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Synergistic regulation of hyphal elongation by hypoxia, CO_2, and nutrient conditions controls the virulence of *Candida albicans*

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Summary

Candida albicans reversibly switches between yeast and hyphal morphologies, with hyphae being associated with virulence. Hyphal initiation and maintenance depends on host environment sensing. Hyphal maintenance *in vitro* requires chromatin remodeling of hypha-specific gene promoters, although disrupting chromatin-remodeling does not disrupt *C. albicans* hyphal elongation and virulence during invasive infection. We find that the combination of hypoxia and high CO_2, but neither condition alone, maintains hyphal elongation, even in mutants lacking the nutrient responsive chromatin-remodeling pathway. Ume6, the transcriptional activator of hypha-specific genes is stabilized via regulation by Ofd1, a prolyl hydroxylase family member inhibited by hypoxia, and by an uncharacterized pathway that senses high CO_2. Virulence and hyphal elongation *in vivo* are attenuated only when the parallelly acting Ume6 stabilization and chromatin-remodeling pathways are both blocked. The evolution of redundant signaling pathways allowing *C. albicans to* adapt to varied host environments may explain this commensal's success as a pathogen.

Introduction

Fungal pathogens face numerous environmental challenges as they colonize and infect mammalian hosts. Their ability to adapt to environmental changes is critical for their survival and ability to cause disease. As a part of the commensal microbiota, *Candida albicans* colonizes multiple mucosal sites, including the GI tract. It can cause superficial infections of the oropharynx, vagina, skin, and nails. In susceptible patients, *C. albicans* can enter the bloodstream and cause a frequently fatal disseminated infection that is characterized by the formation of microabscesses in most organs. Although significant advances have been made in understanding how *C.albicans* causes disease, the dynamic

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microenvironments it encounters during infection and the mechanisms by which it adapts to these microenvironments are not fully understood.

In the human host, hypoxia is present at most foci of fungal infections. At these sites, the influx of immune effector cells and tissue necrosis caused by the invading pathogen generate hypoxic microenvironments to which both the pathogen and host cells must adapt in order to survive (Ernst and Tielker, 2009; Grahl et al., 2012). The ability to adapt to various levels of hypoxia is an important component of the virulence arsenal of pathogenic fungi (Grahl and Cramer, 2010). Insights into how human pathogenic fungi respond to oxygen limitation first came from studies in the nonpathogenic fission yeast *Schizosaccharomyces pombe*, where the transcription factor Sre1 was found to be essential for hypoxic responses and survival (Hughes et al., 2005). Under low oxygen, Sre1 is cleaved to release the N-terminal transcription factor Sre1N. Sre1N degradation is accelerated by the prolyl 4-hydroxylase-like 2-oxoglutarate-Fe(II) dioxygenase, Ofd1, in the presence of oxygen (Hughes and Espenshade, 2008). This family of enzymes uses oxygen as a substrate to perform hydroxylation, thus providing a natural means of oxygen-dependent control of their functions. The functions and regulation of Sre1 are likely conserved in numerous fungal species, as Sre1 homologs play an oxygen-sensing role that is essential for virulence in the pathogenic fungi *Cryptococcus neoformans* (Chang et al., 2007; Chun et al., 2007) and *Aspergillus fumigatus* (Willger et al., 2008).

In *C. albicans*, little is known about the mechanisms by which this pathogen adapts to hypoxic microenvironments. Although *C. albicans* is capable of anaerobic fermentation in rich medium, oxygen is essential for synthesis of ergosterol, NAD, and heme, which are scarce in the host milieu. Ergosterol biosynthesis is regulated by the transcription factor Upc2 instead of Sre1 in *C. albicans* (MacPherson et al., 2005; Silver et al., 2004). So far, no evidence indicates the existence of an Ofd1 oxygen-sensing pathway for the regulation of ergosterol biosynthesis in *C. albicans*. Although *C. albicans* cells grow exclusively as hyphae in anaerobic conditions (Dumitru et al., 2004), it is not clear how hypoxia governs hyphal development. Furthermore, CO_2, which is frequently present at high concentrations at sites of hypoxia *in vivo*, also influences hyphal development and virulence in *C. albicans* (Klengel et al., 2005). The relationship between O_2 and CO_2 sensing with regard to hyphal development and pathogenicity is poorly understood.

The capacity of *C. albicans* to reversibly switch between yeast and hyphal morphologies is a key virulence factor (Cao et al., 2006; Lo et al., 1997). The yeast-to-hypha transition requires initiation and then maintenance. Hyphal initiation requires a rise in temperature to 37°C and release from quorum sensing molecules, such as farnesol, to temporarily clear the major repressor of hyphal morphogenesis, Nrg1 (Lu et al., 2011). Hyphal maintenance requires active sensing of the surrounding environment. During hyphal initiation when the Nrg1 protein disappears, the expression of a GATA family transcription factor Brg1 is activated in response to starvation or treatment with rapamycin via reduced Tor1 signaling (Lu et al., 2012; Lu et al., 2011; Su et al., 2013). Brg1 expression is also activated by other hyphal inducing conditions, including serum and N-acetylglucosamine (Figure1A). The accumulated Brg1 recruits the Hda1 histone deacetylase to promoters of hypha-specific genes, and Hda1 deacetylates Yng2, a subunit of the NuA4 histone acetyltransferase

module, leading to nucleosome repositioning, obstruction of Nrg1 binding sites, and sustained hyphal development (Figure 1A) (Lu et al., 2012; Lu et al., 2011). Hda1-mediated nucleosome repositioning is essential for hyphal maintenance *in vitro* under all media known to support prolonged hyphal development (Lu et al., 2011). One key downstream target of Hda1-mediated chromatin regulation is Ume6, a hypha-specific transcription factor that controls the level and duration of hypha-specific transcription (Banerjee et al., 2008; Carlisle et al., 2009; Lu et al., 2012; Zeidler et al., 2009).

Given that misregulation of either Nrg1 or Ume6 alters filamentation and attenuates virulence (Banerjee et al., 2008; Saville et al., 2003), one would predict that the Hda1-mediated chromatin-remodeling pathway is also important for virulence. However, disruption of the chromatin-remodeling pathway alone is not sufficient to disrupt *C. albicans* hyphal elongation and pathogenicity during disseminated infection. Here, we show that *C. albicans* has a 2-OG-Fe (II)-dependent dioxygenase, Ofd1. Ofd1 controls Ume6 stability in response to oxygen levels. The nutrient sensing Brg1/Hda1 chromatin remodeling pathway and the oxygen-sensing Ofd1 pathway act in parallel to control Ume6 levels, hyphal development, and virulence.

Results

Hda1-mediated hyphal maintenance is not essential for *C. albicans* virulence

Hda1 regulates sustained hyphal development through deacetylation of Yng2K175 (Lu et al., 2011). To determine the importance of this regulatory pathway in virulence, we infected mice intravenously with *C. albicans* cells carrying a wild-type copy of *YNG2*, *yng2*^{K175R}, or *yng2*^{K175Q} (a mutation mimicking constitutive acetylation). Other than the K175 residue of Yng2, the three strains had identical genotypes, and all strains had similar growth rates. As shown in Figure 1B, the survival of mice infected with either the *yng2/yng2*^{K175R} or *yng2/yng2*^{K175Q} mutants was similar to that of mice infected with the *yng2/YNG2* strain. The finding Yng2 acetylation status did not affect virulence was unexpected, as this regulation of Yng2 was important for hyphal elongation in all known media that favored sustained hyphal growth *in vitro* (Lu et al., 2011). Histopathologic examination of the kidneys of mice infected with the *yng2/yng2*^{K175Q} mutant revealed that they contained a mixture of hyphal and pseudohyphal filaments, similar to kidneys of mice infected with the *yng2/YNG2* strain (our unpublished data). Therefore, although Hda1-mediated deacetylation of Yng2 at K175 is essential for hyphal extension *in vitro*, it is not essential for hyphal development and virulence during disseminated infection in mice. The *brg1* mutant was found to have normal virulence in screening competition assay (Noble et al., 2010), but showed some attenuation in virulence when tested by itself (Du et al., 2012b). Therefore, Brg1 may influence virulence by mechanisms in addition to chromatin modification (Cleary et al., 2012; Nobile et al., 2012). Nevertheless, our results suggest that conditions to which *C. albicans* is exposed within the host must activate a signaling pathway that is independent of the Hda1-mediated hyphal elongation pathway.

Hypoxia plus 5% CO_2 can induce sustained hyphal development in *brg1* and *yng2/yng2^{K175Q}* mutants

When *C. albicans* disseminates hematogenously, it forms microabscesses in most organs, including the kidney. In these foci of infection, fungal cells are exposed to both hypoxia and hypercarbia relative to standard *in vitro* growth conditions. Therefore, we hypothesized that the combination of hypoxia and elevated CO_2 would induce hyphal elongation and virulence of wild-type as well as *yng2/yng2^{K175Q}* cells. As postulated, we found that hypoxia, together with high CO_2 (0.2% O_2, 5% CO_2), induced robust hyphal elongation. In wild-type cells, hyphal development was sustained for at least 12 hours under these conditions at 37°C (Figure 1C). The effect of low oxygen plus high CO_2 on hyphal maintenance was only observed after hyphal formation was initiated by a brief exposure to 37°C. Cells exposed to 0.2% O_2 and 5% CO_2 at 30°C did not form hyphae (our unpublished data). Importantly, both the *brg1* and *yng2/yng2^{K175Q}* mutants were able to maintain hyphal growth in 0.2% O_2 and 5% CO_2 (Figure 1C). But deletion of *UME6* resulted in a profound defect in hyphal maintenance in hypoxia with 5% CO_2, with only a few cells forming hyphae and the majority of cells growing as either elongated yeast or pseudohyphae (Figure 1C). In contrast, in rapamycin-containing medium (which mimics a starvation response), the defect in hyphal maintenance of the *ume6* mutant was less severe as about 25% cells formed hyphae (Figure 1C). Our data clearly show that the combination of low oxygen plus high CO_2 can bypass the requirement of Brg1 and Hda1-mediated nucleosome repositioning for hyphal elongation, but not the need for Ume6. These results provide an explanation for why virulence was not significantly affected by Yng2 acetylation status whereas the virulence was attenuated when *UME6* is deleted. Therefore, we suggest that hypoxia plus 5% CO_2 mimics the host microenvironment encountered by *C. albicans* cells during infection and provides a key signal for hyphal maintenance.

Ume6 is stabilized in hypoxia plus 5% CO_2

Several lines of evidence led us to predict that hypoxia plus 5% CO_2 may promote hyphal elongation by regulating Ume6. First, Ume6 is required for hyphal elongation in hypoxia plus 5% CO_2 (Figure. 1C). Second, constitutively expressing Ume6 rescues the hyphal growth defect of the *brg1* and *hda1* mutants (Lu et al., 2012). Third, we detected much higher levels of Ume6 protein after 8 h of exposure to hypoxia plus 5% CO_2 compared to rapamycin-containing medium in air (Figure S1A). The high levels of Ume6 were also observed in the *brg1* and *hda1* mutant cells in 0.2% O_2 with 5% CO_2, but not in air (Figure S1A). These data suggest that hypoxia plus 5% CO_2 increases Ume6 levels through a pathway that is independent of the Brg1/Hda1-mediated regulation of Ume6 expression. One possible mechanism of Ume6 regulation is at the level of protein stability. We hypothesized that Ume6 is continuously degraded in air but stable in hypoxia plus 5% CO_2. To test this possibility, we examined Ume6 turnover in air and in hypoxia plus 5% CO_2 by promoter shut off assays. A gene encoding Ume6$_{C778/785S}$, which had the Cys778 → Ser and Cys785 → Ser substitutions in the Gal4 DNA binding domain of Ume6, was expressed under the control of the *MET3* promoter. The DNA binding domain of Ume6 was mutated in the construct to disrupt its affinity for DNA as we found that *MAL2* or *MET3* expression could not be shut off completely when wild-type Ume6 was expressed (our unpublished

data). With the *MET3* promoter shut off assay, we found that Ume6$_{C778/785S}$ was unstable in air regardless of temperature or rapamycin, as it disappeared at around 30 min after adding methionine (Figure 2A & our unpublished data). Interestingly, Ume6$_{C778/785S}$ was partially stabilized in either low oxygen or high CO_2, but was completely stable under low oxygen combined with 5% CO_2 (Figure 2A). In control experiments, we verified that the *MET3* promoter was properly shut off by methionine when the organism was exposed to hypoxia with 5% CO_2 (Figure S1B). These results indicated that both low oxygen and high CO_2 contributed to Ume6 stabilization, but neither alone was sufficient.

Hgc1, a hypha-specific G_1 cyclin-like protein required for hyphal morphogenesis (Zheng et al., 2004), is another hypha-specific regulator that is unstable in air (Wang et al., 2007). Using a *MAL2* shut off assay, we found that Hgc1 was stabilized in hypoxia plus 5% CO_2 (Figure 2A). These results suggest that *C. albicans* uses a common pathway to stabilize both the Ume6 and Hgc1 hyphal regulators in hypoxia plus high CO_2.

We further predicted that stabilized Ume6 would bind to its own promoter to activate *UME6* transcription, leading to robust hyphal maintenance via a positive feedback loop. A previous study demonstrated that *UME6* expression levels determine *C. albicans* morphology (Carlisle et al., 2009). We found that constitutive expression of Ume6 was sufficient to sustain hyphal development (Figure S1C) and hypha-specific transcription (Figure S1D) in wild-type cells under conditions that normally did not sustain hyphal elongation. Interestingly, the *UME6* promoter is required for sustained hyphal development, as hyphae could not be maintained in the *ume6* mutant when an ectopic copy of *UME6* was constitutively expressed (Figures S1C and S1D). We next used ChIP to determine if Ume6 binds to its own promoter. The intergenic region upstream of *UME6* contains only one Ume6-binding motif, GCGG, based on the DNA binding motif of *S. cerevisiae* Ume6 (Zhu et al., 2009). As shown in Figure 2B, in hypoxia plus 5% CO_2, Ume6 bound directly to its own promoter, and the levels of promoter-bound Ume6 remained high for at least 12 h. In rapamycin-containing medium in air, the level of promoter-bound Ume6 was lower and peaked earlier than that in hypoxia plus 5% CO_2 (Figure 2B). We consistently detected higher expression levels of hypha-specific genes, *UME6* and *HWP1*, in hypoxia plus 5% CO_2 than in rapamycin-containing medium in air (Figure 2C). Unlike the hypha-specific transcription program in air, hypoxia plus 5% CO_2 did not activate the Brg1 pathway, as the expression level of *BRG1* did not increase significantly under this condition (Figure 2C). Furthermore, the expression of *NRG1* was much lower in hypoxia plus CO_2 compared to air (Figure 2C). The low level of *NRG1* in hypoxia plus CO_2 could be due to the high level of Ume6, as overexpressing Ume6 could repress *NRG1* expression (Figure S1D) (Banerjee et al., 2008). The hypha-specific transcriptional program could not be maintained in either hypoxia or 5% CO_2 alone, as partial stabilization of Ume6 was not sufficient to maintain the levels of promoter-bound Ume6 and therefore the expression of hypha-specific genes (Figures 2B and 2C). Our data demonstrated that when Ume6 is stabilized in hypoxia plus 5% CO_2, it can generate a much stronger positive feedback loop via its own promoter, thus bypassing the requirement for Brg1/Hda1 in hyphal maintenance. We suggest that preventing Ume6 degradation is the underlying mechanism for sustained hyphal development in hypoxia plus 5% CO_2.

Deletion of Ofd1 stabilizes Ume6 in air with 5% CO_2

In fission yeast, the prolyl 4-hydroxylase-like 2-oxoglutarate-Fe(II) dioxygenase, Ofd1, mediates the oxygen-dependent degradation of Sre1N in the presence of oxygen (Hughes and Espenshade, 2008). *C. albicans* has one uncharacterized ORF, orf19.1802, that shares the highest similarity with *S. pombe* Ofd1 (53% identical), which we have thereafter designated as *OFD1*. Similar to *S. pombe OFD1*, the expression level of *C. albicans OFD1* increased when cells were exposed to 0.2% O_2, but was not affected by CO_2 levels or temperature (Figure S2). To determine whether a similar molecular mechanism is used by *C. albicans* to regulate Ume6 stability under hypoxic conditions, we constructed an *ofd1/ofd1* null mutant by sequential gene disruption. If Ofd1 negatively regulates Ume6 stability, a strain lacking Ofd1 would be expected to maintain hyphal elongation even in air and nutrient-rich media. Indeed the mutant exhibited a higher tendency to develop filaments, as about 11% of the *ofd1* mutant cells grew as hyphae in an overnight culture at 30°C (Figure 3A). Unexpectedly, the *ofd1* mutant could not sustain hyphal development. A significant percentage of the mutant cells had converted to yeast cells by 8 h, although nearly all grew as elongated hyphae at 4.5 h (Figure 3A). Consistent with the cell morphology results, Ume6 degradation was only partially blocked when the *ofd1* mutant was exposed to air (Figure 3B). Therefore, deletion of *OFD1* is not sufficient to completely stabilize Ume6. The stability of Ume6 in the *ofd1* mutant in air was reminiscent of its stability in wild-type cells exposed to hypoxia in the absence of 5% CO_2 (Figure 2A). We reasoned that Ofd1 senses oxygen concentration to regulate Ume6 stability; but in parallel to Ofd1, additional regulator(s) that senses high CO_2 may exist and further stabilizes Ume6. Therefore, we examined the morphology of the *ofd1* mutant in high CO_2. Indeed, in 5% CO_2, hyphale longation was fully maintained and Ume6 was completely stable in the *ofd1* mutant (Figures 3A and 3B). Oxygen depletion had no effect on Ume6 stability and hyphal elongation in the *ofd1* mutant (Figures 3A and 3B, air versus 0.2% O_2). Our results indicate that Ume6 stability is regulated by two parallel pathways in *C. albicans*.Ofd1 regulates Ume6 stability in response to oxygen concentration, and high CO_2 stabilizes Ume6 through an unknown mechanism (Figure 3C).

C. albicans Ofd1 has two functional domains: Ofd1C promotes Ume6 degradation and Ofd1N inhibits the activity of Ofd1C in hypoxia

S. pombe Ofd1 has two functionally distinct domains: the N-terminal dioxygenase domain is required for oxygen sensing, and the C-terminal domain accelerates Sre1N degradation. Also, the N-terminal domain of Ofd1 inhibits the activity of the C-terminal domain in an O_2-dependent manner (Hughes and Espenshade, 2008). To determine whether *C. albicans* Ofd1 uses the same strategy to regulate Ume6 degradation, we expressed the full length, the N-terminus (aa 1-260), and the C-terminus (aa 261-617) of Ofd1 under the control of the *ACT1* promoter in an *ofd1* mutant transformed with MET3p-Ume6$_{C778/785S}$-Myc. As shown in Figure 4A, ectopically expressing Ofd1C but not Ofd1N in the *ofd1* mutant led to Ume6 degradation even in hypoxia with 5% CO_2, a condition under which Ume6 was stabilized with the presence of a full-length Ofd1. This result suggests that the removal of the N-terminal dioxygenase domain created a constitutively active Ofd1 that was no longer inhibited by hypoxia. Because the dioxygenase domain of *S. pombe* Ofd1 functions as an

oxygen sensor that regulates the activity of Ofd1C in Sre1N degradation (Hughes and Espenshade, 2008), we next examined whether the dioxygenase domain in *C. albicans* Ofd1 has a similar function. Protein sequence alignment revealed that the characteristic HXD…H of the Fe^{2+} binding motif (Henri et al., 2010) that is essential for the enzymatic activity of dioxygenases is present in *C. albicans* Ofd1. Using this information, we constructed a *C. albicans* $Ofd1_{H162A,D164A}$ strain in which Ofd1 was mutated at the conserved Fe^{2+} binding residues (162H→A and 164D→A). This mutant failed to stabilize Ume6 in hypoxia plus 5% CO_2 (Figure 4A). Consistent with the Ume6 degradation results, we found that neither Ofd1C nor $Ofd1_{H162A,D164A}$ could sustain hyphal development in hypoxia plus 5% CO_2 (Figure 4B). Thus, our data are consistent with the model that Ofd1C is sufficient to promote Ume6 degradation, whereas the Ofd1 N-terminal dioxygenase domain functions as an oxygen sensor that regulates the activity of the C-terminal domain in response to hypoxia (Figure 4C). Based on the proposed mode of action shown in Figure 4C, Ofd1C is expected to have a dominant effect over the full-length wild-type Ofd1 on Ume6 degradation under hypoxic conditions, leading to impaired hyphal maintenance. As expected, constitutive expression of Ofd1C ($Ofd1_{261\text{-}617}$, designated *OFD1-1*) in wild-type cells led to impaired hyphal elongation in hypoxia plus 5% CO_2 (Figure 5A).

Parallel pathways to hyphal elongation: a change in *UME6* promoter chromatin or a change in Ume6 stability

Two lines of evidence suggest that *C. albicans* employs two different strategies to maintain hyphal elongation in air versus hypoxia. *brg1* and *yng2/yng2*^{K175Q} mutants had defective hyphal elongation in air but not in hypoxia plus 5% CO_2 (Figure 1C). Conversely, *OFD1-1* specifically impaired hyphal maintenance under hypoxia plus 5% CO_2, but had no effect in rapamycin in air (Figure 4B). To further demonstrate that hyphal elongation is regulated by two parallel pathways, we constructed a double *yng2/yng2*^{K175Q} *OFD1-1* mutant by transforming the $Ofd1_{261\text{-}617}$ construct into the *yng2/yng2*^{K175Q} mutant. We also combined the two hyphal elongation conditions by placing rapamycin-containing medium in a hypoxic chamber containing 0.2% O_2 and 5% CO_2. As predicted, mutants that were blocked in only one pathway, the *yng2/yng2*^{K175Q} mutant and *OFD1-1*, were defective in hyphal elongation in their respective inducing condition, but grew as elongated hyphae in the condition that combined both rapamycin and hypoxia with 5% CO_2 (Figure 5A). In contrast, the *yng2/yng2*^{K175Q} *OFD1-1* double mutant could not maintain hyphal development under all hyphal elongation conditions, including exposure to rapamycin, hypoxia, and 5% CO_2 (Figure 5A). Levels of *UME6* expression in these mutants and hyphal growth conditions closely correlated with cell morphology (Figure 5B). The *yng2/yng2*^{K175Q} mutant had low levels of *UME6* expression in rapamycin in air, but had sustained *UME6* expression similar to that of wild-type cells in hypoxia plus 5% CO_2. The *OFD1-1* mutant failed to sustain *UME6* expression when exposed to hypoxia plus 5% CO_2, but was able to maintain *UME6* expression in the rapamycin-containing medium. In contrast, the *yng2/yng2*^{K175Q} *OFD1-1* double mutant was unable to sustain *UME6* expression under single or combined hyphal elongation conditions of rapamycin and hypoxia plus 5% CO_2 (Figure 5B). Altogether, our data demonstrate that there are two redundant pathways for hyphal elongation in *C. albicans*, and either one is sufficient to sustain hyphal elongation during exposure to rapamycin, 0.2% O_2, and 5% CO_2.

Synergy between two hyphal elongation pathways for *C. albicans* virulence

Having identified an oxygen-sensing pathway that regulates hyphal elongation in parallel to the Brg1/Hda1 regulation *in vitro*, we next investigated whether the two pathways act in parallel during experimental hematogenously disseminated infection. As shown in Figure 6A, the virulence of the *OFD1-1* mutant was similar to that of the control strain carrying the full-length *OFD1*. The *yng2/yng2*^{K175Q} mutant showed reduced virulence in comparison to the wild-type strain ($p < 0.0001$, log-rank test). As the *yng2/yng2*^{K175Q} showed a similar level of virulence to the *yng2/YNG2* strain (Figure. 1B), the reduced virulence of the *yng2/yng2*^{K175Q} mutant was likely due to the copy number of *YNG2*. This reasoning is consistent with the slow growth phenotype observed with the *yng2/yng2* mutant (Lu et al., 2008), indicating additional functions of Yng2 in cellular growth and virulence. Significantly, the *yng2/yng2*^{K175Q} *OFD1-1* double mutant showed greatly attenuated virulence in comparison to the *yng2/yng2*^{K175Q} single mutant ($p < 0.0001$, log-rank test) (Figure 6A), which indicates synergy between the two parallel pathways of hyphal elongation in systematic infection. Furthermore, in studies in which mouse kidneys were fixed, sectioned, and stained to visualize fungal cells, we found that kidneys of mice infected with the *yng2/yng2*^{K175Q} *OFD1-1* double mutant contained shorter cells that appeared to be pseudohyphae, whereas a mixture of extended hyphal and pseudohyphal filaments were observed in kidneys of mice infected with the wild-type strain, and the *yng2/yng2*^{K175Q} and *OFD1-1* single mutants (Figure 6B). These data suggest that hyphal elongation *in vivo* is regulated by two parallel pathways that share overlapping functions in hyphal elongation and pathogenesis.

Discussion

Using single and double *C. albicans* mutants grown under defined *in vitro* conditions, we are able to dissect the multiple signaling pathways that are required for hyphal elongation and virulence during systemic infection. In this study, we show that *C. albicans* uses two parallel pathways to sustain hyphal development in air and in hypoxia plus CO_2 (Figure 7A). Serum, N-acetylglucosamine, or nutrient limitation activates the expression of Brg1, leading to sustained expression of hypha-specific genes through changes in promoter chromatin that occlude Nrg1 binding (Lu et al., 2012; Lu et al., 2011; Su et al., 2013). In response to hypoxia and 5% CO_2, Ofd1 and an uncharacterized CO_2 sensing pathway stabilize Ume6, leading to sustained hyphal development through a positive feedback loop in which Ume6 binds to its own promoter (Figure 7A). Disrupting one pathway blocks hyphal elongation only in response to its corresponding inducing conditions. Both hyphal elongation pathways require the hyphal initiation step, which temporally down-regulates the level of Nrg1 and up-regulates the expression of Brg1 and Ume6. This window of opportunity allows remodeling of promoter chromatin at *UME6* and other hyphal genes by Brg1 and Hda1 in response to nutrient limitation, or initiating a positive feedback loop by stabilizing Ume6 in response to hypoxia plus high CO_2 (Figure 7A). Both pathways down-regulate Nrg1 activity/level and up-regulate Ume6, consistent with the current understanding of the key functions of Nrg1 and Ume6 in dimorphism in *C. albicans*. Importantly, Ofd1-mediated regulation also functions in parallel to the Brg1/Hda1 pathway in controlling *C. albicans* hyphal elongation and virulence *in vivo*. *OFD1-1* single mutant does not show defect in hyphal maintenance and virulence compared to wild-type *OFD1* during

disseminated infection; but the *yng2/yng2*^{K175Q} *OFD1-1* double mutant displayed a profound defect in hyphal elongation and attenuated virulence in comparison to the *yng2/yng2*^{K175Q} single mutant (Figure 7B). Therefore, during disseminated candidiasis in mice, *C. albicans* is exposed to nutrient limitation, which activates the Brg1/Hda1 pathway, and to hypoxia plus elevated CO_2, which blocks Ume6 degradation via Ofd1 and another mechanism. The synergistic regulation of hyphal elongation and virulence *in vivo* by two independent pathways provides the underlying mechanism for how different host signals are integrated in the regulation of dimorphism in *C. albicans*.

We show that Ofd1, a prolyl 4-hydroxylase-like 2-oxoglutarate-Fe(II) dioxygenase, acts as an oxygen sensor that regulates hyphal development in a mechanism similar to that of the *S. pombe* Ofd1. Both Ofd1 proteins have two functional domains: an N-terminal dioxygenase domain with the conserved Fe^{2+} binding motif essential for O_2 sensing and a C-terminal domain that promotes protein degradation. The N-terminal domain inhibits the activity of the C-terminal degradation domain in hypoxia (Henri et al., 2010; Hughes and Espenshade, 2008). Our study provides a second example of a dioxygenase domain functioning in the autoregulation of protein activity in response to O_2 levels. The closest structural homolog to the N-terminal dioxygenase domain is human $PHD2_{cat}$, a prolyl 4-hydroxylase that acts as an oxygen-sensing component and post-transcriptionally hydroxylates the hypoxia-inducible transcription factor HIF1α in the presence of oxygen, thus leading to its degradation by the proteasome (Henri et al., 2010; Ozer and Bruick, 2007; Schofield and Ratcliffe, 2005). Ofd1 orthologs with sequence conservation in both N- and C-terminal domains are found only in fungi, including *C. neoformans* and *A. fumigatus*, which have the hypoxia-responsive Sre1 pathway. *S. cerevisiae* does not contain a conserved Sre1 pathway, but has an Ofd1 ortholog, Tpa1. Tpa1 is involved in translation termination, mRNA deadenylation and turnover (Henri et al., 2010; Keeling et al., 2006). As in *S. cerevisiae*, ergosterol biosynthesis in *C. albicans* is regulated by Upc2, and there is no evidence for a Sre1 pathway in *C. albicans*. Instead of regulating ergosterol biosynthesis, the C-terminal domain of *C. albicans* Ofd1 functions by accelerating the turnover of Ume6 and Hgc1, linking the Ofd1 oxygen-sensing pathway to hyphal development. Therefore, Ofd1 orthologs function in distinct pathways in different organisms.

In addition to hypoxia-responsive regulation by Ofd1, Ume6 stability in *C. albicans* is also under the control of CO_2 by an as yet uncharacterized pathway. Hypoxia or high CO_2 alone is not sufficient for Ume6 stabilization and sustained hyphal elongation. Such synergy between hypoxia and CO_2 for hyphal development has also been observed on solid media when cells were treated with rapamycin, or with deletion mutants of the Sch9 kinase, a major target of Tor1 (Stichternoth et al., 2011; Urban et al., 2007). The mechanism of CO_2 sensing and components involved in Ume6 turnover are not clear. It is known that CO_2 is hydrolyzed into HCO_3^- inside the cell naturally and through the activity of carbonic anhydrase. HCO_3^- then regulates hyphal morphogenesis through the activation of the adenylyl cyclase Cyr1, resulting in activation of the cAMP-PKA pathway (Klengel et al., 2005). HCO_3^- also signals independently of Cyr1 to regulate levels of carbonic anhydrase (Cottier et al., 2012) and promote hyphal development and cell-fate transition (Du et al.,

2012a). Stabilization of Ume6 by CO_2 is likely mediated through a Cyr1-independent pathway, as we found that CO_2 and hypoxia promote hyphal elongation, not initiation.

Pathogens must be proficient at sensing their surroundings and responding to cues to survive in the face of a changing microenvironment in the host. This is particularly important for commensal-pathogenic organisms such as *C. albicans*. For example, *C. albicans* cells can repress iron-uptake for commensalism in the gastrointestinal tract where levels of iron are high, but activate iron uptake during bloodstream infections where free iron is limited (Chen et al., 2011). Hyphal development represents a major response of *C. albicans* in adapting to changing host environments during infection. Hyphal maintenance through Brg1/Hda1 mediated chromatin remodeling requires active sensing of complex growth environments, including starvation, serum and N-acetylglucosamine (Lu et al., 2011). In parallel, hypoxia plus high CO_2 stabilizes Ume6 to sustain hyphal development. Only when both pathways are blocked do the *C. albicans cells* display a profound decrease in pathogenicity during disseminated infection. This synergy between two pathways of hyphal elongation for virulence indicates that nutrient limitation, as well as hypoxia and high CO_2, must all exist at the same time during disseminated infection, likely at the infected sites of internal organs. The multitude of host signals and the redundancy for hyphal regulation may explain why some *C. albicans* mutants have profound defects in hyphal formation and elongation *in vitro*, yet have normal virulence in mice (Noble et al., 2010). The concept of nutrient limitation here is broad, as many nutritional and stress signals, as well as pH, are integrated and signaled through the Brg1/Hda1 mediated pathway (Su et al., 2013). In addition, nutrient uptake and utilization seem to be co-regulated with hyphal growth. For example, Als3 and Rbt5, which are preferentially expressed in hyphal cells, are important for iron acquisition from host ferritin and hemoglobin, respectively (Almeida et al., 2008; Weissman and Kornitzer, 2004). Ume6 not only sustains the expression of hyphal genes but also genes involved in metabolism of nutrients, as it prevents the complete shut off of expression from the *MAL2* and *MET3* promoters (our unpublished data). From the point of evolution in fungal pathogenicity, the regulatory network for sustained hyphal development seems to have evolved recently, as the key regulators are either “young” or rewired (Nobile et al., 2012). Brg1 is only detected in species closely related to *C. albicans* (Nobile et al., 2012); Ume6 functions in different pathways in *C. albicans* versus *S. cerevisiae* (filamentation vs. meiosis); the oxygen sensor Ofd1 is rewired to control the stability of Ume6 in *C. albicans* instead of Sre1N in *S. pombe*. The evolution of redundant signaling pathways that govern hyphal elongation in *C. albicans* may reflect how a commensal organism has evolved to survive in complex and changing host microenvironments in order to become a successful pathogen.

Materials and Methods

Media and growth conditions

C. albicans strains were routinely grown at 30°C in YPD (2% Bacto peptone, 2% dextrose, 1% yeast extract). Transformants were selected on synthetic medium (2% dextrose, 0.17% Difco yeast nitrogen base w/o ammonium sulfate, 0.5% ammonium sulfate and auxotrophic supplements). Hyphal inductions were performed as follows. Strains were grown overnight

in liquid YPD at 30°C, pelleted, washed twice in PBS, resuspended in an equal volume of PBS and diluted 1:250 in YPSucrose medium (2% Bacto peptone, 2% sucrose, 1% yeast extract) with or without rapamycin at 37°C. For hyphal induction in hypoxia, experiments were carried out using a Proox P110 hypoxic chamber (BioSpherix). The oxygen concentration was maintained at 0.2% O_2 by varying the concentration of nitrogen or the gas mixture containing 5% carbon dioxide and 95% nitrogen. 250 μl of prewarmed YPSucrose medium with or without 10 nM rapamycin was added to each well of a 24-well plate, and 1 μl of overnight culture was inoculated into each well. The plate was placed into the chamber at 37°C immediately. After 12 h, cells were collected for morphological analysis.

Promoter shut down assays

C. albicans strains containing Ume6$_{C778/785S}$-Myc under the regulation of the *MET3* promoter were grown in SCD (-Met, -Cys) for 2 h to induce the expression of Ume6$_{C778/785S}$-Myc at room temperature. 25 ml of medium were transferred from the culture to a petri dish (150×15 mm), and placed it into air, a hypoxic chamber, or a CO_2 incubator as indicated. After incubation at room temperature for 4 h, 5 mM methionine was added to shut off the promoter. Aliquots were collected after the times indicated. Ume6$_{C778/785S}$-Myc protein levels were analyzed via Western blotting. The expression of Myc-Hgc1 under the regulation of the *MAL2* promoter was induced in yeast extract-peptone plus 2% maltose (YPM). The promoter shut down assay was performed as mentioned above. Glucose (2%) was added to shut off the promoter.

Virulence studies

The virulence of the various strains was analyzed in the mouse model of hematogenously disseminated candidiasis as described previously (Sanchez et al., 2004; Sun et al., 2010). Briefly, 8 male ICR mice (Taconic Farms) per strain were injected with 7.5×10^5 organisms via the lateral tail vein. The inoculum was verified by quantitative culture. In the survival experiments, the mice were monitored at least three times daily, and moribund animals were humanely euthanized. Differences in survival of mice infected with the various strains were analyzed by the log-rank test. To analyze the morphology of the different strains *in vivo*, the mice were sacrificed after 4 days of infection. Three kidneys per strain were fixed in zinc buffered formalin and embedded in paraffin, after which thin sections were cut and stained with periodic acid Schiff. All animal experiments were approved by the Los Angeles Biomedical Research Institute Animal Care and Use Committee and as outlined in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Hypoxia plus high CO_2 induce *C. albicans* hyphal elongation without chromatin remodeling
- Hyphae activator Ume6 is stabilized via Ofd1 prolyl hydroxylase in response to hypoxia
- Ume6 stabilization during hypoxia also requires high CO_2, sensed by an unknown pathway
- Ume6 stability and chromatin remodeling act parallelly for hyphal elongation and virulence

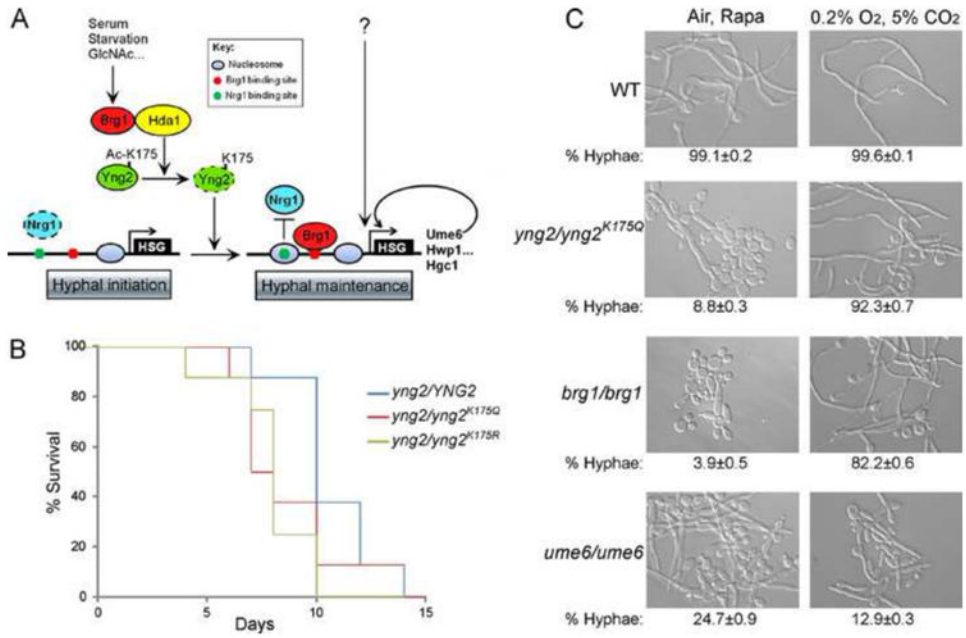

Figure 1. Hypoxia plus 5% CO_2 sustains hyphal elongation independent of the Brg1 and Hda1-mediated chromatin remodeling pathway

(A). Schematic model for regulation of hyphal maintenance in *C. albicans* by nucleosome repositioning. Dashed circles represent degraded proteins. (B). Survival of mice infected with indicated *C. albicans* strains: *yng2/YNG2* (HLY4035), *yng2/yng2*^{K175R} (HLY4036) and *yng2/yng2*^{K175Q} (HLY4037). Results are from 8 mice per strain. (C). Overnight cultures of wild type, *yng2/yng2*^{K175Q}, *brg1/brg1*, and *ume6/ume6* mutant cells were diluted into YPSucrose medium at 37°C. Half of the samples were put into the hypoxic chamber immediately. Cells were incubated for 12 h for cell morphology analysis. 10 nM rapamycin was added after 1 hour to the samples in air. Cells were collected at 6 h for cell morphology analysis. The percentage of hyphal cells was determined by counting at least 200 cells/sample. The data show the average of three independent experiments with the standard error. Cells which had a length-width ratio of >4.5 and characteristic shape were considered as hyphae. The data are represented as mean ± SEM of three independent experiments.

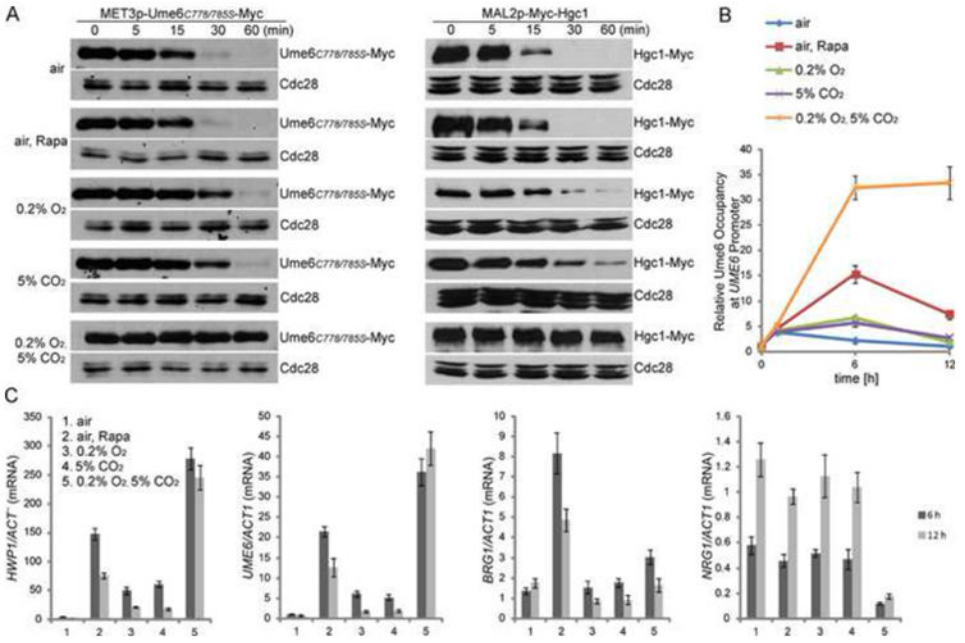

Figure 2. Ume6 is unstable in air, but stable in hypoxia plus 5% CO_2

to sustain its own transcription (A). Stability of Ume6 and Hgc1 was monitored by a *MET3* or *MAL2* promoter shut down. Western blot analysis in *C. albicans* wild-type cells expressing Ume6$_{C778/785S}$-Myc from the *MET3* promoter or expressing Myc-Hgc1 from the *MAL2* promoter. (B). Ume6 binds to its own promoter. ChIP analysis of Ume6-Myc in wild-type cells (HLY4078) under indicated conditions. The region -2967 ∼ -2715 upstream of *UME6* was used for qRTPCR. The 0 hour value was set to 1.00. Mean data ± SEM from three independent qPCR experiments was plotted. (C). Comparison of hyphal transcription programs under different hyphal elongation conditions. Wild-type cells were diluted into pre-warmed YPSucrose medium at 37°C under the indicated conditions. *HWP1, UME6, BRG1,* and *NRG1* mRNA levels were determined by qRT-PCR. The signal obtained from *ACT1* mRNA was used for normalization. The 0 h values were set to 1.00. Mean data ± SEM from three independent qRT-PCR experiments was plotted. See also Figure S1.

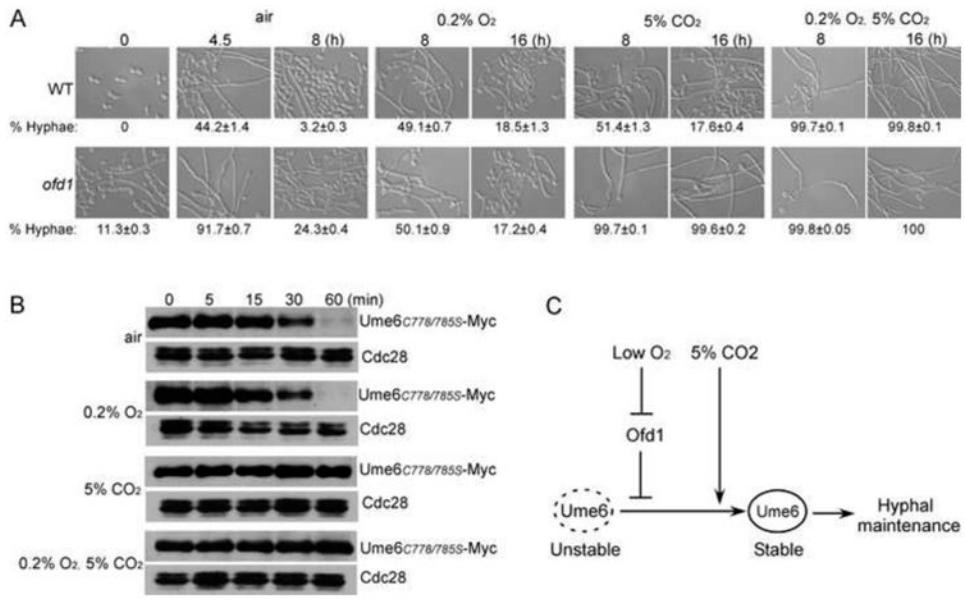

Figure 3. *C. albicans ofd1* mutant exhibits sustained hyphal elongation in 5% CO_2

(A). Overnight cultures of wild-type and *ofd1* mutant cells were diluted into YPSucrose medium at 37°C under the indicated conditions. Cells were collected at the indicated time points for cell morphology analysis. The percentage of hyphal cells was determined as described in Figure 1C. (B). Ume6 protein is stable in 5% CO_2 in the *ofd1* mutant. The protein stability of Ume6$_{C778/785S}$-Myc in the *ofd1* mutant was monitored by *MET3* promoter shut down. (C). Model for regulation of Ume6 stability by low oxygen and high CO_2. See also Figure S2.

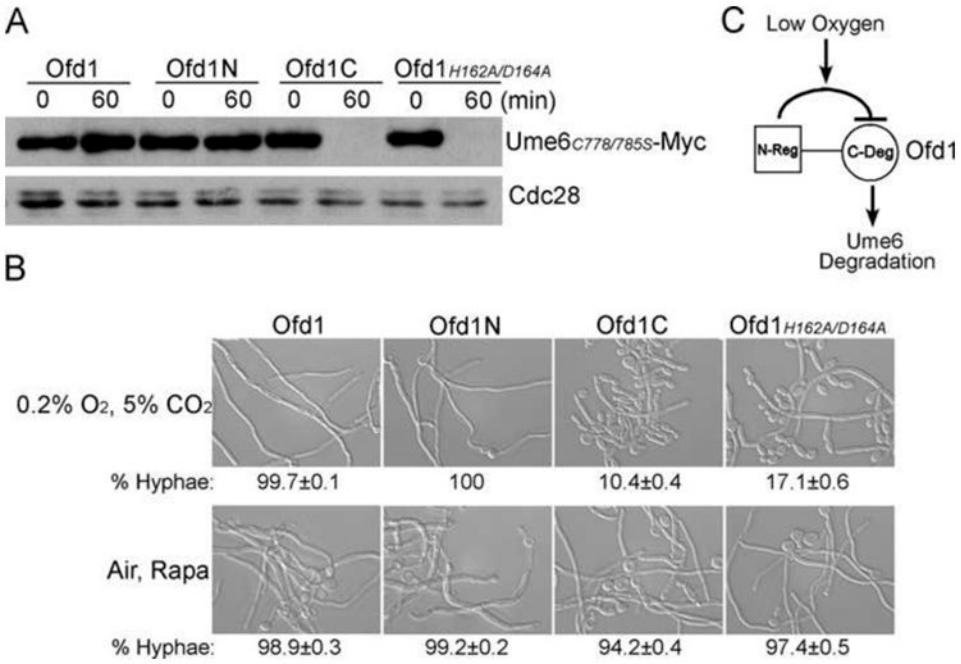

Figure 4. Constitutively expressed C-terminal domain of Ofd1 blocks hyphal elongation in hypoxia plus 5% CO_2 through destabilizing Ume6

(A). Functional analysis of Ofd1 domains in Ume6 stability. The *ofd1* mutant was transformed with Ofd1, N-terminal (aa 1-260) or C-terminal (aa 261-617) of Ofd1, or with Ofd1$_{H162A/D164A}$. Ume6 stability was monitored as described in Figure 2A. (B). Cell morphology of strains in Figure. 4A grown in YPSucrose medium at 37°C under indicated conditions. The percentage of hyphal cells was determined as described in Figure 1C. (C). Model for Ofd1 regulation.

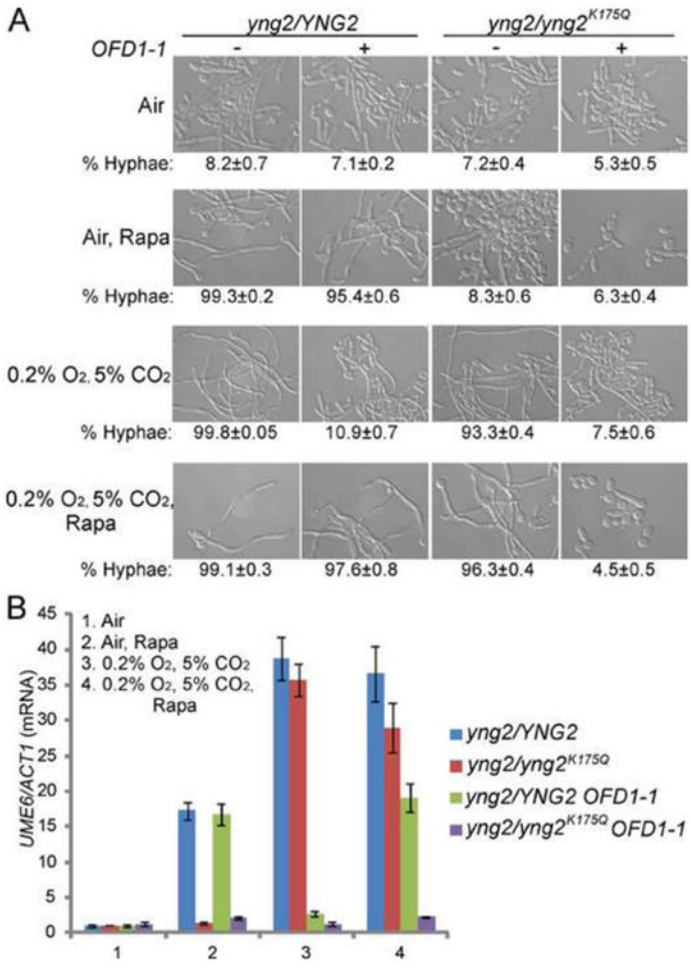

Figure 5. Two pathways regulate hyphal elongation

(A). Overnight cultures of *yng2/YNG2*(HLY4035) and *yng2/yng2*^{K175Q} (HLY4037) mutant cells transformed with or without *OFD1-1* were diluted into YPSucrose medium at 37°C under indicated conditions. 10 nM rapamycin was added to the medium as indicated. Cells were collected at 6 h (air and air plus rapamycin) or 12 h (0.2% O_2, 5% CO_2 and 0.2% O_2, 5% CO_2 plus rapamycin) for morphological analysis. The percentage of hyphal cells was determined as described in Figure 1C. (B). Overnight cultures of indicated strains were incubated as described in Figure 5A. Cells were collected at 6 h. *UME6* expression levels were quantified by qRT-PCR and normalized with *ACT1*. The value of wild-type cells in YPSucrose medium without rapamycin in normoxia was set to 1.00. Mean data ± SEM from three independent qRT-PCR experiments was plotted.

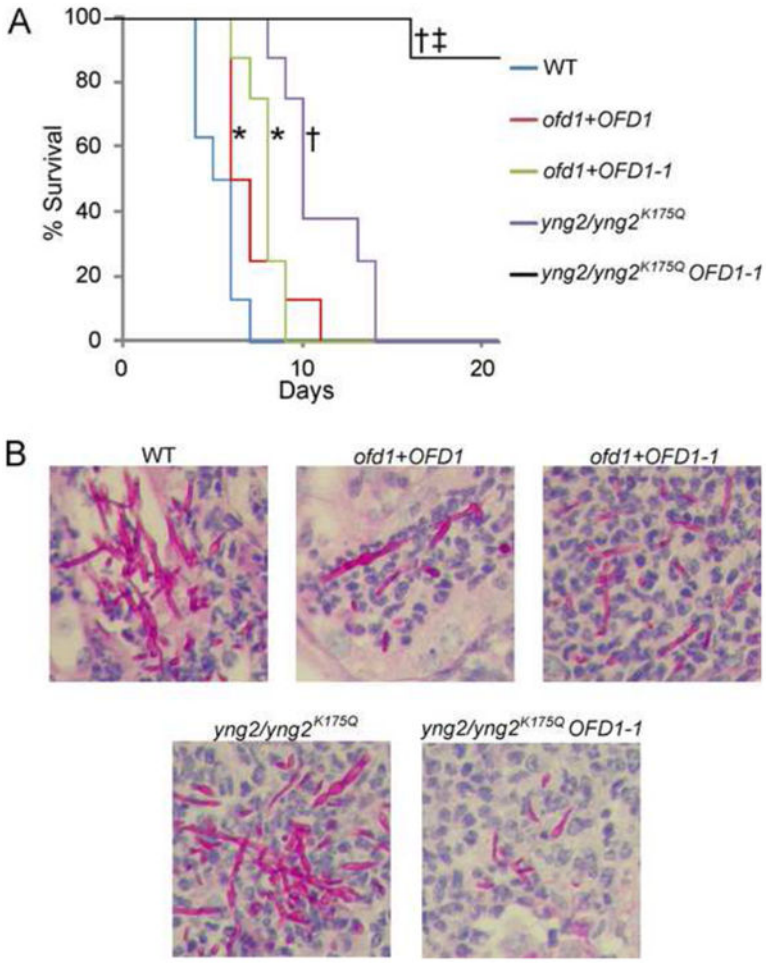

Figure 6. Blocking both pathways of hyphal elongation leads to a dramatically decreased virulence in *C. albicans*

(A). Survival of mice infected with indicated *C. albicans* strains: WT (DAY185), *ofd1+OFD1* (HLY4114), *ofd1+OFD1-1* (HLY4116), *yng2/yng2*^{K175Q} (HLY4037) and *yng2/yng2*^{K175Q} *OFD1-1* (HLY4119). Results are from 8 mice per strain. *$P < 0.02$ compared to WT; $^{\dagger}P < 0.0001$ compared to WT. $^{\ddagger}P < 0.0001$ compared to *yng2/yng2*^{K175Q}. (B). Kidney tissues from the mice infected with the same strains used in Figure 6A were fixed, sectioned, and stained to visualize fungal cells.

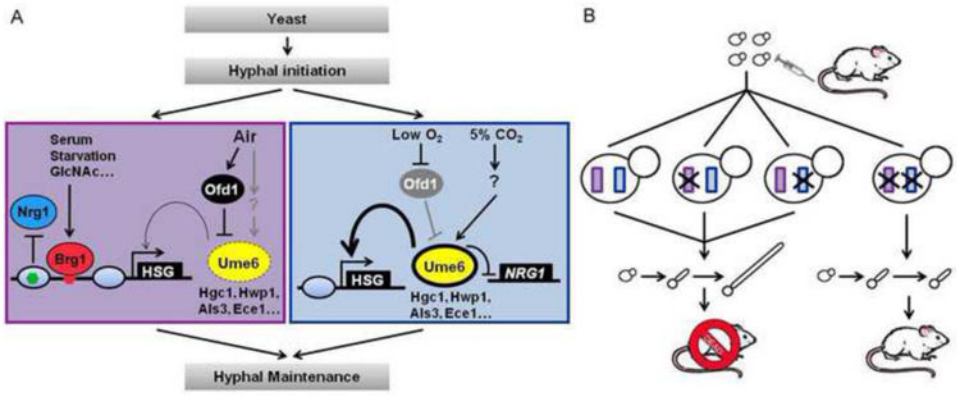

Figure 7. A schematic diagram depicting the synergy between two hyphal elongation pathways for *C. albicans* pathogenesis

(A). Two pathways for hyphal maintenance. Black lines represent active regulatory relationships; grey lines represent inactive relationships. Dashed circles represent degraded proteins. (B). Virulence is attenuated significantly when both hyphal elongation pathways are blocked. The colored rectangles in yeast cells represent the two hyphal elongation pathways of corresponding colors in Figure 7A.