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Sch9 Kinase Integrates Hypoxia and CO_2 Sensing To Suppress Hyphal Morphogenesis in *Candida albicans*▽†

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The yeast-hypha transition is an important virulence trait of *Candida albicans*. We report that the AGC kinase Sch9 prevents hypha formation specifically under hypoxia at high CO_2 levels. *sch9* mutants showed no major defects in growth and stress resistance but a striking hyperfilamentous phenotype under hypoxia (<10% O_2), although only in the presence of elevated CO_2 levels (>1%) and at temperatures of <37°C during surface growth. The *sch9* hyperfilamentous phenotype was independent of Rim15 kinase and was recreated by inhibition of Tor1 kinase by rapamycin or caffeine in a wild-type strain, suggesting that Sch9 suppression requires Tor1. Caffeine inhibition also revealed that both protein kinase A isoforms, as well as transcription factors Czf1 and Ace2, are required to generate the *sch9* mutant phenotype. Transcriptomal analyses showed that Sch9 regulates most genes solely under hypoxia and in the presence of elevated CO_2. In this environment, Sch9 downregulates genes encoding cell wall proteins and nutrient transporters, while under normoxia Sch9 and Tor1 coregulate a minor fraction of Sch9-regulated genes, e.g., by inducing glycolytic genes. Other than in *Saccharomyces cerevisiae*, both *sch9* and *rim15* mutants showed decreased chronological aging under normoxia but not under hypoxia, indicating significant rewiring of the Tor1-Sch9-Rim15 pathway in *C. albicans*. The results stress the importance of environmental conditions on Sch9 function and establish a novel response circuitry to both hypoxia and CO_2 in *C. albicans*, which suppresses hypha formation but also allows efficient nutrient uptake, metabolism, and virulence.

Candida albicans is a normal inhabitant of human mucosal surfaces, but lowering of immune defenses can cause tenacious localized and severe systemic fungal disease. *C. albicans* parasitism depends on its ability to change cellular morphologies, especially to transform its spherical yeast to a filamentous true hyphal form (dimorphism). During recent years numerous regulators of the yeast-hypha transition have been described that are required to regulate dimorphism in general or only under specific environmental conditions (reviewed in reference 8). Under normoxia a main signaling pathway of hypha formation is triggerd by molecules present in serum, such as muramyl peptides, and by carbon dioxide (20, 43). This pathway involves an increased cyclic AMP (cAMP) level that relieves the repression of protein kinase A (PKA) catalytic subunits Tpk1 and Tpk2 (3, 35), which in turn activate downstream transcriptional regulators, including, as the global regulator, Efg1. Efg1 and Efg1-dependent transcription factors regulate expression of target genes required for hyphal morphogenesis, e.g., by inactivating negative regulators Tup1 and Nrg1 and by recruiting histone-modifying activities that close or open up chromatin structures (23, 39). Under hypoxia, the ability of *C. albicans* to form hyphae is effectively repressed (4, 35). Interestingly, some positive regulators of hypha formation under normoxia, including Efg1 and Flo8, are also needed as repressors of filamentation under hypoxia (6, 10, 33, 36). Past results (18) and data presented here indicate that relief of repression rather than induction is a crucial mechanism that allows expression of hypha-specific genes under several environmental conditions.

The PKA isoforms Tpk1 and Tpk2 are members of the so-called AGC group of kinases in eukaryotes, including PKA, PKB, PKC, and PKG, as well as phosphoinosite-dependent kinase (PDK) and ribosomal protein S6 kinase (reviewed in reference 17). AGC proteins have a common domain organization consisting of a central catalytic domain flanked by an N-terminal lipid-binding domain of pleckstrin homology and a C-terminal regulatory domain containing a conserved hydrophobic sequence that can be phosphorylated by an upstream kinase. Fungal AGC kinases respond primarily to the availability of sugars and nitrogen. An example is the PKB protein Sch9 in the yeast *Saccharomyces cerevisiae* (homolog of Akt in mammalian cells), which in rich media is phosphorylated and thereby activated by the target of rapamycin, TORC1 (40). Both Sch9 and TORC1 proteins colocalize to the vacuolar membrane, presumably to sense and/or to mobilize intracellular nutrients (32). In *S. cerevisiae*, *sch9* mutants form smaller colonies and grow at a lower rate (because of a prolonged G_1 phase), presumably because Sch9 is a positive regulator of transcription, especially of genes encoding ribosomal proteins and genes involved in nitrogen metabolism (9, 16, 42). Recent results indicate that Tor1 in *C. albicans* has a similar function but also regulates additional genes, including genes encoding

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TABLE 1. *C. albicans* strains used in the study

Strain	Genotype	Reference
CAF2-1	*URA3*/*ura3*::*imm434*	13
CAI4	*ura3*::*imm434*/*ura3*::*imm434*	13
CAS4	As CAI4 but *sch9*::*hisG*/*sch9*::*hisG*	This work
CCS3	As CAS4 but *URA3*/*ura3*::*imm434*	This work
CCS4	As CAS4 but *LEU2*/*leu2*::[*ACT1p-SCH9 URA3*]	This work
CCS5	As CAS4 but *leu2*::[*URA3*]	This work
CAR23-7-5-1	As CAI4 but *rim15*::*hisG*/*rim15*::*hisG*	This work
CCS6	As CAR23-7-5-1 but *URA3*/*ura3*::*imm434*	This work
AF1002	As CAI4 but *sch9*::*hisG*/*sch9*::*hisG-URA3-hisG rim15*::*hisG*/*rim15*::*hisG*	This work
IIHB6	As CAI4 but *tpk1*Δ::*hisG-URA3-hisG*//*tpk1*Δ::*hisG*	3
HPY300U (tpk1mut)	As CAI4 but *tpk1*Δ::*hisG*/*tpk1*Δ::*hisG URA3*/*ura3*::*imm434*	26
TPO7.4	As CAI4 but *tpk2*Δ::*hisG*/*tpk2*Δ::*hisG-URA3-hisG*	35
HPY400U (tpk2mut)	As CAI4 but *tpk2*Δ::*hisG*/*tpk2*Δ::*hisG URA3*/*ura3*::*imm434*	26
JLC19	As CAI4 but *cph1*Δ::*hisG*/*cph1*Δ::*hisG-URA3-hisG*	21
CKY230	As CAI4 but *czf1*Δ::*hisG*/*czf1*Δ	4
MK106	*ace2*Δ::*FRT*/*ace2*Δ::*FRT*	19

adhesins (2). In *S. cerevisiae* Sch9 was also found to be required for hyperosmotic stress resistance by direct transcriptional activation of osmostress-responsive genes (27). On the other hand, under nutrient-rich conditions, Sch9 was found to collaborate with PKA to reduce stress sensitivity by blockage of the AGC kinase Rim15 that activates transcription factors Gis1 and Msn2/4, driving expression of stress protection genes (5, 28). In *sch9* mutants or under nutrient-poor conditions, i.e., low Sch9 activity, these transcription factors are activated via Rim15 and increase stress protection and significantly prolong the chronological life span of yeast (12, 41).

In *C. albicans*, a homolog of *S. cerevisiae* Sch9 has been partially characterized, and it was shown that it is required for efficient growth, stress resistance, and filamentation under certain conditions (22, 25). Here we describe additional functions of the *C. albicans* Sch9 homolog and show that it is a component of a regulatory circuit sensing oxygen and CO_2. Specifically, Sch9 is a positive regulator of yeast growth, in all environments, while under hypoxia and high carbon dioxide levels it strongly represses hypha formation. We demonstrate that transcriptomal effects of Sch9 are hypoxia and CO_2 dependent and that other Sch9 functions, including its contribution to antifungal resistance and longevity, are dependent on this gaseous environment.

MATERIALS AND METHODS

Strains and growth conditions. *C. albicans* strains are listed in Table 1. Strains were grown in yeast extract-peptone-dextrose (YPD) medium, YPS medium (1% yeast extract, 2% peptone, 2% sucrose), or in supplemented SD minimal medium at 30°C (33); solid media contained, in addition, 2% agar. Hypoxic growth under defined gaseous conditions was carried out using an Invivo200 hypoxia chamber (Ruskinn) with attached flasks of nitrogen, CO_2, and compressed air (36). Liquid media were preequilibrated overnight under hypoxia/CO_2 conditions before inoculation. Transformation of *C. albicans* strains was carried out using the spheroplast method as described previously (33). For hyphal induction in liquid, the strains were grown at 37°C in YP medium with 20% horse serum. To induce chlamydospore formation, cells were streaked out lightly on chlamydospore induction medium (cornmeal agar [Merck]–0.33% Tween 80), covered by a coverslip, and incubated for 5 days at 20°C (34).

Construction of *sch9* and *rim15* mutants. Both alleles of *SCH9* (ORF19.829/ORF19. 8449) were deleted in strain CAI4 by using the URA blaster method (13). A 1.0-kb sequence upstream of the open reading frame (ORF) was amplified by genomic PCR using primers CaSCH9-dis1/for and -rev; likewise, a 0.55-kb fragment of the 3′-unstranslated region (3′-UTR) was amplified using CaSCH9disII-for and -rev. Oligonucleotides used for PCR in this paper are listed in Table S1 of the supplemental material. Using BamHI/BglII sites at the fragment ends of both fragments were inserted into the BamHI/BglII sites flanking the *hisG-URA3-hisG* cassette in p5921. The BamHI/BglII 5.6-kb fragment of the resulting plasmid p5921-SCH9dis was used to sequentially disrupt both *SCH9* alleles as described previously (13). Correct positioning of the *SCH9* disruption cassette was verified by colony PCR and by genomic Southern blotting assays (data not shown).

In a similar approach, both alleles of *RIM15* (ORF19.7044) were deleted in strain CAI4. A 1.1-kb fragment upstream of the ORF was amplified by genomic PCR using primers CaRIM15disI-for and -rev; likewise, a 1.1-kb fragment partially encompassing 500 bp of the ORF and 500 bp of the 3′-UTR was amplified using CaRIM15disII-for and -rev. Both flanking fragments were inserted into p5921 as described above. The BamHI/BglII 6.2-kb fragment of the resulting plasmid pCaRIM15/Blaster was then used to sequentially disrupt both *RIM15* alleles, and correct integration of the disruption cassette was verified by genomic Southern blot assays (data not shown). The resultant strain was named CAR23-7-5-1. In addition, a *sch9 rim15* double mutant strain (AF1002) was constructed by disrupting *SCH9* in the background of strain CAR23-7-5-1, as described above.

Because the presence of *URA3* at ectopic loci may influence *C. albicans* phenotypes, we reconstituted *URA3* at its authentic locus in *sch9* and *rim15* mutants, as described previously (29). The resulting prototrophs were named CCS3 (*sch9* mutant) and CCS6 (*rim15* mutant).

Construction of *SCH9* and *RIM15* expression plasmids. The *SCH9* ORF was PCR amplified using genomic DNA of strain CAI4 using primers CaSCH9/BHI/for and -rev, which generate BamHI and BglII sites flanking the ORF. The 2.4-kb *SCH9* ORF fragment was inserted into the BglII site downstream of the *PCK1* promoter in pBI-1 (37) to generate pBI-CaSCH9 and downstream of the *ACT1* promoter in pYW105 to construct pYW-CaSCH9. Both expression vectors also contain *URA3* as a selection marker and the Ca*ARS* replicator. Likewise, the *RIM15* ORF was amplified primers RIM15-Start and -Stop, which generate flanking BamHI sites. The 5.8-kb BamHI fragment was also cloned downstream of the *ACT1* promoter in plasmid pDS1044-I to generate p2297R1.

Transcriptome of *sch9* mutant. Strains CAF2-1 (*SCH9*/*SCH9*) and CCS3 (*sch9*/*sch9*) were grown at 25°C in liquid YPS medium under an atmosphere of (i) air, (ii) 0.2% O_2 (no CO_2), or (iii) 0.2% O_2 plus 6% CO_2. Three independent cultures were used for each strain to isolate total RNA in the exponential growth phase and generate Cy3- or Cy5-labeled cDNAs as described previously (10). Labeled cDNAs representing wild-type and mutant transcripts were hybridized to genome-wide *C. albicans* microarrays (Eurogentec) representing all ORFs in duplicate, as described previously (10). Spot intensities were evaluated using program GenePix 6.0 for background substraction and GeneSpring 10.2 for normalization (LOWESS) and determination of the mean values (ratios of transcript level in the *sch9* mutant relative to the *SCH9* strain). Results are available at ArrayExpress under accession number E-MEXP-2407.

Virulence tests. Female, 8- to 10-week-old, outbred CD1 mice were obtained from Harlan Nossan Laboratories (Milan, Italy) and housed at the Animal Facilities of the University of Perugia, Perugia, Italy. Procedures involving animals and their care were conducted in conformity with national and international

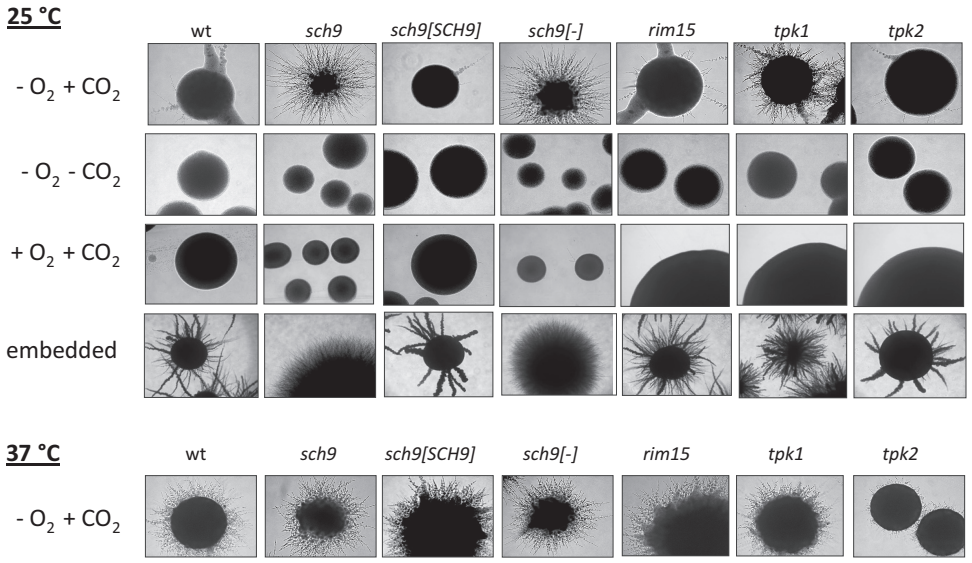


FIG. 1. Colony phenotypes of *C. albicans* strains. Wild-type and mutant strains were grown on YPS agar for 3 days (25°C) or 2 days (37°C), and colonies were photographed. Strains were grown in an atmosphere of 0.2% O_2 containing 6% CO_2 (- O_2 + CO_2) or lacking CO_2 (- O_2 - CO_2) or under 20% oxygen (normoxia) containing 6% CO_2 (+ O_2 + CO_2). In addition, cells were grown embedded in YPS agar under air. Strains used were CAF2-1 (wild-type [wt]), CCS3 (*sch9*), CCS4 (*sch9*[*SCH9*]), CCS5 (*sch9*[-]), CCS6 (*rim15*), IIHB6 (*tpk1*), and TPO7.4 (*tpk2*).

laws and policies. For systemic infection, mice were inoculated intravenously on day zero with *C. albicans* CAF2-1, CCS3, or CCS4 strains (1×10^6/mouse in 0.5 ml saline). Infected animals were monitored daily for survival. The experiments were repeated 3 times by using 4 animals/experimental group. Differences between survival data for the groups were analyzed by using the Mann-Whitney U test.

RESULTS

***sch9* and *rim15* mutants.** In *S. cerevisiae* the Tor1-Sch9-Rim15 kinase cascade regulates various functions, including metabolism, stress response, and longevity (reviewed in reference 31). The B-type kinase Sch9 is known to repress the activity of Rim15, which positively regulates stress responses and chronological life span (38). The *C. albicans* genome contains genes (*ORF19.829* and *ORF19.7044*) encoding close homologs of Sch9 and Rim15 (1). CaSch9 and ScSch9 have 66% overall identity, which rises to near-complete identity in the kinase catalytic domain; Rim15 homologs in both species share 35% overall identity, which increases to 60% between residues 1263 and 1408 in the kinase domain (see Fig. S1 in the supplemental material). Using the URA blaster protocol we disrupted both alleles of *SCH9* and *RIM15* in *C. albicans* CAI4. In the resulting homozygous mutants we reinserted the *URA3* selection marker at its authentic locus to construct strains with an identical *URA3* status as the control strain, CAF2-1 (7). Furthermore, mutant strains were reconstituted by integrating plasmids carrying the respective wild-type *SCH9* or *RIM15* ORFs.

Filamentation phenotypes of the *sch9* mutant. In previous reports the *sch9* mutant was shown to be completely defective in chlamydospore formation (25) and partially defective in growth and filamentation (22). We reassessed these phenotypes by using the *URA3* reconstituted *sch9* mutant CCS3 and the *SCH9 URA3* reconstituted mutant CCS4. As described previously (22), colony sizes of the *sch9* mutant on YPD agar were reduced and the doubling time of mutant CCS3 was increased to 1.65 h, compared to 1.2 h of the CAF2-1 control strain in YPD medium at 30°C. Standard plate tests using Lee's, Spider, or serum medium showed a slight delay, which presumably was related to reduced growth, but no major defects in hypha formation at 37°C (data not shown).

The *sch9* mutant developed slighly smaller colonies than the control strain under hypoxia (0.2% O_2) on YPS medium at 25°C and generated few or no hyphae emanating from colonies. However, strikingly, the presence of a high CO_2 concentration under hypoxia (0.2% O_2, 6% CO_2) led to strongly increased filamentous growth of the *sch9* mutant but not of the control strain or the *SCH9* reconstituted mutant CCS4 (Fig. 1A, top panel). Hyperfilamentation of the *sch9* mutant was also observed during YPS-embedded growth, a condition presumably lowering oxygen and increasing CO_2 levels (Fig. 1, top panel). To assess the threshold O_2 and CO_2 concentrations for this phenotype we carried out experiments at various concentrations of both gases. Under high CO_2 (6%), hypha formation of the *sch9* mutant declined gradually with increasing O_2 concentrations and was almost completely absent at 10% O_2 (Fig. 2, top). At low O_2 levels (0.2% O_2), hypha formation started at 0.5% CO_2, and it was clearly apparent at 1% CO_2 (Fig. 2, bottom). Unexpectedly, the ability of the *sch9* mutant to hyperfilament under hypoxia at elevated CO_2 levels was strongly influenced by temperature. At 37°C filamentation of the *sch9* mutant was moderate and equaled the control and *SCH9* reconstituted strains under all gaseous conditions (Fig. 1, bottom panel, shows results for the hypoxia–high-CO_2 condition). We conclude that temperatures of <37°C, O_2 concentrations of $<10\%$, and CO_2 concentrations of $>1\%$ trigger strong hypha formation of the *sch9* mutant. Conversely, it follows that *C. albicans* wild-type cells use Sch9 to effectively suppress hypha formation under these growth conditions. Opposite to these

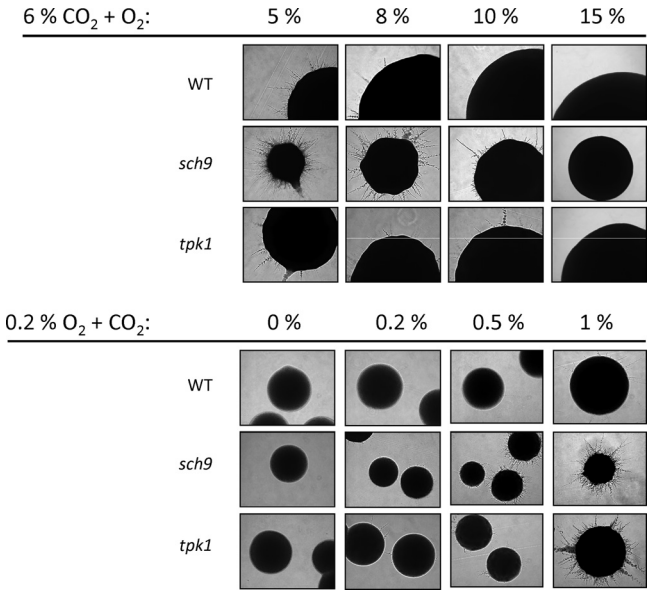


FIG. 2. Oxygen- and CO_2-dependent filamentation. Strains CAF2-1 (wt), CCS3 (*sch9*), and IIHB6 (*tpk1*) were grown at 25°C on YPS agar either under 6% CO_2 and the indicated concentrations of oxygen or under 0.2% oxygen and the indicated concentrations of CO_2. Colony phenotypes were photographed after 3 days of growth.

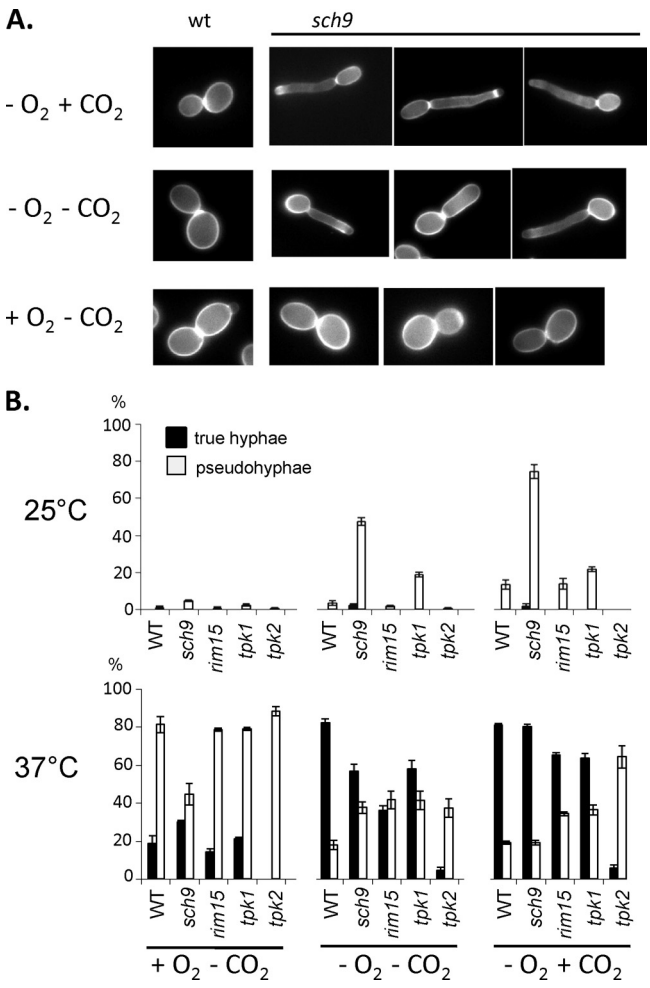


FIG. 3. Dependence of cellular morphologies on hypoxia and CO_2. (A) Cells of *sch9* mutant CCS3 and control strain CAF2-1 (wild type [wt]) were grown in liquid YPS for 4 h at 25°C. Cells were grown for 4 h in low oxygen (0.2% O_2) containing or not containing 6% CO_2. Chitin was stained with calcofluor white (2 μg/ml), and cells were inspected by fluorescence microscopy. (B) Distribution of cell forms in various strains. Cells were grown as for panel A, and at least 100 cells were classified according to their cell form. Percentages of pseudohyphal and true hyphal forms are indicated by the white and black bars, respectively, while the remaining percentage is due to yeast-form cells. For strain designations, see Fig. 1.

results, filamentation of mutants lacking the regulator Efg1 was greatly induced under hypoxia, even in the absence of CO_2 (33).

In liquid YPS medium, a particular *sch9* phenotype was observed under hypoxia, since cells formed pseudohyphae while the control strain grew in yeast form (Fig. 3A). This phenotype occurred irrespective of CO_2 levels at 25°C under hypoxia but not under normoxia. At 37°C, under any gaseous condition, no major differences in filamentation were observed, since both the *sch9* mutant and the control strain formed true hyphae, as well as pseudohyphae (Fig. 3B). Thus, in liquid media Sch9 suppresses pseudohypha formation under hypoxia. Comparison of *sch9* mutant phenotypes during growth in liquid or on agar suggests that surface contact is yet another parameter influencing the activity of the Sch9 protein.

Derepressed filamentation of the *sch9* mutant does not require Rim15. Because of the Tor1-Sch9-Rim15 cascade known in *S. cerevisiae*, we assumed that elevated activity of Rim15 was the cause for elevated filamentation of the *sch9* mutant. To clarify this notion we constructed *rim15* single and *rim15 sch9* double mutants and examined their phenotypes.

In *S. cerevisiae*, Sch9 represses the function of Rim15 to activate genes involved in the stress response, sporulation, transition into stationary phase, and longevity (28). Unexpectedly, the *C. albicans rim15* mutant CCS6 did not show any significant phenotypic differences compared to the control strain CAF2-1 or the reconstituted mutant CAR23-7-5-1[p2297R1]. No phenotypic abnormalities were observed with respect to growth rate, budding index in stationary phase, heat shock (53°C) sensitivity, osmotolerance, glycogen accumulation, or sensitivity to several antifungal agents; furthermore, hyphal development in liquid or on solid media with the inducers serum and GlcNAc was similar in *rim15* and wild-type strains (data not shown). The only slight mutant phenotype arose during growth under hypoxia (with or without CO_2), where filamentation was slightly increased in the *rim15* mutant compared to control strains (Fig. 1, top panel). We conclude that Rim15 in *C. albicans* has a significantly different function than its homolog in *S. cerevisiae* and that it does not have a major role in growth or hypha formation under normoxia or hypoxia. Importantly, the *sch9 rim15* mutant strain showed an identical phenotype under hypoxia and elevated CO_2 as the *sch9* single mutant, i.e., vigorous hypha formation (Fig. 4A). Thus, released activity of Rim15 in cells lacking Sch9 is not the reason for the hyperfilamentous phenotype.

Tor1 inhibition generates *sch9* mutant-like hyperfilamentation. Tor1 activity is inhibited by rapamycin and caffeine (2, 16, 41). Because Tor1 functions upstream of Sch9, we asked if

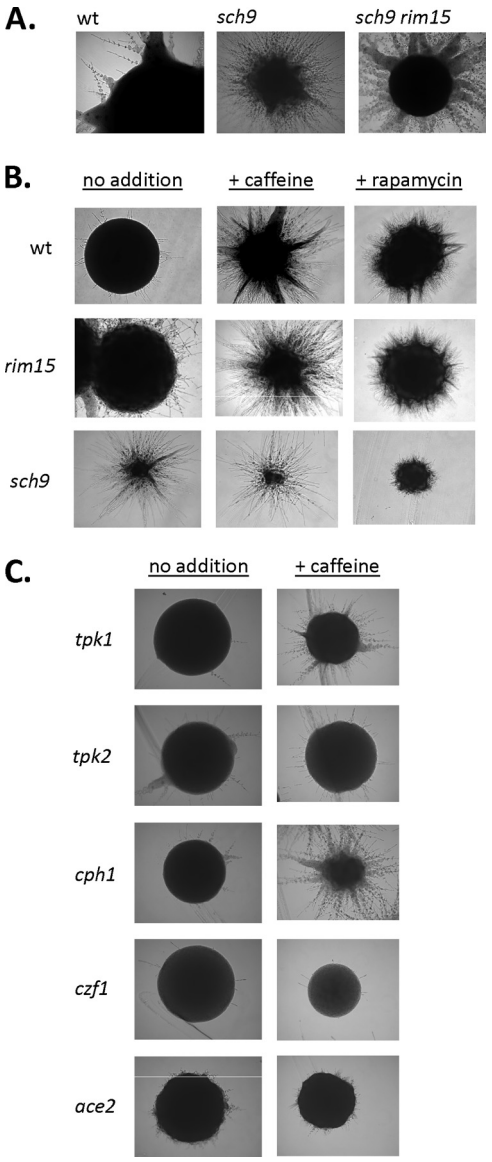


FIG. 4. Hypoxic filamentation by *C. albicans* mutants. Single colonies of strains were grown on YPS agar at 25°C under an atmosphere of 0.2% O_2 and 6% CO_2; colonies were photographed after 3 days of growth. (A) Control strain CAF2-1, *sch9* mutant CCS3, and *sch9 rim15* double mutant AF1002 were compared. (B and C) Strains were grown on YPS medium without additions or in YPS medium containing 2 nM rapamycin or 1 mM caffeine. Strains included control strain CAF2-1, *rim15* mutant CCS6, *sch9* mutant CCS4, *tpk1* mutant HPY300U, *tpk2* mutant HPY400U, *cph1* mutant JLC19, *czf1* mutant CKY230, and *ace2* mutant MK106.

Tor1 inhibition can generate a *sch9* mutant phenotype in wild-type strains. This was indeed the case, since both rapamycin and caffeine induced strong hypha formation in the control strain and the *rim15* mutant (Fig. 4B). Hyperfilamentation of the *sch9* mutant was not stimulated further by either of these inhibitors, suggesting that Tor1 and Sch9 function in the same pathway. Importantly, rapamycin/caffeine-induced filamentation only occurred under conditions known to reveal the *sch9* mutant hyperfilamentation phenotype, i.e., during hypoxic growth with elevated levels of CO_2 (data not shown). Thus, these results indicate that the Tor1-Sch9 pathway strongly suppresses filamentation of *C. albicans* under specific environmental conditions.

Since Tor1 inhibition was able to generate a *sch9* mutant-like phenotype, we were able to conveniently test if other suspected regulators of hypoxic filamentation are required for this phenotype. In both *tpk1* and *tpk2* mutants, hyperfilamentation under hypoxia and high CO_2 was greatly reduced compared to the control strain or the *cph1* mutant known to be filamentation defective on agar surfaces (21) (Fig. 4C). Likewise, in the *czf1* and *ace2* mutants, known to be defective in hypoxic filamentation (4, 14, 19, 24), caffeine was unable to trigger hyperfilamentation. These results indicate that Tpk1, Tpk2, Czf1, and Ace2 allow hypha formation under hypoxia and elevated CO_2, if repression by the Tor1-Sch9 pathway does not occur.

Antifungal resistance and longevity of *sch9* and *rim15* mutants. Standard drop dilution tests were performed to evaluate the sensitivity of the *sch9* mutant CAS4 and the control strain CAI4 in different oxygen and temperature environments (Fig. 5A). The *sch9* mutant was significantly more resistant to SDS and fluconazole than the control strain, especially at 25°C. In turn, the *sch9* mutant was significantly more sensitive than the control strain to hygromycin B, especially under hypoxia. We conclude that lack of Sch9 leads to increased or reduced sensitivity, depending on the type of antifungal agent. Furthermore, we note that the oxygen level and temperature have significant effects on antifungal susceptibilities.

The yeast *S. cerevisiae* has been used as a model system to study chronological cellular aging, and nutrient-rich environments were shown to reduce longevity by activating Sch9 via Tor1, which inactivates Rim15 and prevents its role in stress responses (12, 41). In spite of these antagonistic roles of Sch9 and Rim15, both *C. albicans sch9* and *rim15* were unable to survive for long in stationary phase in cultures grown under normoxia, compared to the control strain (Fig. 5B). Unexpectedly, however, following hypoxic growth in stationary phase, both mutants survived, similar to the control strain (Fig. 5B). This protective effect of hypoxia also occurred in the absence of elevated CO_2 levels (data not shown). The results suggest that both Sch9 and Rim15 are required for longevity specifically under normoxia, while they are dispensable for survival under hypoxia. Furthermore, these results again indicate different regulatory wiring of Sch9 proteins in *C. albicans* and *S. cerevisiae.*

Transcriptome of *sch9* mutant. In *S. cerevisiae* Sch9 is a target of the Tor kinase (40), and inhibition of Tor1 in *C. albicans* by rapamycin indicated that Tor1 function is largely conserved between both fungal species (2). To explore if the functions of Tor1 are mediated via Sch9 in *C. albicans*, we performed transcriptomal comparisons of the *sch9* mutant CCS3 and the control strain CAF2-1. These comparisons were carried out for cells growing under normoxia but also under hypoxia (with or without CO_2), because the above phenotypes had suggested specific roles for Sch9 under this condition.

In all environments, levels of 310 transcripts were regulated at least 2-fold in the *sch9* mutant relative to the control strain. Venn diagrams and functional categories are shown in Fig. 6. Regulated genes are listed in Table S2 of the supplemental material. Surprisingly, the majority of Sch9-dependent up- or

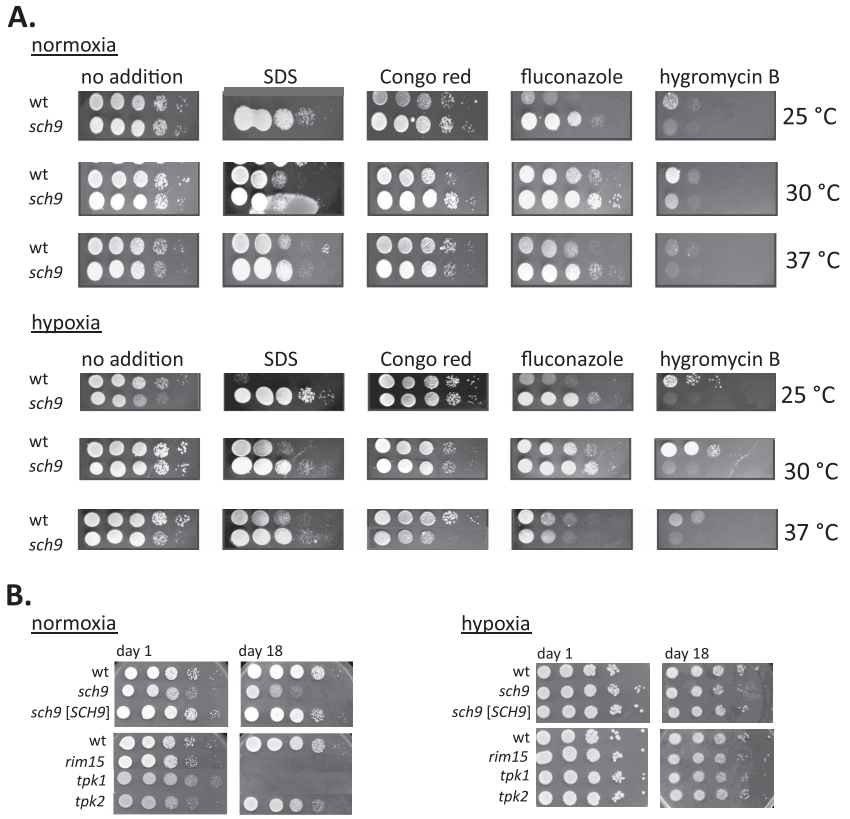


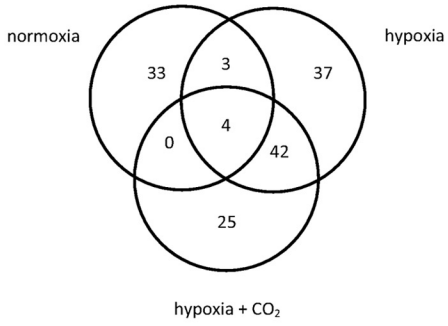
FIG. 5. Environment-dependent sensitivities of the *sch9* mutant. (A) Sensitivities to antifungals. Strains were grown in air (normoxia) or under hypoxia (99.9% nitrogen) at the indicated temperatures. Five-microliter drops of 10-fold dilutions containing 10^5 to 10^1 cells of strain CAI4 (wild type [wt]) and *sch9* mutant CAS4 were spotted on YPD agar containing 0.06% SDS, 200 μg/ml Congo red, 5 μg/ml fluconazole, or 200 μg/ml hygromycin B. (B) Longevity experiment. Strains were grown in liquid YPS medium at 25°C under normoxia or under hypoxia (0.2% O_2, 6% CO_2) for 1 day or 18 days. Viability of cells after this time was assayed by a drop dilution test as for panel A. Strains tested were control strain CAF2-1, *sch9* mutant CCS5, reconstituted *sch9* mutant CCS4, *rim15* mutant CCS6, *tpk1* mutant IIHB6, and *tpk2* mutant TPO7.4.

downregulation genes were detected solely under hypoxia. For example, 33 transcripts were downregulated in the *sch9* mutant only under normoxia, 104 transcripts under hypoxia, and only 7 transcripts were downregulated under both conditions (Fig. 6). Interestingly, about one-third of genes down- or upregulated by Sch9 under hypoxia depended on the presence of CO_2. This finding demonstrates the function of Sch9 under hypoxia and shows its importance for CO_2-dependent induction and repression of gene expression. To confirm some of the transcriptomal data, we compared levels of specific transcripts in the *sch9* mutant CCS3 to the reconstituted mutant CCS4 by quantitative. The results demonstrated a largely similar Sch9-mediated environmental regulation compared to the array experiments for 3 key transcripts, including *ERG11*, *ECE1*, and *RNR22*, while the *CZF1* and ORF19.5831 transcripts showed a different regulation (see Table S3 in the supplemental material). The aberrant result of the latter transcripts is probably due to the fact that in CCS4 a single *SCH9* allele driven by the *ACT1* promoter was used for complementation; thus, the different dosage and regulation of Sch9 may partially alter its transcriptomal effects.

Almost none of the genes regulated in the *sch9* mutant were found to be downregulated in the presence of rapamycin, i.e., by Tor1 inactivation (2). While Tor1 activity was required to induce a large set of genes encoding components of the translational machinery, none of these genes was Sch9 dependent for expression. Twelve genes involved in translation are even repressed under hypoxia, since their levels increased in the *sch9* mutant under this condition (e.g., *RPS20*/*24*, *RPL6*/*12*/*23*/*24*/*30*/*82*). The only similarity of rapamycin treatment with *sch9* mutation was downregulation of genes involved in glucose metabolism (e.g., of the glycolytic genes *CDC19*, *GPM2*, *PDC11*, and *PFK2*). Sch9 deficiency also downregulated additional genes involved in sugar metabolism but only under hypoxia, e.g., the *GRA1* gene encoding glucoamylase and strongly the *MAL2* and *MAL31* genes involved in maltose metabolism.

A total of 166 genes were upregulated in the *sch9* mutant, thus reflecting a repressive function of Sch9 on these genes. The only similarities of these genes to genes regulated in rapamycin-treated cells were genes involved in cell wall structure and morphogenesis, including *ALS1*, *ALS2*, *ALS3*, *ECE2*, *RBT1*, and *CZF1* (marked in Fig. 6). Upregulation of the latter 3 genes was observed only in hypoxic cells supporting increased pseudohyphal growth of the *sch9* mutant under hypoxia. Surprisingly, Sch9 represses a large number of genes involved in metabolite transport, since genes encoding transporters for hexoses, ions, iron, amino acids, and polyamine, which were upregulated in the *sch9* mutant. The suppression of transport

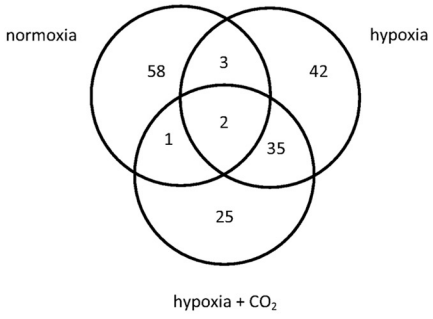
A. Downregulated transcripts in *sch9* mutant



growth condition and functional category (numbers)	*gene*
all conditions (4)	
amino acid biosynthesis (1)	*ARO3*
formiate catabolism (1)	*ORF19.1774*
glyoxylate catabolism (1)	*ORF19.2989*
unknown function (1)	*ORF19.2048*
normoxia and hypoxia/no CO_2 (3)	
isoleucine biosynthesis (1)	*ORF19.1997*
unknown function (1)	*ORF19.1257, ORF19.3968*
normoxia and hypoxia/+ 6 % CO_2 (0)	
none	
normoxia only (33)	
glucose metabolism (6)	*CDC19*, GPM2*, PDC11*, PFK1, PFK2*, PGI1*
chromatin/chromosome structure (2)	*RNR22*, ORF19.6910*
various/unknown functions (25)	*COX15*, HEM13, RHR2*, TPO3, QDR1*, ZRT2, MSM1, CWH8, LYS12, LEU2, MEP1, GSG1, SUR2*, OSM1, RSN1, ORF19.6816*, ORF19.4617*, ORF19.4581, ORF19.5136*, ORF19.5534, ORF19.1691, ORF19.2804, ORF19.5103, ORF19.6834, ORF19.4620*
hypoxia with or without CO_2 (42)	
sugar metabolism (3)	*GCA1, MAL2,MAL31, NTH1*
amino acid transport (2)	*CAN2**, ORF19.7566***
arginine biosynthesis (2)	*ARG3, CPA1*
proline utilization (2)	*PUT1, PUT2*
various/unknown functions (33)	*ASR1, PHO84, ADH2, CLN3, MRF1, MLS1, OYE23, CSH1, UGA11, FMP45, PST1, ORF19.2296, ORF19.822, ORF19.1172, ORF19.5975, ORF19.1117, ORF19.7491, ORF19.6592, ORF19.566, ORF19.823, ORF19.7283, ORF19.4735, ORF19.7437, ORF19.803, ORF19.7490, ORF19.3976, ORF19.4128, ORF19.320, ORF19.2168, ORF19.5295, ORF19.3434, ORF19.77.1*
hypoxia + 6 % CO_2 (25)	
arginine biosynthesis (3)	*ARG1, ARG2, ORF19.3418***
chromatin/chromosome structure (2)	*CDC54, ORF19.810*
stress response (2)	*GAD1, HSP70*
various/unknown functions (18)	*FAA2-3, FOX2, HGT17,, HMX1, TCC1, STE2, ILV5*, PLC1, IFD6, ORF19.553, ORF19.7513, ORF19.2065**, ORF19.1072, ORF19.670.2, ORF19.40, ORF19.6004, ORF19.4834, ORF19.994*
hypoxia only (37)	
stress response (6)	*SOD1, SOD2, ORF19.345, FRE30, ASR2, DDR48*
sugar metabolism (3)	*AMS1, GPH1, ORF19.3982*
hypha specific genes (2)	*CHA1, UCF1*
ATP biosynthesis (2)	*ATP1, ATP2*
various/unknown functions (24)	*CYB2, ARG8, PRC2, ARO10**, OSM2, HGT6*, XYL2, CDR4, ORF19.5245, ORF19.7310, ORF19.2591, ORF19.4121*, ORF19.1584, ORF19.5614, ORF19.1775, ORF19.682, ORF19.6877, ORF19.3435, ORF19.1473, ORF19.1469, ORF19.1368, ORF19.3372, ORF19.4636, ORF19.6342*

* downregulated or ** upregulated by rapamycin

B. Upregulated transcripts in *sch9* mutant



growth condition and functional category (numbers)	*gene*
all conditions (2)	
chromatin/chromosome structure (1)	*HTA2*
hypha specific gene (1)	*ORF19.4459*
normoxia and hypoxia/no CO_2 (3)	
ion transport (1)	*SEQ1*
iron uptake (1)	*FRE10*
unknown function (1)	*ORF19.699*
normoxia and hypoxia/+ 6 % CO_2 (1)	
sugar uptake (1)	*HGT12*
normoxia only (58)	
cell wall (6)	*ALS1**, ALS2**, ALS3**, ALS4, ORF19.1098, ORF19.2121*
hexose transport (5)	*HGT2, HGT17, SNF3, HXT5, ORF19.4386*
ion transport (5)	*PMC1, FRP3, ORF19.5170, ORF19.6070, ORF19.2959.1*
transcription factor (4)	*UCF5, MDM34, GAT2, ORF19.5026*
sugar metabolism	*GAL1*, GAL7*, GAL10, ARD1*
amino acid transport (3)	*DIP5**, ORF19.2943, ORF19.7566*
various/unknown functions (31)	*GPR1, CTN1, HMX1, HSP12, CIT1, ANT1, DPP3, CUP9, HAP41, PXA1, RGS2, PDK2, ORF19.4445, ORF19.6169, ORF19.215, ORF19.6996, ORF19.6281, ORF19.4531**, ORF19.1225, ORF19.1344, ORF19.2030, ORF19.2496, ORF19.3073, ORF19.3029, ORF19.3439, ORF19.3984, ORF19.6169, ORF19.5125, ORF19.5843, ORF19.6311, ORF19.79*
hypoxia with or without CO_2 (35)	
translation (5)	*ASC1, RPL12, RPL82, RPS24, ORF19.3690.2*
iron uptake (5)	*FET34, FET35, FTR1, PGA10*, SIT1*
ergosterol biosynthesis (5)	*ERG1*, ERG5, ERG6, ERG11, ERG251*
chromatin/chromosome structure (4)	*HHF1, HHT21, HTA1, ORF19.1052*
hypha specific gene (3)	*PTP3, FGR51, RFX2*
cell wall (3)	*ECE1**, ORF19.3384, PHR1, RBT1***
various/unknown functions (10)	*CAR1, ORF19.1074, ORF19.5193, ORF19.7296, orf19.2426, ORF19.5137, ORF19.1982, ORF19.7070, ORF19.7455, ORF19.2452*
hypoxia + 6 % CO_2 (25)	
transcription factor (2)	*ZCF4, ORF19.3088*
ergosterol biosynthesis (2)	*ERG10, ERG26*
polyamine transport (2)	*UGA4, UGA6*
various/unknown functions (19)	*HGT7, SOD6, RPS1, EXG2, ORF19.1583, ORF19.2583.2, ORF19.4982, ORF19.47, ORF19.5831, ORF19.7147, ORF19.3903, ORF19.6518, ORF19.68.2, ORF19.3387, ORF19.2734, ORF19.7273, ORF19.467, ORF19.3118, ORF19.2350*
hypoxia only (42)	
translation (7)	*RPS20*, RPL6, RPL23A, RPL29, RPL30, RNR1, YST1**
cell wall (6)	*HWP1, HYR1, SAM2, PGA39, PGA45, RBT5*
chromatin/chromosome structure (5)	*CTF8, HHO1, HTA3, SET3, ORF19.581*
microtubuli processes (5)	*MLC1, TIB1, TIB4, ORF19.708, ORF19.5008.1*
hypha specific gene (3)	*CZF1**, CDC10, CDC11*
ergosterol biosynthesis (2)	*ERG2, ERG3*
various/unknown functions (14)	*CFL2, MYO2, STE18, ORF19.6184, ORF19.5735,3, ORF19.2397.3, ORF19.5491, ORF19.5069, ORF19.6579, ORF19.453, ORF19.6837, ORF19.5012, ORF19.1116, ORF19.1691*

* downregulated or ** upregulated by rapamycin

TABLE 2. Virulence of *C. albicans* strains in a mouse model of hematogenously disseminated infection

C. albicans strain[a]	Relevant genotype	MST[b]	D/T[c]	Survival range (days)
CAF2-1	*SCH9/SCH9 ura3/URA3*	5.5	12/12	2–8
CCS3	*sch9/sch9 ura3/URA3*	27*	7/12	7 to >60
CCS4	*sch9/sch9 ura3/ura3 LEU2/leu2*::[*ACT1p-SCH9 URA3*]	7	12/12	2–10

[a] CD1 mice were infected intravenously with 1×10^6 *C. albicans* cells.
[b] Median survival time (in days). *, $P < 0.01$ (mutant versus wild-type strain CAF2-1).
[c] Number of dead mice (D) at 60 days over the total number of animals infected (T).

genes may be related to the assumption that increased activity of Sch9 reflects high nutrient levels, and high-affinity uptake systems may not be needed under this condition. Furthermore, several *ERG* genes were upregulated in the *sch9* mutant under hypoxia (*ERG10* and *ERG26*, only in the presence of CO_2). Because these genes are known to be upregulated under hypoxia (33), it appears that Sch9 dampens this response, presumably to prevent membranes from losing fluidity, which is already compromised by lowered biosynthesis of unsaturated fatty acids.

Finally, we also note that some genes were regulated oppositely in the *sch9* mutant and by rapamycin treatment of the control strain, including the *CAN2* permease gene and *GAL1*/*GAL7* genes, which were down- and upregulated, respectively. We conclude that only a minor subset of genes regulated by Sch9 is also regulated via Tor1.

Virulence of the *sch9* mutant. To confirm the contribution of Sch9 to *C. albicans* virulence, which was suggested previously (22), we used strains containing *URA3* at its native locus to exclude any effects due to extragenic *URA3* localization (7). Mice were infected intravenously with control strain CAF2-1, *sch9* mutant strain CCS3, or the *SCH9* reconstituted strain CCS4. Median survival times of mice infected with the control CAF2-1 strain and mutant CCS3 were 5.5 days and 27 days, respectively (Table 2). Forty percent of mice infected with the mutant survived until the end of the observation period (60 days). In contrast, after inoculation of the reconstituted mutant CCS4, survival of mice was as short as that of animals infected with the control strain. Thus, we confirmed that Sch9 is essential for virulence of *C. albicans.* Reduced virulence appears not to be caused by impaired biofilm formation, since in a static model of biofilm formation in normoxia or hypoxia with and without CO_2 (36) the *sch9* mutant did not show significant defects (data not shown).

DISCUSSION

The human body contains numerous niches that are low in oxygen but increased in CO_2 levels that may become colonized by *C. albicans* and other pathogens (reviewed in reference 11). We report here that *C. albicans* has a high inherent propensity to filament under this condition but that this property is repressed by the Sch9 kinase. At oxygen levels of $<10\%$ O_2 and $>1\%$ CO_2, the action of Sch9 is needed to downregulate hypha formation, possibly to evade immune responses directed against the hyphal growth form. Recently, glucan unmasked in hyphae and other hypha-associated components has been shown to directly interact with host components (30, 38). Thus, it appears that Sch9 activity could be an important requirement for commensal yeast growth of *C. albicans*, e.g., within phagocytotic vacuoles or in the gut, which contains low oxygen and high CO_2 levels. Hyperfilamentation of the *sch9* mutant was detected experimentally during growth on agar and at temperatures of $<37°C$. Agar is a polysaccharide that in an actual infection event may mimic glycostructures on the surface of host cells or in the extracellular matrix. The fact that hyperfilamentation of the *sch9* mutant was observed at lower temperatures may reflect our present ignorance of additional factors regulating hypha formation under hypoxic conditions and high CO_2 levels. For example, switching to and stabilization of the opaque growth form was found to occur only at lower temperatures and only recently was shown to depend on elevated CO_2 levels even at 37°C (15). Some *C. albicans* components, including Efg1 and Flo8 transcription factors, have already been described that repress hypha formation during embedded growth or under hypoxia at lower temperatures (6, 33), but repression by these proteins did not require CO_2, as Sch9 does. We conclude that Sch9 has a novel regulatory function, since it integrates sensing of hypoxia, CO_2, and surface contact to supppress filamentation of *C. albicans.*

The fact that the repressive function of Sch9 is apparent only at high CO_2 levels under hypoxia raises a question about the Sch9 signaling pathway. Interestingly, we also obtained a *sch9* mutant-like hyperfilamentation phenotype by repressing Tor1 activity by rapamycin or caffeine (2, 41). Because Tor1 is a known upstream activator of Sch9 in *S. cerevisiae* (40), it can be assumed that a Tor1-Sch9 pathway acts as a repressor of filamentation. On the other hand, Rim15, the AGC kinase downstream of Sch9 in *S. cerevisiae* (28, 41), was not required for the *sch9* mutant phenotype, as shown by *sch9 rim15* double mutants and by rapamycin/caffeine-induced hyperfilamentation of a *sch9* mutant. Deletion of Rim15 in *C. albicans* also did not generate growth or stress defects known for *S. cerevisiae rim15* mutants (28), indicating that the roles of Rim15 have diverged significantly in both fungal species. While Rim15 was dispensable for hypoxia/CO_2-triggered filamentation, the transcription factors Czf1 and Ace2, which had previously been shown to be involved in filamentation under hypoxia or agar embedding (14, 24), appeared to be required for hyperfilamention in the case of the nonfunctional Tor1-Sch9 suppression pathway. Czf1 has been suggested to downregulate Efg1, which under hypoxia is a strong repressor of hypha formation, independent

FIG. 6. Down- and upregulated transcription in the *sch9* mutant CCS3 relative to wild-type strain CAF2-1. The Venn diagrams depict numbers of genes regulated at least 2-fold under the 3 conditions (air, 0.2% O_2, or 0.2% O_2 plus 6% CO_2); affected genes are listed below. A total of 144 and 166 transcripts were down- and upregulated, respectively, in the *sch9* mutant. (A) Downregulated genes; (B) upregulated genes. Genes also regulated by rapamycin (2) are marked with asterisks (*, downregulated; **, upregulated genes).

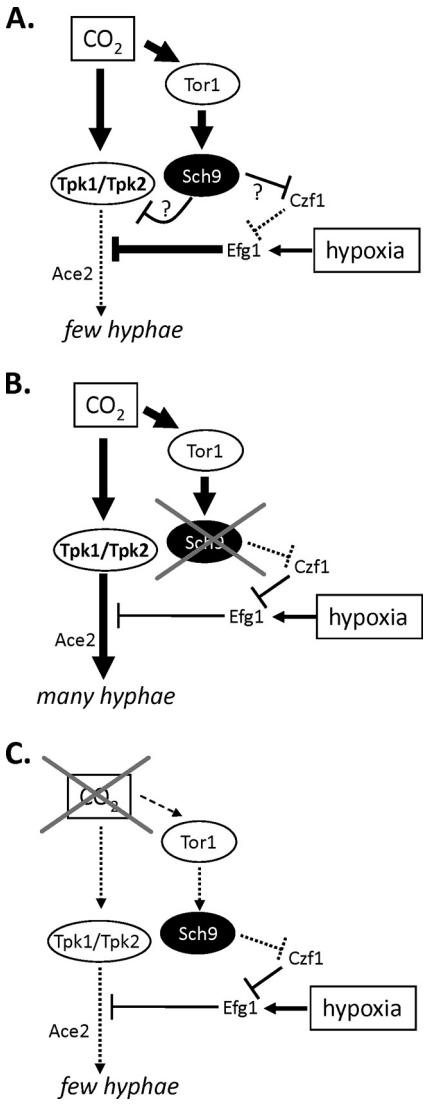


FIG. 7. Model for the regulation of filamentation by Sch9. (A) Wild-type cells grown under hypoxia at high CO_2 levels. Under hypoxia, Efg1 acts as a repressor of filamentous growth (14, 33, 34), but Czf1 limits its repressor activity (4, 14). Under high CO_2 levels, the PKA isoforms Tpk1 and Tpk2 are activated, presumably by bicarbonate-mediated activation of adenylate cyclase (20). It is proposed that PKA is downregulated in certain environments (surface growth, temperatures of <37°C) by the Tor1-Sch9 pathway to prevent hypha formation. It is yet unclear if Sch9 increases Efg1 repressor activity by inactivation of Czf1, as shown, or by another mechansm, e.g., by blocking PKA activity. Ace2 is required for hypoxic filamentation (24) by a yet-unknown mechanism. (B) Lack of Sch9 or Tor1 activity during hypoxic growth under high CO_2 fails to repress the CO_2-triggered increase in PKA activity, leading to hyperfilamentation. (C) A moderate level of Efg1 repressor activity under hypoxic growth and low CO_2 levels suffices to block hypha formation at low PKA activity.

of elevated CO_2 levels (14, 33, 34). The model in Fig. 7 involves the Tor1-Sch9 pathway in the Efg1 circuitry by proposing that in wild-type cells Sch9 inactivates/downregulates Czf1, thereby increasing the repressor activity of Efg1. This process appears necessary to counteract the bicarbonate-stimulated increase in adenylate cyclase activity, cAMP levels, and PKA activity, which stimulate filamentation (20). Although other mechanisms are possible, the proposed pathway can explain that *C. albicans*, in specific hypoxic/CO_2-containing environments, prevents filamentation and favors growth in the yeast form.

Sch9 appears to be a general regulator of growth and morphogenesis of *C. albicans* because of additional phenotypes and aberrant gene regulation in the *sch9* mutant. Mutants showed increased pseudohyphal growth in liquid medium that occurred only under hypoxia but was independent of CO_2 levels. Furthermore, in the *sch9* mutant, sensitivity to hygromycin B but also resistance to fluconazole, SDS, and Congo red were increased. The latter two resistance properties we observed are at variance with results of Liu et al. (22), who used different drug concentrations and strain backgrounds. Interestingly, resistance properties of the *C. albicans* control strain were significantly influenced by oxygen levels and temperature, e.g., because hygromycin, SDS, and fluconazole resistance increased under hypoxia and higher temperatures. Increased fluconazole resistance may be due to elevated ergosterol levels in the *sch9* mutant under hypoxia, in which transcripts of several *ERG* genes are increased. A surprising phenotype of the *sch9* mutant was its short chronological life span in the stationary growth phase. This finding is opposite to results for *S. cerevisiae*, for which Rim15 has been established as the key kinase to allow long-term survival by inducing stress response genes; the upstream Tor1-Sch9 kinases inactivate Rim15 during nutrient-rich growth, but they release Rim15 in stationary phase because they are downregulated (12, 41). Thus, it appears that Sch9 is not involved in regulating chronological life span in *C. albicans*, whereas the decreased life span of a *rim15* mutant is consistent with the *S. cerevisiae* results and identifies the only significant phenotype of Rim15 depletion in *C. albicans.* Unexpectedly, the dependence of *C. albicans* longevity on Sch9 and Rim15 under normoxia was not paralleled under hypoxia, where both proteins were not needed for long-term survival. This result again stresses the different regulatory circuitries operating under hypoxia and normoxia, which in the past were reported apparent with regard to filamentation and biofilm formation (10, 33, 36).

The transcriptomal analyses revealed that 310 genes were up- or downregulated more than 2-fold in the *sch9* mutant. Sch9 regulates only a fraction of genes that are dependent on the Tor1 kinase. Such genes include genes involved in the metabolism of sugars, which are both Sch9 and Tor1 dependent, and genes encoding cell wall components, which are repressed by both proteins (2). Sch9 also represses numerous transport activities of *C. albicans* under various conditions, reflecting the role of Sch9 in an abundance metabolism, which is similar to the functions of PKA isoforms in the presence of nutrients. Importantly, we discovered that under hypoxia Sch9 regulates many genes that are unaffected under normoxia and that the presence of CO_2 elicits yet another pattern of Sch9-dependent gene activities. Genes that are up- or downregulated by Sch9 only under hypoxia in the presence of 6% CO_2 may be responsible for the hyperfilamentation phenotype of the *sch9* mutant. The functions of such genes are largely unknown or represent various functional classes. Therefore, it remains to be determined how many of the respective 25 up- or 25 downregulated genes are involved in a CO_2-sensing pathway that comprises Sch9. Future experiments will clarify whether, for example, transcription factors encoded by the

ZCF4 and ORF19.3088 genes regulate hypoxia- and CO_2-dependent genes.

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