-hisG* and *TUP1/tup1Δ::hisG* and homozygous *tup1Δ::hisG/tup1Δ::hisG* mutants were verified by PCR on whole yeast cells by using oligonucleotides hisG_{up} and Tup_V5 or hisG_{down} and Tup_V3 (Table 2) and by Southern analysis as described previously (Braun and Johnson, 1997).

Biofilm Formation

Biofilms were produced in microfermentors as described previously (Garcia-Sanchez *et al.*, 2004). Biofilms were formed on plastic slides (Thermanox; Nalge Nunc, Rochester, NY). After contact with the plastic slide, cells adherent to the surface were incubated at 37°C with a continuous flow of YNB medium containing 0.4% glucose, 0.1 g/l arginine, 0.1 g/l histidine, 0.2 g/l methionine, and 0.01 g/l uridine and air. Under these conditions, growth of the planktonic cells is minimized. After 41 h of growth, the biofilm in each microfermentor was resuspended in water, filtered, and dried 3 d at 70°C. Quantification of the biofilm dry mass was done using a precision scale, and the data presented are representative of three independent experiments that used two microfermentors per strain. Adherence of yeast cells to Thermanox was tested as previously described (Peltroche-Llacsahuanga *et al.*, 2006).

Scanning Electron Microscopy

Biofilms formed on Thermanox by strains SC5314 (YAK1/YAK1; Table 1) and CEC362 (*yak1Δ/yak1Δ*; Table 1) were analyzed with a JEOL JSM-6700F apparatus, which is an ultrahigh-resolution field emission scanning electron microscope (FESEM) equipped with a cold-field-emission gun and a strongly excited conical lens. The secondary-electron-image resolution was 1 nm at 15 kV and 2.2 nm at 1 kV. Biofilms were frozen using a Gatan Alto 2500 cryo-stage and cryo-preparation chamber (Gatan UK, Oxford, United King-

Table 2. Oligonucleotides used in this study

Oligonucleotide name	Sequence (5'-3')
YAK1.V5	AATTCTGATCCTCCCCCTTTTCC
YAK1.V3	ATATAAACCATATTCAGTCTTAAC
YAK1.5	CAAACAAAACGCAAGAGATCACATACCATTAATAATATATAAGACCAACCATTGTAACCACACAAAAGTATC ACAGTATCACCGACAAATTTATACATAGAAGCTTCGTACGCTGCAGGTC
YAK1.5	CAAACAAAACGCAAGAGATCACATACCATTAATAATATATAAGACCAACCATTGTAACCACACAAAAGTATC ACAGTATCACCGACAAATTTATACATAGAAGCTTCGTACGCTGCAGGTC
YakLocus SALI	ACGGTTCGACGAATGGAATGATAACGAATG
YakLocusECORV	GATATCGATTTATTGTTTTCTGCCAAATG
yakintF	GATCATCTGTAGGGGTATTAC
yakintR	GTTCCAGTGGTTTAGTTAATACTCG
YakF1	CCCTCAAGTTCAAACAAAACGCAAGAG
YakR1	GAGTACCAGCAATTCAGCAGGACCAGAAGG
YakF3	CATATATCACCAAGTCAATGCCTTATCG
YakR3	CTGAAGTTCAGGAAACATTGGTAAACC
YakA1	GCATAAAGAACATCTTTGTTTAGTAGCTGAAATCTTGGCATCG
YakA2	CGATGCCAAGATTTTCAGTACTAAACAAAAGATGTTCTTTATG
hisG_up	CCGGGGGCTTTCTCATGCGTTTCATGCACCA
Tup_V5	TGTGATAACTAGCCCCAACACGAACTAAT
hisG_down	GATGGAGTAAAGACCATGAGCTTCAATACC
Tup_V3	CGTAACAACCCACCATCAATGTACTCTGCT
HWP1F	CTCCAGCTGGCTCAAGTGGT
HWP1R	TGGCAGATGGTTGCATGAGT
HYR1F	CTCCGGTTCAGGCTCACAAT
HYR1R	CGTTTGATCCGTTACCAGCA
EFT3F	CAAGAAATGTCCATCTGCTCAATC
EFT3R	TGTTTAGTTTTAACCCCTTCCAAGA
BCR1F	CTTCAGCAGCTTCATTAACACCTA
BCR1R	TCTTGGATCAGGTGTACTTTTCAA
TEC1F	ACCTAATATGTATGAGAACGGCAAC
TEC1R	TAAGCACCAAGTAGGTACATGTGAAA
ALS3F	AATGGTCCTTATGAATCACCATCTACTA
ALS3R	GAGTTTTTCATCCATCTTGATTTCACAT
RBT5F	GCCAGAATGTGCCAAAGAAT
RBT5R	CACCAGCAAATTGAGGCATA

dom) dedicated for use in FESEM. First, samples were frozen in slush nitrogen and cryo-transferred under vacuum to the preparation chamber for ice sublimation and metal shadowing with argon. Material was then transferred (under secondary vacuum) from the Gatan preparation chamber to the scanning electron microscope (SEM) stage for sample observation. The working temperature with the SEM stage module was -110°C . SEM working conditions were as follows: accelerating voltage, 1 or 2 kV; probe current, 30 pA; semi-in-lens secondary electron image; and working distance 21 nm.

Transcriptome Analysis by Microarray

Total RNA was prepared from 70-ml cultures of strain SC5314 (Table 1, WT) and CEC362 (Table 1, *yak1Δ/yak1Δ*). Saturation cultures grown in YPD were diluted to 2×10^6 cells ml^{-1} and grown for 1 h in YPD at 30°C (yeast phase) or grown for 1 h in Lee's medium at 37°C (hyphal phase). Total RNA was isolated using RNeasy according to the manufacturer's instructions (QIAGEN, Valencia, CA). Total RNA samples (5 μg) were indirectly labeled using Atlas PowerScript Fluorescent Labeling kit (Clontech, Mountain View, CA) with 1 μg of oligo(dT) (Invitrogen), according to the conditions recommended by the manufacturer. cDNAs were coupled with cyanines by using Cy3 Mono-Reactive Dye or Cy5 Mono-Reactive Dye (GE Healthcare, Chalfont St. Giles, United Kingdom). Fluorescent cDNAs were then purified with the Fluorotrap matrix (Clontech). Equal quantities of Cy3- and Cy5-labeled cDNAs were mixed and concentrated by Microcon YM-30 (Millipore, Billerica, CA). Purified cDNAs were hybridized with microarrays containing ≈ 6000 *C. albicans* probes representing nearly the whole genome according to the conditions recommended by the manufacturer (Eurogentec, Seraing, Belgium). Arrays were scanned with an Axon 4000a scanner with fixed photomultiplier tube (550 for Cy3 and 650 for Cy5). Four comparisons were performed: hyphal phase wild-type cells versus yeast phase wild-type cells; hyphal phase mutant cells versus yeast phase mutant cells; hyphal phase wild-type cells versus hyphal phase mutant cells; and yeast phase wild-type cells versus yeast phase mutant cells. For each comparison, two biological replicates were used and each biological replicate was subjected to technical replicates with dye-swaps. Data were acquired and analyzed by Genepix Pro5.0 (Axon Instruments,

Foster City, CA). Data normalization using Lowess and analysis were performed using GeneSpring (Silicon Genetics, Redwood City, CA). Raw data are available at <http://www.galarfungail.org/data/Goyard/data.htm>. Normalized data are available in Supplemental Table 15. The p values for differentially expressed genes between the compared strains or conditions were adjusted with Benjamini-Hochberg's transformation. A list of genes that show statistically significant >3 -fold differences in the hyphal phase wild-type cells versus hyphal phase mutant cells or yeast phase wild-type cells versus yeast phase mutant cells comparisons ($p < 0.01$) is available in Supplemental Table 25. A list of genes that show statistically significant >3 -fold differences in the hyphal phase wild-type cells versus yeast phase wild-type cells comparison ($p < 0.01$) along with corresponding ratio and p values for the three other comparisons is available in Supplemental Table 35.

Quantitative Reverse Transcription (RT)-PCR

Total RNA was isolated from cultures of strain CEC362 (*yak1Δ/yak1Δ*), CEC366 (*yak1Δ/yak1Δ/YAK1*), and BWP17AHU (*YAK1/YAK1*) that had been grown to saturation in YPD, diluted to 2×10^6 cells ml^{-1} , and grown for 0, 15, 30, 60, or 180 min in Lee's medium at 37°C . Total RNA was isolated using RNeasy (QIAGEN) including a first step of DNase treatment according to the manufacturer's instructions. Purified RNA was DNase digested a second time using the DNA-free kit (Ambion, Austin, TX) to ensure the absence of genomic DNA contamination.

All RNA samples were verified as DNA free by using them directly as template in a PCR assay. Lack of a PCR product in the DNase-treated sample indicated that products amplified in the subsequent assay were not derived from trace amounts of genomic DNA. RNA concentration was measured spectrophotometrically and 500 ng from each sample was used for cDNA synthesis with the iScript kit according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Quantitative (q)RT-PCR was carried out on an iCycler (Bio-Rad). Oligonucleotide primers were designed with OligoPerfect Designer (Invitrogen), and they were 22–25 bases (Table 2). Primer pairs had melting temperatures of 60°C , and they resulted in amplicons of 120–150 base pairs. All primer pairs produced products of the expected sizes. For qRT-PCR,

ABsolute* QRT-PCR (ABgene, Epsom, Surrey, United Kingdom) was used according to the manufacturer's recommendations. Before transcript quantification, efficiency values were determined for all primer pairs, efficiencies of 90% or greater were observed. Each reaction mixture contained a 200 nM concentration of each primer and 10 μ l of cDNA product diluted 1:50. Each sample was run in duplicates and between duplicates the variation of the two independent CT was <0.5. Reactions were run in sealed 96-well plates (ABgene) by using recommended cycle times and temperatures. Relative expression levels were calculated using the delta-delta Ct method, with *C. albicans* translation elongation factor *EFT3* transcripts as standard. The relative expression was calculated as $2^{(Ct \text{ target yeast} - Ct \text{ EFT3 yeast}) - (Ct \text{ target hyphae} - Ct \text{ EFT3 hyphae})}$. All the data were normalized to the expression level of cells of strain CEC366 (*yak1Δ/yak1Δ/YAK1*) at time zero.

Animal Models of Infection

The virulence of *C. albicans* strains SC5314 (*YAK1/YAK1*), CEC362 (*yak1Δ/yak1Δ*), and CEC366 (*yak1Δ/yak1Δ/YAK1*) in mouse models of hematogenously disseminated and oropharyngeal candidiasis were determined as described previously (Cornet *et al.*, 2005; Park *et al.*, 2005). In the disseminated candidiasis models, the mice were infected via the tail vein. Two different inocula were tested (2×10^5 and 2×10^6 organisms per mouse), and the mice were monitored for survival for 14 d. In the oropharyngeal candidiasis model, the mice were immunosuppressed with cortisone acetate, and then they were inoculated orally with each of the strains. After 5 d of infection, the oral fungal burden of the mice was determined by quantitative culture. In all experiments, each strain of *C. albicans* was tested in seven mice.

RESULTS

Characterization of the *C. albicans* *YAK1* Gene

A search of assembly 19 of the *C. albicans* genome sequence (Jones *et al.*, 2004) was performed to identify ORFs encoding proteins homologous to *S. cerevisiae* and *C. glabrata* Yak1. Two contiguous open reading frames, namely, orf19.148 and orf19.147, were identified (Figure 1). In the course of assembly 19 annotation (Braun *et al.*, 2005), these ORFs have been merged through an artificial frame-shift to generate a unique ORF, referred to as orf19.147 (Figure 1). The same frame-shift was observed for the two allelic ORFs, namely, orf19.7788 and orf19.7787 (Figure 1). The region encompassing this frame-shift was amplified from genomic DNA of *C. albicans* strain SC5314 and the amplification product directly sequenced. The resulting sequence (GenBank accession no. EU443248) differed from that in assembly 19 at six positions, yielding a unique 2427-nt ORF (Figure 1). This ORF was identical to that of *YAK1* in *C. albicans* WO-1 (http://www.broad.mit.edu/annotation/genome/candida_albicans/Home.html), except for several microsatellite-like regions. When the deduced amino acid sequence was used to search the *S. cerevisiae* or *C. glabrata* proteome, it identified the two Yak1 pro-

teins, suggesting that it is orthologous to *S. cerevisiae* and *C. glabrata* Yak1.

Figure 1 shows a schematized organization of the *C. albicans* Yak1 protein, similar to that observed for *S. cerevisiae* and *C. glabrata* Yak1 and other Yak1 proteins identified in different yeast genomes (data not shown). Yak1 proteins share a poorly conserved low-complexity amino-terminal domain of ≈ 400 amino acids. In species of the CTG clade of Saccharomycotina (Fitzpatrick *et al.*, 2006), this domain includes two well-conserved regions (Figure 1). The carboxy-terminal domain of Yak1 proteins harbors a conserved (>60% identity) catalytic domain typical of Ser/Thr protein kinases. This probable catalytic domain is separated by a low-complexity region from a second but partial Ser/Thr protein kinase catalytic domain. Four conserved putative PKA phosphorylation sites were identified in the *C. albicans* Yak1 protein, three sites (K₁₃₆RLS, R₂₀₉RMS, and R₂₅₈RSS) in the amino-terminal low-complexity region, and one site (R₃₅₁RCS) in a domain that is conserved among all yeast Yak1 proteins and located upstream of the catalytic domain (Figure 1). The corresponding four PKA phosphorylation sites in the *S. cerevisiae* protein have been shown to be phosphorylated (Zappacosta *et al.*, 2002). The conserved tyrosine residue that is subject to autophosphorylation in other kinases of the DYRK family is located at position 588. Low-complexity regions in the *C. albicans* Yak1 proteins show intraspecific variations at the level of amino acid repeats (data not shown).

The *C. albicans* *YAK1* Gene Is Necessary for Biofilm Formation

C. albicans is an obligate diploid and auxotrophies have been associated with altered virulence (Lay *et al.*, 1998; Brand *et al.*, 2004). To evaluate gene function in this species, it is necessary to perform two transformations to successively inactivate both alleles by gene replacement. It is also important to correct all auxotrophies. Furthermore, this organism has significant genomic instability, which can be induced by transformation (Selmecki *et al.*, 2005). Therefore, it is necessary to reintegrate a functional wild-type allele of the gene of interest back into the homozygous null mutant to ensure that the wild-type phenotype is restored and that the observed phenotype is not the result of a secondary mutation that was inadvertently introduced during transformation (Noble and Johnson, 2007).

Figure 1. Organization of the *YAK1* gene and gene product. The *C. albicans* *YAK1* gene is divided in two ORFs in assembly 19 of the *C. albicans* strain SC5314 genome, namely orf19.148 and orf19.147, that have been merged through an artificial nucleotide duplication (gray background). orf19.148 and orf19.147 are allelic to orf19.7788 and orf19.7787, respectively. Sequencing of the junction between orf19.147 and orf19.148 indicated sequencing errors and showed that *YAK1* encoded a 2427-nt ORF. The Yak1 protein contains a catalytic domain typical of Ser/Thr protein kinases (amino acids [aa] 429–640; highlighted in black) and a partial Ser/Thr catalytic domain (aa 738–801).

Other regions are poorly conserved and of low complexity with single amino acid repeats except for three domains that are conserved in all Yak1 proteins (dark gray) or in Yak1 proteins of yeast species of the CTG clade only (light gray). Putative PKA phosphorylation sites at positions 136, 209, 258, and 351 are indicated by asterisks. The conserved tyrosine residue (Y) at position 588 that is subject to autophosphorylation in *S. cerevisiae* and the phenylalanine residue at position 507 that was converted into an alanine residue to yield a 1-NMPP1-sensitive *yak1* allele (F $\rightarrow$ A) are shown. Nucleotide positions are indicated in regular font, whereas amino acid positions are shown in italics.

To investigate the function of *C. albicans* *YAK1*, strains deleted for one allele (CEC364, *yak1Δ::ARG4/YAK1* and CEC365, *yak1Δ::HIS1/YAK1*; Table 1) or two alleles of *YAK1* (CEC362, *yak1Δ::HIS1/yak1Δ::ARG4* and CEC363, *yak1Δ::ARG4/yak1Δ::HIS1*; Table 1) were constructed through targeted replacement of the *YAK1* ORF by the *HIS1* or *ARG4* genes (Gola *et al.*, 2003). Replacement of the *YAK1* ORF and its absence in homozygous null mutants was confirmed by PCR and Southern blot analysis (data not shown). All strains were rendered prototroph through reconstitution of the *HIS1* or *ARG4* loci or integration at the *RPS10* locus of the *URA3* gene carried by the *Clp10* plasmid (Murad *et al.*, 2000). In addition, a derivative of strain CEC359 carrying a wild-type copy of *YAK1* at the *RPS10* locus was generated and is referred to as CEC366 (*yak1Δ::HIS1/yak1Δ::ARG4 RPS10/RPS10::Clp10-URA3-YAK1*; Table 1). No difference in growth rates were observed when the CEC362 (*yak1Δ./yak1Δ*) and CEC366 (*yak1Δ/yak1Δ/YAK1*) were grown in rich medium at 30°C or in minimal medium at 30°C or 37°C, indicating that *YAK1* is not necessary for normal planktonic growth of *C. albicans* yeast cells (data not shown).

C. glabrata yak1 null mutants showed a fivefold reduction in their ability to form biofilms in a microtiter plate assay for biofilm formation (Iraqi *et al.*, 2005). Whereas wild-type *C. glabrata* formed biofilms consisting of colonies of up to 100 cells adhering to plastic, biofilms of the *C. glabrata yak1* null mutants consisted of single cells or colonies of <10 cells, with no alteration of initial adherence. Therefore, we tested the ability of the different *C. albicans yak1Δ* mutants to form biofilms. For this purpose, a continuous-flow microfermenter model (Garcia-Sanchez *et al.*, 2004) was used and the biofilm biomass formed after 41 h of biofilm growth at 37°C was measured. Data presented in Figure 2A show that strain CEC362 that lacked the two *YAK1* alleles was impaired for biofilm formation, with a biomass representing ≈20% of that of a biofilm formed by *YAK1* wild-type *C. albicans* strains.

Figure 2. The *C. albicans* *YAK1* gene is necessary for biofilm formation. (A) Biofilms of *C. albicans* strains SC5314 (*YAK1/YAK1*), BWP17AHU (*YAK1/YAK1*), CEC364 (*YAK1/yak1Δ*), CEC362 (*yak1Δ/yak1Δ*) and CEC366 (*yak1Δ/yak1Δ/YAK1*) were grown on Thermanox plastic slides in a microfermentor model under a continuous flow of YNB medium for 41 h at 37°C. Dry biomasses of the biofilms were quantified. Values correspond to the mean of the data obtained for two microfermentors and are representative of three separate experiments. (B) Scanning electron microscopy of cryo-fixed biofilms formed by *C. albicans* strain SC5314 (*YAK1/YAK1*) and CEC362 (*yak1Δ/yak1Δ*). Two magnifications are shown. Matrix material in the wild-type biofilm can be seen as filaments linking cells in the bottom left panel.

Similar results were obtained with an independent *yak1Δ/yak1Δ* mutant strain (CEC363; data not shown). The heterozygous *YAK1/yak1Δ* strain CEC364 as well as the complemented *yak1Δ/yak1Δ/YAK1* strain CEC366 exhibited a biofilm biomass close to that observed for the SC5314 or BWP17AHU control strains (Figure 2A), indicating that the biofilm formation defect of the CEC362 strain was a consequence of *YAK1* inactivation.

This defect in biofilm formation might be due to a defect in adherence of the mutant yeast cells to the plastic surface used to grow *C. albicans* biofilms. Adherence to Thermanox plastic of the SC5314 wild-type and CEC362 *yak1Δ/yak1Δ* strains were compared. No significant difference could be observed indicating that the defect in biofilm formation was not due to a lack of initial adherence to the plastic surface (data not shown). Cryo-scanning electron microscopy was used to assess the architecture of the biofilm formed by the *yak1Δ/yak1Δ* null mutant. Although the biofilm formed by *C. albicans* wild-type strain SC5314 consisted of a very dense and organized structure of intertwined long hyphal elements that seemed embedded in a matrix, such a structure was not observed for the CEC362 *yak1Δ/yak1Δ* mutant strain and only microcolonies consisting mostly of yeast or pseudohyphal cells and apparently devoid of matrix material could be seen (Figure 2B).

The *C. albicans* *YAK1* Gene Is Necessary for Hyphal Differentiation

C. albicans mutants with defects in filamentation are unable to form a dense and mature biofilm (Ramage *et al.*, 2002; Richard *et al.*, 2005). Because the thin biofilm formed by the CEC362 *yak1Δ/yak1Δ* mutant strain consisted mostly of pseudohyphal cells despite the presence of some hyphae (Figure 2B), we hypothesized that the defect in biofilm formation of the mutant strain could reflect a general defect in morphogenesis. The ability of the different *C. albicans yak1* mutants to produce hyphae was first examined on solid agar media under Efg1-dependent—YPD FCS 20% and RPMI—and Efg1-independent—embedded YPS—hypha-inducing conditions. Results presented in Figure 3A show that the CEC362 *yak1Δ/yak1Δ* strain exhibited a strong filamentation defect in all three media. Whereas the wild-type strain formed wrinkled colonies (pseudohyphal and mycelial cells) on YPD FCS 20% and developed peripheral long hyphae, strain CEC362 formed smooth colonies and peripheral hyphae could not be detected. Reintegration of a wild-type copy of *YAK1* resulted in a wrinkled colony but with fewer peripheral hyphae than wild type (Figure 3A). On RPMI medium, the *yak1Δ/yak1Δ* strain showed some filamentation, but uniform filamentation around the colony was never seen, and filaments seemed shorter than those observed for the wild-type or reconstituted strains (Figure 3A). Moreover, agar invasion was abolished on YPD FCS 20% and RPMI solid medium when the *YAK1* gene was inactivated (data not shown). In embedded conditions, the wild-type strain formed homogeneous fluffy, furry colonies, whereas no filamentation was observed with strain CEC362 (Figure 3A). These results indicated that the *C. albicans* *YAK1* gene was necessary for efficient filamentation on different solid media.

The ability of the *yak1Δ/yak1Δ* strain to form filaments was also assessed in liquid media under Efg1-dependent hypha-inducing conditions—Lee's medium and YPD FCS 10%. Results presented in Figure 3A show that the *YAK1* gene was also necessary for efficient filamentation in Lee's medium, because strain CEC362 was unable to form true hyphae with nearly 90% cells forming pseudohyphae. In contrast, when yeast cells of the *yak1Δ/yak1Δ* mutant were

Figure 3. The *C. albicans* *YAK1* gene is necessary for hyphal differentiation in vitro. (A) *C. albicans* strains BWP17AHU (*YAK1/YAK1*), CEC362 (*yak1Δ/yak1Δ*), and CEC366 (*yak1Δ/yak1Δ/YAK1*) were plated on different solid medium or grown in liquid Lee's medium. YPD FCS 20% and RPMI plates were incubated at 37°C for 7 d. Cultures in embedded conditions were incubated at 22°C for 7 d. Cultures in Lee's medium were at 37°C for 4 h. (B) Log-phase cells of *C. albicans* strains SC5314 (*YAK1/YAK1*) and CEC362 (*yak1Δ/yak1Δ*) were incubated in liquid YPD FCS 10% for 2.5 and 6 h. Representative pictures are shown. (C) Periodic acid Schiff staining of mice tongues after 5 d of oro-pharyngeal infection with *C. albicans* strains BWP17AHU, CEC362, and CEC366. Representative pictures are shown at different magnitudes (100×, 200×, and 400×).

exposed to 10% FCS, they originally underwent hyphal differentiation in a manner indistinguishable of the wild-type strain, but they eventually exhibited abnormal patterns of filamentation, including lateral and apical budding (Figure 3B). These results indicated that the Yak1 protein is dispensable for the initiation of hyphal morpho-

genesis in response to serum but necessary for hyphal maintenance in this condition.

A *C. albicans* Strain with a 1-NMPP1-sensitive *yak1* Allele Shows Conditional Hyphal Emergence and Elongation Defects

Shokat and colleagues have shown that the replacement in the ATP binding pocket of protein kinases of a conserved bulky residue—the so-called gate-keeper residue—by a short chain amino acid such as glycine or alanine results in a kinase that is sensitive to inhibition by derivatives of the tyrosine kinase inhibitor PP1 (Bishop *et al.*, 2000; Bishop *et al.*, 2001). We used this chemical genetics strategy to generate a *C. albicans* strain with conditional Yak1 function. On the basis of an alignment of the Yak1 amino acid sequence with sequences in the kinase database available at <http://sequoia.ucsf.edu/ksd>, a mutation that increased the size of the ATP binding pocket of Yak1 was introduced by substituting a phenylalanine at position 507 with an alanine (Figure 1A).

A copy of the *yak1*^{F507A} allele was introduced at the *RPS10* locus in the CEC359 *yak1Δ/yak1Δ* strain. As shown in Figure 4A, the resulting CEC382 *yak1Δ/yak1Δ/yak1*^{F507A} strain showed wild-type filamentation when incubated in Lee's medium, suggesting that the F507A mutation does not alter the activity of the Yak1 kinase in the absence of PP1 derivatives. In contrast, when 1.5 μM 1-NMPP1 was added to Lee's medium, hyphal differentiation of the *yak1Δ/yak1Δ/yak1*^{F507A} strain was abolished, whereas this drug had no impact on the filamentation of a *C. albicans* wild-type strain (Figure 4A). Addition of 5 μM 1-NMPP1 to RPMI 1640 agar plates inhibited filamentation and agar invasion of the *yak1Δ/yak1Δ/yak1*^{F507A} strain, but it had no effect on the filamentation and agar invasion of a *yak1Δ/yak1Δ/YAK1* strain (data not shown).

We took further advantage of the conditional *yak1* allele to probe the role of Yak1 during hyphal elongation. Addition of 1-NMPP1 at different time points after transfer of yeast cells of the CEC382 *yak1Δ/yak1Δ/yak1*^{F507A} strain into Lee's medium resulted in an arrest of hyphal elongation and promoted lateral and apical bud formation from hyphae as illustrated in Figure 4B. Together, these results indicated that the Yak1 protein kinase activity is necessary for the initiation of hyphal formation as well as for hyphal elongation and maintenance.

The *YAK1* Gene Is Not Required for *C. albicans* Virulence

The ability to make the transition from the yeast to hyphal forms has been considered a major requirement for *C. albicans* virulence (Saville *et al.*, 2003). Our observation that the *C. albicans* *YAK1* gene is required for hyphal emergence and/or elongation in vitro led us to examine whether it is also required for *C. albicans* virulence. The virulence of *C. albicans* strains SC5314 (*YAK1/YAK1*), CEC362 (*yak1Δ/yak1Δ*), and CEC366 (*yak1Δ/yak1Δ RPS10/RPS10::YAK1*) was examined in murine models of systemic candidiasis (Cornet *et al.*, 2005) and oropharyngeal candidiasis (Park *et al.*, 2005). Similar mortality rates were observed for the three strains upon systemic candidiasis (data not shown). In the murine model of oropharyngeal candidiasis no significant difference in oral fungal burden and histopathology of oral tissues were observed between the three strains (Figure 3C; data not shown). Together, these data indicated that *YAK1* was dispensable for virulence during systemic and oropharyngeal candidiasis mediated by *C. albicans*.

Figure 4. The conditional *yak1^{F507A}* allele reveals a requirement for the Yak1 protein kinase at the onset and for maintenance of hyphal growth. (A) *C. albicans* strains BWP17AHU (*YAK1/YAK1*) and CEC382 (*yak1Δ/yak1Δ/yak1^{F507A}*) were grown in liquid Lee's medium in the presence or absence of 1.5 μM 1-NMPP1 for 4 h at 37°C. (B) After growth of *C. albicans* strain CEC382 (*yak1Δ/yak1Δ/yak1^{F507A}*) for 3 h at 37°C in liquid Lee's medium, 1.5 μM 1-NMPP1 was added and cells were observed at the indicated time. Representative pictures of calcofluor-stained cells are shown and highlight the pseudohyphal morphology of branching cells.

The Yak1 Kinase Is Necessary for the Up-Regulation of a Subset of Hypha-induced Genes

For further insight into the function of Yak1 in hyphal morphogenesis, transcript profiling of *C. albicans* strains SC5314 (*YAK1/YAK1*) and CEC362 (*yak1Δ/yak1Δ*) cultivated 1 h in YPD at 30°C, i.e., in the yeast phase, or incubated 1 h in Lee's medium at 37°C, i.e., in the hyphal phase, was performed. Comparison of the transcript profiles obtained for each strain in the two culture conditions or for both strains cultivated in the same condition was achieved.

Data presented in Figure 5A and Supplemental Table 2S show that Yak1 functionality was associated with greater or equal to threefold up-regulation of 42 genes and lesser or equal to threefold down-regulation of 18 genes in the hyphal

phase compared with wild-type cells. In the yeast phase, Yak1 functionality was associated with ≥3-fold up-regulation of nine genes and ≤3-fold down-regulation of six genes, the expression of most of which was similarly altered in the hyphal phase (Figure 5A).

That inactivation of *YAK1* resulted in alterations of gene expression mostly in the hyphal phase suggested that Yak1 might be involved in regulating the differential expression of genes during the yeast-to-hypha transition. Therefore, we investigated whether the 42 genes that show Yak1-dependent increased expression in the hyphal phase were also up-regulated upon the yeast-to-hypha transition in wild-type *C. albicans*. Data presented in Supplemental Table 3S show that 178 genes were up-regulated by more than threefold when *C. albicans* strain SC5314 was induced to undergo the yeast-to-hypha transition in Lee's medium. Genes that have been previously described as being up-regulated upon hyphal growth were found among these 178 genes, including *ALS3*, *HWP1*, *HYR1*, *ECE1*, *IHD1*, *HGC1*, *RBT1*, *RBT5*, *RBT4*, *SOD5*, *SNZ1*, *PHR1*, *SEC24*, *CHA1*, and *RD11* (Birre *et al.*, 1993; Hoyer *et al.*, 1998; Staab and Sundstrom, 1998; Sharkey *et al.*, 1999; Braun *et al.*, 2000; Murad *et al.*, 2001a; Nantel *et al.*, 2002; Martchenko *et al.*, 2004; Zheng *et al.*, 2004). As shown in Figure 5B, of the 42 genes that show Yak1-dependent increased expression in the hyphal phase, 32 were also found among the 178 yeast-to-hypha up-regulated genes. A list of these 32 genes is shown in Table 3, and it includes the hypha-specific genes *ALS3*, *HYR1*, *HWP1*, *ECE1*, *IHD1*, *HGC1*, and *RBT1*. The remaining 146 yeast-to-hypha up-regulated genes showed less than threefold differences between wild-type and mutant hyphae (see Table 4 for examples and Supplemental Table 3S). Consistently, most of these 146 genes were up-regulated when the *C. albicans* strain CEC362 (*yak1Δ/yak1Δ*) was induced to undergo the yeast-to-hypha transition in Lee's medium (see Table 4 for examples and Supplemental Table 3S). Together, these data indicated that although up-regulation of the group of 32 genes in response to hypha-inducing conditions is dependent on the function of Yak1, this is not the case for the group of 146 genes.

Further examination of the group of 32 genes showed that although several genes did not show increased expression when the *yak1Δ/yak1Δ* strain was induced to undergo the yeast-to-hypha transition (see for examples *HYR1*, *HGC1*, *CFL11*, and *CAS4*; Table 3), others had expression levels significantly higher in hyphal than yeast cells of the mutant strain (see for examples *ECE1*, *ALS3*, *HWP1*, and *HGT1*;

Figure 5. Overlaps between the significant sets of differentially expressed genes. (A) Intersection between the sets arising from wild-type versus *yak1Δ* mutant comparisons in the yeast and hyphal phases. Yak1-up hypha, genes up-regulated in the wild-type hypha versus mutant hypha comparison; Yak1-up yeast, genes up-regulated in the wild-type yeast versus mutant yeast comparison; Yak1-down hypha, genes down-regulated in the wild-type hypha versus mutant hypha comparison; and Yak1-down yeast, genes down-regulated in the wild-type yeast versus mutant yeast comparison. (B) Intersection between the set of 42 genes up-regulated in the wild-type hypha versus *yak1Δ* hypha comparison (Yak1-up hypha) and the set of 178 genes up-regulated in the wild-type hypha versus wild-type yeast comparison. The number of genes that show greater or equal to threefold difference ($p < 0.01$) between the two conditions are shown and the data for gene sets defined using a twofold cut-off are indicated in parentheses.

Table 3. *C. albicans* genes whose up-regulation during the yeast-to-hypha transition is dependent upon the function of Yak1

Systematic name	Gene name	Function ^a	Tup1-regulated ^b	WT Y → H ^c	Hypha WT vs. <i>yak1Δ</i> ^c	<i>yak1Δ</i> Y → H ^c	Yeast WT vs. <i>yak1Δ</i> ³
Hypha-specific genes							
orf19.3374	<i>ECE1</i>	Protein composed of eight 34-residue repeats; expression specific to hyphae	+	41.4	57.6	17.1	43.4
orf19.4975	<i>HYR1</i>	Nonessential, GPI-anchored, predicted cell wall protein	+	18.3	25.6	1.0	1.4
orf19.1816	<i>ALS3</i>	Adhesin; ALS family; role in epithelial adhesion, endothelial invasiveness	+	15.3	46.0	2.5	12.1
orf19.1321	<i>HWP1</i>	Hyphal cell wall protein; covalently crosslinked to epithelial cells by host transglutaminase	+	14.2	55.6	6.5	46.9
orf19.5760	<i>IHD1</i>	Putative GPI-anchored protein of unknown function	+	8.2	20.3	1.4	4.1
orf19.6028	<i>HGC1</i>	Hypha-specific G1 cyclin-related protein involved in regulation of hyphal morphogenesis	+	6.6	11.4	1.1	1.6
orf19.1327	<i>RBT1</i>	Putative cell wall protein with similarity to Hwp1p, required for virulence	+	4.1	23.8	1.6	10.7
Transcription factors and other regulatory proteins							
orf19.4056	<i>GAT2</i>	Putative DNA-binding transcription factor; similar to <i>S. cerevisiae</i> Gat2p	+	10.9	8.5	5.0	4.0
orf19.1822		Putative transcription factor with zinc cluster DNA-binding motif similar to <i>S. cerevisiae</i> Ume6p	+	8.0	19.0	0.9	2.8
orf19.6705		Protein described as a putative guanyl nucleotide exchange factor with Sec7 domain	+	4.7	3.4	2.1	1.6
orf19.1693	<i>CAS4</i>	Protein similar to <i>S. cerevisiae</i> Tao3p		4.0	3.4	1.4	1.2
orf19.5102	<i>PLB5</i>	Putative phospholipase B		3.7	3.6	1.1	1.0
orf19.1702	<i>ARF3</i>	Transcription is filament induced; Tup1p regulated	+	3.1	3.2	1.2	1.4
Stress response							
orf19.85	<i>GPX2</i>	Similar to glutathione peroxidase	+	7.9	5.6	3.1	2.7
Transporters							
orf19.4527	<i>HGT1</i>	High-affinity glucose transporter, member of major facilitator superfamily	+	12.7	3.2	6.0	1.9
orf19.3668	<i>HGT2</i>	Putative glucose transporter of the major facilitator superfamily	+	9.3	3.3	4.4	1.6
Iron metabolism							
orf19.701	<i>CFL11</i>	similar to ferric reductase Fre10p	+	4.9	7.4	1.0	1.4
Unknown function							
orf19.7350		Predicted ORF in assemblies 19 and 20	+	17.3	3.5	2.7	0.7
orf19.2457		Predicted ORF in assemblies 19 and 20		7.8	23.9	0.8	2.5
orf19.2210		Predicted ORF in assemblies 19 and 20	+	7.7	7.7	1.2	1.3
orf19.7456		Predicted ORF in assemblies 19 and 20		7.0	4.0	2.2	0.9
orf19.6864		Predicted ORF in Assemblies 19 and 20		6.3	3.7	1.4	1.2
orf19.2691		Predicted ORF in Assemblies 19 and 20	+	5.5	4.5	1.3	1.3
orf19.815		Predicted ORF in assemblies 19 and 20		5.3	3.7	1.8	1.4
orf19.4044	<i>MUM2</i>	Predicted ORF in assemblies 19 and 20		5.2	3.6	1.5	1.7
orf19.1277		Predicted ORF in assemblies 19 and 20	+	4.9	3.5	2.2	1.6
orf19.1120	<i>FAV2</i>	Predicted ORF in assemblies 19 and 20	+	4.3	3.1	1.8	1.3
orf19.7305		Predicted ORF in assemblies 19 and 20		3.7	3.0	1.0	0.9
orf19.4590		Predicted ORF in assemblies 19 and 20; regulated by Nrg1p		3.4	9.3	0.8	2.6
orf19.6027		Predicted ORF in assemblies 19 and 20		3.2	3.6	1.3	1.3
orf19.1203.1		ORF predicted by Annotation Working Group		3.2	3.0	1.4	1.3
19766.1		Predicted ORF absent of assemblies 19 and 20		3.0	8.6	0.9	4.0

^a Functional assignment was based on annotation available at the *Candida* Genome Database (Arnaud *et al.*, 2007).

^b + indicates Tup1-negative regulation as inferred from transcript profiling data available in Garcia-Sanchez *et al.* (2005) and Kadosh and Johnson (2005).

^c Ratio between expression levels are indicated for the following comparisons: WT Y → H, wild-type hypha-induced cells versus wild-type yeast cells; hypha WT versus *yak1Δ*, wild-type hypha-induced cells versus *yak1Δ* hypha-induced cells; *yak1Δ* Y → H, *yak1Δ* hypha-induced cells versus *yak1Δ* yeast cells; yeast WT versus *yak1Δ*, wild-type yeast cells versus *yak1Δ* yeast cells. Ratios are indicated in italics when the *t* test *p* value is >0.01.

Table 4. Examples of *C. albicans* genes whose yeast-to-hypha up-regulation is independent of the function of Yak1

Systematic name	Gene name	Function ^a	Tup1-regulated ^b	WT Y → H ^c	Hypha WT vs. <i>yak1Δ</i> ^c	<i>yak1Δ</i> Y → H ^c	Yeast WT vs. <i>yak1Δ</i> ^c
orf19.5636	<i>RBT5</i>	GPI-anchored cell wall protein	+	60.0	1.3	22.4	0.4
orf19.7114	<i>CSA1</i>	Surface antigen on elongating hyphae and buds		59.5	1.4	31.8	0.8
orf19.2947	<i>SNZ1</i>	Similar to stationary phase-related proteins		58.5	0.8	9.8	0.2
orf19.5635	<i>PGA7</i>	Protein described as a putative precursor of a hyphal surface antigen	+	58.4	1.1	28.3	0.8
orf19.7585	<i>INO1</i>	Inositol-1-phosphate synthase	+	57.4	2.1	24.2	0.6
orf19.5674	<i>PGA10</i>	Plasma membrane protein involved in heme-iron use	+	55.7	1.3	20.1	0.4
orf19.1264	<i>CFL2</i>	Iron use protein	+	53.6	0.8	19.1	0.3
orf19.3117		Predicted ORF in Assemblies 19 and 20	+	52.6	1.3	20.3	0.4
orf19.4445		Predicted ORF in Assemblies 19 and 20		28.4	0.7	24.2	1.0
orf19.7469	<i>ARG1</i>	Similar to argininosuccinate synthase	+	24.0	2.9	6.1	0.9
orf19.4784	<i>CRP1</i>	Copper transporter of the plasma membrane	+	22.9	1.1	18.1	1.3
orf19.7112	<i>FRP2</i>	Protein described as ferric reductase		21.1	0.6	17.8	0.6
orf19.2877	<i>PDC11</i>	Protein similar to pyruvate decarboxylase		17.9	1.3	7.5	0.9
orf19.2432	<i>HAC1</i>	Putative transcription factor with bZIP DNA-binding motif		14.9	2.9	6.0	1.3
orf19.5634	<i>FRP1</i>	Protein similar to ferric reductases	+	14.1	0.9	11.3	0.8
orf19.5908	<i>TEC1</i>	TEA/ATTS transcription factor involved in regulation of hypha-specific genes		8.1	2.0	6.0	1.7
orf19.723	<i>BCR1</i>	Transcription factor required for wild-type biofilm formation		2.8	1.3	1.9	1.0

^a Functional assignment was based on annotation available at the *Candida* Genome Database (Arnaud *et al.*, 2007).

^b + indicates Tup1-negative regulation as inferred from transcript profiling data available in Garcia-Sanchez *et al.* (2005) and Kadosh and Johnson (2005).

^c Ratio between expression levels are indicated for the following comparisons: WT Y → H, wild-type hypha-induced cells versus wild-type yeast cells; hypha WT versus *yak1Δ*, wild-type hypha-induced cells versus *yak1Δ* hypha-induced cells; *yak1Δ* Y → H, *yak1Δ* hypha-induced cells versus *yak1Δ* yeast cells; yeast WT versus *yak1Δ*, wild-type yeast cells versus *yak1Δ* yeast cells. Ratios are indicated in italics when the *t* test *p* value is >0.01.

Table 3). For these latter genes, we observed that 1) the activation ratio was always lower in the mutant strain than in the wild-type strain and 2) the level of expression in wild-type yeast cells was higher than that in yeast cells of the *yak1Δ/yak1Δ* mutant strain (Table 3). Consequently, despite up-regulation of these genes in response to hypha-inducing conditions, their expression was always significantly diminished in hypha of the mutant strain relative to the wild type. These data suggested that Yak1 contributes to the transcriptional regulation of a subset of hypha-induced genes in the yeast and hyphal forms.

C. albicans genes that are differentially regulated at the yeast-to-hypha transition are the subject of complex positive and negative regulation by an array of transcription factors, including Efg1, Tec1, Bcr1, Rim101, Tup1, Nrg1, and Rfg1 (Eckert *et al.*, 2007; Whiteway and Bachewich, 2007). Interestingly, we noted a significant enrichment for Tup1-regulated genes in the group of 32 Yak1-dependent, hypha up-regulated genes in contrast to the group of 146 Yak1-independent, hypha up-regulated genes. Indeed,

62.5% of the genes in the former group have been reported to be under Tup1-negative regulation, whereas this is the case for 24.7% of the genes in the latter group. Tup1-negatively regulated genes account for 6.5% of the whole *C. albicans* gene set (Garcia-Sanchez *et al.*, 2005; Kadosh and Johnson, 2005).

To confirm the role of Yak1 in the hypha-induced transcriptional activation of hypha-specific genes, real-time PCR was used to examine the expression of *HWP1*, *HYR1*, *ALS3*, and *RBT5* during a time course experiment for hyphal differentiation. These genes were chosen because three (*HWP1*, *HYR1*, and *ALS3*) showed Yak1-dependent hypha up-regulation in our transcript profiling experiment, whereas the latter (*RBT5*) showed Yak1-independent hypha up-regulation (Tables 3 and 4). Data presented in Figure 6A show that mRNA levels of the four genes increased during hyphal differentiation of the CEC366 *yak1Δ/yak1Δ/YAK1* strain. The same observation was made when mRNA levels of *RBT5* were monitored during hyphal differentiation of the CEC362 *yak1Δ/yak1Δ* strain, indicating that *RBT5* up-regulation is

Figure 6. Quantitative RT-PCR analysis of hypha-induced genes. Total RNA of *C. albicans* strains CEC366 (*yak1Δ/yak1Δ/YAK1*), CEC362 (*yak1Δ/yak1Δ*) and BWP17AHU (*YAK1/YAK1*) grown in Lee's medium for the indicated times (0, 15, 30, 60, and 180 min.) were reverse transcribed and the expression of *HWP1*, *HYR1*, *ALS3* and *RBT5* was quantified by real-time PCR. Data were normalized for each strain by using data obtained at time zero for the CEC366 strain as indicated in *Materials and Methods*, and relative expression values at the other time points are indicated. Values are means and standard deviations for data of two biological replicates and two technical replicates, except those obtained for *HYR1* and *RBT5* at time 60 min for which only one biological replicate was tested. Please note the difference in scale between the different panels.

independent of the function of Yak1. In contrast, mRNA levels of *HYR1*, *HWP1*, and *ALS3* did not increase in the CEC362 strain to the same extent as that seen in the CEC366 strain (Figure 6), consistent with the microarray data presented above and the Yak1-dependent hypha up-regulation of *HWP1*, *HYR1*, and *ALS3*.

The *C. albicans* Yak1 kinase does not regulate the cAMP-PKA-Efg1 Cascade

The cAMP-PKA pathway plays a key role in the control of morphogenesis through regulation of the activity of the transcription factor Efg1 and subsequent activation of genes encoding transcriptional factors and hypha-specific genes (Eckert *et al.*, 2007; Whiteway and Bachewich, 2007). Results presented above showed that efficient hyphal morphogenesis and hypha-induced expression of several hypha-specific genes, such as *HWP1*, *HYR1*, and *ALS3*, are dependent upon a functional *YAK1* gene. Thus, we investigated whether Yak1 might regulate the cAMP-PKA-Efg1 regulatory cascade.

Addition of cAMP or overexpression of the PKA catalytic subunit Tpk1 can restore filamentation to *C. albicans* strains that have defects in components that act upstream of adenylyl cyclase (Maidan *et al.*, 2005). To test whether the Yak1 kinase is involved in activating hyphal morphogenesis upstream of adenylyl cyclase, the effect of cAMP on the filamentation and agar invasion defects of the *yak1Δ/yak1Δ* CEC362 strain was tested. Addition of 5 mM cAMP to solid

YPD FCS 20% medium or liquid Lee's medium did not restore filamentation and agar invasion of strain CEC362 (data not shown). Moreover, transformation of a *C. albicans yak1Δ/yak1Δ* strain with plasmids allowing overexpression of *TPK1* (Bockmuhl *et al.*, 2001) or *EFG1* (Stoldt *et al.*, 1997) did not alleviate the filamentation and agar invasion defects associated to the deletion of *YAK1* (data not shown). Thus, artificial activation of the cAMP-PKA-Efg1 pathway is not sufficient to suppress the filamentation defect of a *yak1Δ* mutant.

The *TEC1* and *BCR1* genes are targets of the cAMP-PKA-Efg1 pathway, and they show up-regulation upon induction of hyphal growth. Efg1 is responsible for the activation of *TEC1*, whereas Tec1 activates *BCR1* whose product in turn activates *HWP1*, *HYR1*, *ALS3*, and *RBT5* (Nobile and Mitchell, 2005; Cao *et al.*, 2006). Interestingly, *TEC1* was found within the group of 146 genes whose transcriptional activation in response to a hypha-inducing signal did not require a functional *YAK1* gene (Table 4). *BCR1* was not found among the genes that showed more than threefold induction upon hyphal morphogenesis in the wild-type strain. However, as shown in Table 4, *BCR1* was up-regulated 2.8-fold ($p = 2.5 \times 10^{-4}$) in the wild-type strain and 1.9-fold ($p = 0.04$) in the mutant strain undergoing hyphal differentiation, suggesting that transcriptional activation of *BCR1* in response to a hypha-inducing signal did not require a functional *YAK1*. To confirm this observation, the expression of *TEC1* and *BCR1* was monitored using real-time PCR. Quan-

tification of *TEC1* and *BCR1* mRNA levels was associated with important variability between biological replicates, possibly due to the low level of expression of these genes and variations in the kinetics of germ tube emergence between biological replicates. However, we consistently observed up-regulation of both genes upon hyphal induction in the CEC366 (*yak1Δ/yak1Δ/YAK1*) and CEC362 (*yak1Δ/yak1Δ*) strains. These results suggested that the defect in the transcriptional activation of hypha-specific genes such as *HWP1*, *HYR1*, and *ALS3* observed in the *yak1Δ/yak1Δ* mutant is probably not due to defects in the up-regulation of *TEC1* and *BCR1*, and more generally in the activation of the cAMP-PKA-Efg1 pathway.

Deletion of the *TUP1* Gene Can Bypass the Yak1 Requirement for Filamentation

C. albicans yeast-to-hypha transition is the subject of negative regulation by the general repressor Tup1 in association with the DNA-binding proteins Nrg1 and Rfg1 (Braun and Johnson, 1997; Braun *et al.*, 2001; Kadosh and Johnson, 2001; Murad *et al.*, 2001b). Because 63% of the Yak1-dependent, hypha-specific genes have been reported to be negatively regulated by Tup1 (see above), we hypothesized that Yak1 may function in the Tup1 pathway to govern hyphal emergence and maintenance. Inactivation of Tup1 is associated with constitutive filament formation in media that are not normally conducive to filamentation (Braun and Johnson, 1997). Thus, a *C. albicans yak1Δ/yak1Δ tup1Δ/tup1Δ* strain was generated through successive disruption of the two *TUP1* alleles in the *yak1Δ/yak1Δ* CEC359 strain. As shown in Figure 7, the resulting *yak1Δ/yak1Δ tup1Δ/tup1Δ* CEC991 strain showed constitutive filamentation when grown under non-inducing conditions in a manner similar to the *YAK1/YAK1 tup1Δ/tup1Δ* BCA2-10 strain (Braun and Johnson, 1997). Thus, deletion of the *TUP1* gene was able to bypass the requirement for Yak1 in *C. albicans* filamentation.

Figure 7. Deletion of the *TUP1* gene can by-pass the Yak1 requirement for *C. albicans* filamentation. Representative pictures of strains CEC366 (*yak1Δ/yak1Δ/YAK1 TUP1/TUP1*), CEC362 (*yak1Δ/yak1Δ TUP1/TUP1*), and CEC991 (*yak1Δ/yak1Δ tup1Δ/tup1Δ*) grown in YPD medium at 30°C. A picture of strain BCA2-10 (*YAK1/YAK1 tup1Δ/tup1Δ*) (Braun and Johnson, 1997) is shown as a way of illustration for the filamentous phenotype associated to the deletion of the *TUP1* gene.

DISCUSSION

Results presented in this study have identified the Yak1 protein kinase as a novel regulator of hyphal growth in *C. albicans*. Indeed, inactivation of Yak1 through gene deletion or chemical inhibition is associated with defects in 1) the emergence of germ tubes, 2) the maintenance of hyphal growth, and 3) the expression of a number of genes that are normally up-regulated upon hyphal morphogenesis.

We originally investigated the function of *C. albicans* Yak1 because of the role of the orthologous Yak1 kinase in the formation of biofilms by *C. glabrata* (Iraqi *et al.*, 2005). Our results showed that the *C. albicans yak1Δ/yak1Δ* mutants were severely impaired in their ability to form biofilms. This is likely to reflect the contribution of *C. albicans* Yak1 to filamentation, this process being central to the establishment of mature biofilms by *C. albicans* (Ramage *et al.*, 2002; Richard *et al.*, 2005), and to the expression of the Als3 and Hwp1 adhesins that have been proposed to mediate cell-to-cell interactions within biofilms (Nobile and Mitchell, 2005; Nobile *et al.*, 2006a,b). Thus, both *C. glabrata* Yak1 and *C. albicans* Yak1 play important roles in regulating the expression of genes encoding adhesins mediating cell-to-cell interactions and in biofilm formation. *C. glabrata* Yak1 is thought to regulate the expression of the *EPA* genes through negative regulation of the subtelomeric silencing machinery (Iraqi *et al.*, 2005), a process that is unlikely to account for the role of *C. albicans* Yak1 in the regulation of the *ALS3* and *HWP1* genes because these genes are not located at subtelomeric regions (van het Hoog *et al.*, 2007). Yet, a role of *C. albicans* Yak1 in controlling gene expression through regulation of chromatin remodeling cannot be excluded because *S. cerevisiae* Yak1 was recently shown to influence the nucleocytoplasmic distribution of the Msi1 (Cac3) protein and its association in the CAF-I (Pratt *et al.*, 2007). Whether this is also the case for *C. albicans* Yak1 and influences the observed binding of chromatin remodeling proteins to the promoter regions of Yak1-regulated genes will remain to be investigated. In this regard, the *HWP1* control region critical for hypha-induced activation has recently been shown to be bound by proteins involved in chromatin remodeling (Kim *et al.*, 2007) and the Swi/Snf chromatin remodeling complex is essential for hyphal development and expression of hypha-specific genes (Mao *et al.*, 2006).

Our data showed that Yak1 is required at the onset of filamentation under some hypha-inducing conditions. Strikingly, however, the *yak1Δ/yak1Δ* mutants did not show defects in germ tube emergence when serum was used to induce the yeast-to-hypha switch. There, germ tubes formed efficiently but, after a certain time, hypha started to produce lateral and apical buds (Figure 3B), suggesting that Yak1 is also necessary for the maintenance of hyphal growth. This conclusion was reinforced through analysis of a *C. albicans* mutant strain expressing a 1-NMPP1-sensitive Yak1 kinase. Addition of 1-NMPP1 to hypha of this strain promoted the production of lateral branches with pseudohyphal morphology (Figure 4B). Thus, there is a requirement of the Yak1 kinase activity for both the initiation and the maintenance of hyphal growth. Although much is known about the molecular requirements for the initiation of hyphal morphogenesis (Eckert *et al.*, 2007; Whiteway and Bachewich, 2007), the mechanisms that underlie the maintenance of hyphal growth and return to the yeast growth phase remain poorly understood. This in part due to the fact that most *C. albicans* morphogenetic mutants are defective at early stages of hyphal morphogenesis and that promoter-replacement methods that could be used for conditional expression of these

genes are not optimal to survey their contribution to later stages of morphogenesis. In this regard, our chemical genetic approach to the study of Yak1 provides for rapid inactivation of the kinase activity without changes in the growth medium and should be applicable to other kinases that have been implicated in hyphal morphogenesis such as Tpk1, Tpk2 and components of the MAP kinase pathways (Bockmuhl *et al.*, 2001; Alonso-Monge *et al.*, 2006). However, examples of *C. albicans* mutants that show defects in the maintenance of hyphal elongation and production of lateral buds have been reported. For example, overexpression of the transcriptional regulator Efg1 in hypha triggers the production of lateral yeast cells, suggesting that down-regulation of *EFG1* is important for the maintenance of hyphal growth (Tebarth *et al.*, 2003). Inactivation of the G1 cyclin Cln1 is also associated with a defect in hyphal maintenance (Loeb *et al.*, 1999), suggesting that proper control of the cell cycle is an important aspect of hyphal maintenance as it is of other aspects of morphogenesis (Berman, 2006). In *S. cerevisiae*, Yak1 interacts with the Hrt1 component of the Skp1-Cdc53-F-box complex (Uetz *et al.*, 2000) that is involved in ubiquitination of G1 cyclins and their subsequent degradation. It has been proposed that the contribution to pseudohyphal growth of *S. cerevisiae* Yak1 might result from its inhibition of G1 cyclin degradation (Zhang *et al.*, 2001). A similar mechanism might explain the Yak1-dependent regulation of hyphal maintenance in *C. albicans*.

That *yak1Δ/yak1Δ* mutants were not defective for germ tube emergence in the presence of serum, whereas they were in other hypha-inducing conditions is not entirely unexpected. Other *C. albicans* mutants with defects in filamentation and agar invasion under a variety of inducing conditions are still able to form germ tubes when exposed to serum (Loeb *et al.*, 1999; Zakikhany *et al.*, 2007). This is possibly because serum triggers filamentation through multiple signaling pathways, whereas other inducers might be more specific (Harcus *et al.*, 2004). The ability to switch between yeast and hyphal forms is a central component of *C. albicans* virulence as indicated by the reduced virulence of numerous *C. albicans* mutants that are defective for hyphal morphogenesis or that can be locked into one of the two morphotypes (Lo *et al.*, 1997; Gow *et al.*, 2002; Saville *et al.*, 2003). Yet, the *yak1Δ/yak1Δ* mutants did not show any virulence alteration in animal models of systemic and oropharyngeal candidiasis. This suggests that the conditions that are encountered by *C. albicans* in these animal models are—such as serum—sufficient to trigger hyphal morphogenesis in a Yak1-independent manner. Further study of the hyphal differentiation program of *yak1Δ/yak1Δ* mutants exposed to serum or other conditions mimicking the animal environment will be needed to identify the signaling pathways that allow to bypass the Yak1 requirement for filamentation.

The activation of adenylyl cyclase, PKA and the transcriptional regulator Efg1 is at the center of the yeast-to-hypha transition, mediating the response to several environmental stimuli (Lo *et al.*, 1997; Stoldt *et al.*, 1997; Eckert *et al.*, 2007), and we have investigated whether a role of Yak1 in regulating filamentation could be through the cAMP/PKA/Efg1 pathway. Several observations suggest that it is not the case: 1) Yak1 is required for hyphal differentiation in conditions that require Efg1 but also in microaerophilic conditions where Efg1 is dispensable; 2) addition of cAMP or overexpression of *TPK1*, encoding a catalytic subunit of PKA, or *EFG1* does not suppress filamentation defects associated to a *yak1Δ/yak1Δ* mutation suggesting that Yak1 does not act upstream of the cAMP/PKA/Efg1 pathway; and 3) transcriptional targets of Efg1 such as *TEC1* (Schweizer *et al.*,

2000) are efficiently activated in response to hyphal induction in cells that lack Yak1. Moreover, we have observed that up-regulation of the *BCR1* gene is not altered in a *yak1Δ/yak1Δ* mutant exposed to hypha-inducing conditions. Similarly, *RBT5*, a target of the Efg1–Tec1–Bcr1 transcriptional activation cascade (Nobile and Mitchell, 2005), showed Yak1-independent hypha up-regulation (Figure 6). Strikingly however, hypha-induced up-regulation of Bcr1 transcriptional targets such as *HWP1*, *HYR1*, and *ALS3* (Nobile and Mitchell, 2005) was altered when Yak1 is lacking. Together, these data suggest that the filamentation defect associated to the absence of Yak1 is due to defective up-regulation of hypha-induced genes, several of which are necessary for efficient hyphal morphogenesis, independently of the activation of the cAMP/PKA/Efg1 pathway and the Tec1 and Bcr1 transcriptional regulators located downstream of this pathway. Although one cannot exclude that Yak1 may positively regulate morphogenesis through phosphorylation of transcriptional activators such as Tec1 or Bcr1, this seems unlikely because *RBT5*, a downstream target of these activators, is still strongly up-regulated in cells that lack Yak1, and *C. albicans bcr1Δ* mutants are not defective for filamentation (Nobile and Mitchell, 2005).

Hypha-induced genes are also negatively regulated by Tup1 in conjunction with the DNA-binding proteins Nrg1 and Rfg1 (Braun and Johnson, 1997; Braun *et al.*, 2001; Murad *et al.*, 2001b; Kadosh and Johnson, 2005), and there is evidence that chromatin remodeling contributes to their expression (Kim *et al.*, 2007; Mao *et al.*, 2006). Therefore, it is tempting to speculate that Yak1 may modify the activity of the Tup1–Nrg1, Tup1–Rfg1, or chromatin remodeling complexes in response to hypha-inducing signals and consequently allow derepression or activation to take place. A large fraction of the genes that are hypha-induced in a Yak1-dependent manner have been shown to be repressed by Tup1 (Kadosh and Johnson, 2001; Garcia-Sanchez *et al.*, 2005), suggesting that Yak1 may interfere with Tup1-mediated repression. This hypothesis is supported by our observation that inactivation of the *TUP1* gene can bypass the Yak1 requirement for filamentation (Figure 7), suggesting that Yak1 might function as a repressor of Tup1. However, Tup1-repression is also observed for a number of genes that are not dependent upon Yak1 for hyphal-dependent induction such as *RBT5*, and we have not identified features that could distinguish these two groups of genes. Therefore, constitutive filamentation of the *tup1Δ yak1Δ* mutant could also indicate that the derepression of Tup1-repressed genes allows progression of the morphogenetic program independently of any positive regulation that may involve Yak1.

In *S. cerevisiae*, Yak1 has been proposed to act downstream of PKA, regulating different processes related to growth. PKA consensus phosphorylation sites in the *S. cerevisiae* Yak1 protein have been shown to be genuinely phosphorylated (Zappacosta *et al.*, 2002). These phosphorylation sites are conserved in *C. albicans* Yak1, and it is conceivable that PKA-mediated phosphorylation of Yak1 at the onset of filamentation may contribute to its function in filamentation, linking the positive and negative regulatory branches of hypha-induced gene regulation. Yet, there are unexplored PKA-independent regulatory aspects to the function of Yak1 in *S. cerevisiae* (Garrett *et al.*, 1991; Ward and Garrett, 1994; Zappacosta *et al.*, 2002). PKA-independent regulation of Yak1 in *C. albicans* may in particular explain its contribution to filamentation under microaerophilic conditions whose relation to the cAMP/PKA pathway is unclear.

As discussed above, *C. albicans* Yak1 might contribute to the regulation of hyphal emergence and maintenance

through different mechanisms that are not mutually exclusive: 1) inhibition of the repression exerted by Tup1, Nrg1, and Rfg1 on hypha-inducible genes; 2) regulation of chromatin remodeling needed for the expression of hypha-induced genes; and 3) regulation of ubiquitination and in turn G1 cyclin activity. The identification of the *C. albicans* proteins that are phosphorylated by Yak1 is likely to shed light on the actual mechanisms that are involved in regulating filamentation in a Yak1-dependent manner. In this regard, the availability of a 1-NMPP1-sensitive form of Yak1 should facilitate the identification of such targets. Indeed, protein kinases whose “gate-keeper” residue has been modified also use radiolabeled analogues of ATP as phosphate donors in *in vitro* phosphorylation reactions, and this property has been used advantageously to identify their cellular targets (Shah *et al.*, 1997; Bishop *et al.*, 2001; Habelhah *et al.*, 2001; Dephoure *et al.*, 2005).

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