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# Hypoxia and Fungal Pathogenesis: To Air or Not To Air?

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Over the last 3 decades, the frequency of life-threatening human fungal infections has increased as advances in medical therapies, solid-organ and hematopoietic stem cell transplantations, an increasing geriatric population, and HIV infections have resulted in significant rises in susceptible patient populations. Although significant advances have been made in understanding how fungi cause disease, the dynamic microenvironments encountered by fungi during infection and the mechanisms by which they adapt to these microenvironments are not fully understood. As inhibiting and preventing *in vivo* fungal growth are main goals of antifungal therapies, understanding *in vivo* fungal metabolism in these host microenvironments is critical for the improvement of existing therapies or the design of new approaches. In this minireview, we focus on the emerging appreciation that pathogenic fungi like *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* are exposed to oxygen-limited or hypoxic microenvironments during fungal pathogenesis. The implications of these *in vivo* hypoxic microenvironments for fungal metabolism and pathogenesis are discussed with an aim toward understanding the potential impact of hypoxia on invasive fungal infection outcomes.

Molecular oxygen was an important driving force in the evolution of single-cell and complex eukaryotic organisms (36, 50). Molecular oxygen plays an essential role as an electron acceptor in the generation of chemical energy via mitochondrial respiration but is also critical for the biosynthesis of sterols, mono- and polyunsaturated fatty acids, NAD, and porphyrin and in other metabolic and biosynthetic pathways (41, 94, 115). Thus, the amount of available oxygen to eukaryotic cells is a critical factor in determining overall cellular metabolism. As most eukaryotic human fungal pathogens are generally considered obligate aerobes, oxygen availability during fungal pathogenesis may play a critical role in the outcome of infection from the perspective of both the host and the fungus. In this minireview, we focus on research generated to date that largely supports a significant role for *in vivo* oxygen availability in fungal pathogenesis. We seek here to expand upon recent reviews in this area that dealt primarily with either fungal oxygen-sensing mechanisms or hypoxia responses in *Candida albicans* and *Cryptococcus neoformans* and also raise questions regarding how manipulation of fungal and host oxygen responses may be used to improve invasive fungal infection treatment outcomes (34, 42).

## WHAT IS HYPOXIA, AND DOES IT OCCUR DURING HUMAN FUNGAL PATHOGENESIS?

In a given environment, oxygen availability is usually described as anaerobic or anoxic (complete absence of oxygen), hypoxic (reduction in available oxygen compared to atmospheric levels), or normoxic (atmospheric levels of generally 21% O<sub>2</sub> or an O<sub>2</sub> partial pressure [pO<sub>2</sub>] of 159 mm Hg at sea level). In the context of microbial pathogenesis, it is generally accepted that hypoxia occurs at sites of infection, thus generating significant environmental stress on most host and microbial pathogen cells (25, 80, 84, 88). An exact oxygen level that defines localized hypoxia *in vivo* is difficult to pinpoint and will likely vary with anatomical location and distinct pathologies. However, the mammalian hypoxic response starts (as monitored through induction of the mammalian hypoxia transcription factor hypoxia-inducible factor 1 [HIF-1]) in most cells at an oxygen level of ~6% (pO<sub>2</sub>, 40 mm Hg) (110). In healthy tissues in the human body, oxygen levels of 2.5% to 9% are

considered normal, while oxygen levels of ≤1% that have been described in tumors and wounds are typically considered hypoxic (1, 28, 77, 80, 109, 120). In the healthy human lung, the initial deposition site of many human fungal infections, alveolar pO<sub>2</sub> is around 100 to 110 mm Hg (~14% O<sub>2</sub>) (57).

When an invading microbe interacts with host cells, tissue damage due to inflammation, thrombosis, and necrosis is thought to decrease available oxygen concentrations due to decreased tissue perfusion at the site of infection (32, 80). For example, it has recently been observed that production of the nonribosomal peptide gliotoxin and other potential secondary metabolites by the mold *Aspergillus fumigatus* contributes to the inhibition of angiogenesis in the lung (7). Inhibition of neovascularization at the site of infection by the fungus is likely to cause significant tissue necrosis, prevent tissue repair, and thus contribute to the development of localized and systemic hypoxia. Moreover, it is likely that oxygen-dependent metabolism of both pathogen and host cells also contributes to the rapid utilization of available oxygen, though this remains to be definitively confirmed. Importantly, at least within the lung, the co-occurrence of microbial infection and hypoxia is often associated with poor clinical outcomes (3, 12, 101).

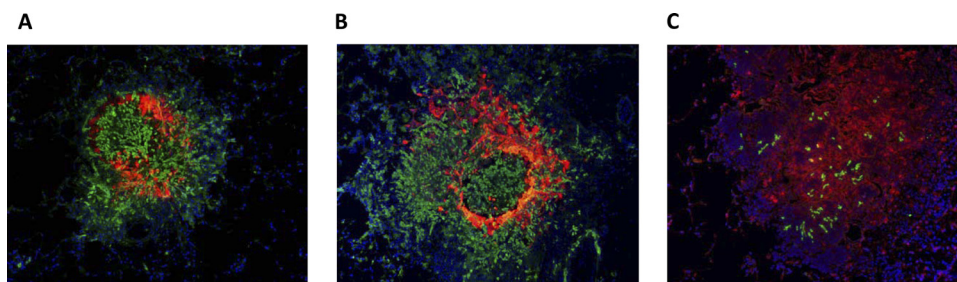
Oxygen concentrations at sites of human fungal infection have not been measured directly *in vivo*, though hypoxemia is often described as part of the clinical picture associated with these infections, even in the lung, that may require invasive or noninvasive oxygen therapy (13, 14, 53, 64, 70, 98). In addition, CO<sub>2</sub> production is directly coupled to oxygen consumption of eukaryotic cells and sites of hypoxia *in vivo* often contain increased levels of this gas, whose sensing has been linked to fungal virulence (31, 67). The interconnections and relationship between oxygen and

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**FIG 1** Hypoxia occurs in murine models of IPA. The hypoxia detection agent pimonidazole hydrochloride, Hypoxyprobe-1, was used to monitor *in vivo* hypoxia in three immunologically distinct murine models of invasive pulmonary aspergillosis. Green, *A. fumigatus*; blue, 4',6-diamidino-2-phenylindole (DAPI)-stained host cells; red, hypoxia as detected by Hypoxyprobe-1. All images are from day 4 after inoculation and represent merged images as described in reference 44. (A) Chemotherapy model, outbred CD1 mice immunosuppressed with cyclophosphamide and triamcinolone and inoculated with *A. fumigatus* conidia. (B) Corticosteroid model, outbred CD1 mice immunosuppressed with a single dose of triamcinolone and inoculated with *A. fumigatus* conidia. (C) Chronic granulomatous disease model, gp91phox<sup>-/-</sup> mice deficient in NADPH oxidase activity and inoculated with *A. fumigatus* conidia. Data are from experiments in reference 44.

carbon dioxide sensing with regard to fungal pathogenesis is an exciting area for further investigation (113).

With regard to *Candida albicans*, one of the most frequently occurring human fungal pathogens, its normal anatomical location is the human gastrointestinal tract, which contains significant regions of hypoxia (49, 63). For cryptococcal meningitis caused by *Cryptococcus neoformans*, oxygen concentrations in the human brain are also significantly lower than in the atmosphere, indicating that *C. neoformans* is also faced with reduced oxygen levels during infection (33, 106). In further support of the idea that fungal pathogens face significant oxygen depletion during pathogenesis, hypoxia at the site of infection has recently been confirmed in murine models of invasive pulmonary aspergillosis (IPA). In two related studies of murine IPA models, a luciferase-producing *A. fumigatus* strain showed decreased luminescence *in vivo* after reaching a maximum at day 1 postinfection (despite an increase in fungal burden). The authors hypothesized that this observation was due to severe tissue damage caused during infection, which may decrease oxygen availability. Thus, the lack of luminescence may be attributable to hypoxia at the site of infection, as oxygen is essential for the light-producing luciferase reaction (15, 55).

Additional indirect evidence that hypoxia is an important component of the *in vivo* microenvironment during a pulmonary fungal infection is the recent detection of ethanol production in bronchoalveolar lavage fluid from a chemotherapeutic murine model of IPA. It was further found that, under normoxic conditions, *A. fumigatus* did not produce detectable ethanol levels in culture supernatants but, upon exposure to hypoxia, *in vitro* culture supernatants from shake flask cultures contained significant amounts of ethanol (44). These data suggest that in response to hypoxia *A. fumigatus* can ferment glucose or other fermentable carbon sources into ethanol. In addition to *in vivo* ethanol detection, hypoxia was directly monitored in the lung in three immunologically distinct murine models of IPA using the hypoxia detection agent pimonidazole hydrochloride (Hypoxyprobe-1) (Fig. 1) (44). The results of this study suggest that the influx and activity of host immune cells are strong contributors to the development of hypoxia during an invasive pulmonary fungal infection, as more-extensive hypoxia was detected in murine models characterized by strong inflammatory responses (steroid treatment and chronic granulomatous disease) than those characterized by fun-

gal proliferation and tissue invasion (chemotherapeutic model). However, the persistence and occurrence of hypoxia in the chemotherapeutic model also suggests that direct actions of the invading fungus are important contributors to *in vivo* hypoxia. Studies of the occurrence of hypoxia in other models of fungal infections await to be undertaken but are essential to understanding when and where different fungal pathogens are exposed to oxygen limitation. In summary, these observations suggest that human fungal pathogens are faced with rapidly changing oxygen availability during fungal pathogenesis, which may suggest that the ability to adapt to low-oxygen environments is critical for fungal virulence. In the following sections, we review our understanding of how normally aerobic fungal organisms adapt to hypoxia and whether these mechanisms are linked to the ability of these organisms to cause lethal disease.

## FUNGAL ADAPTATION TO HYPOXIA

As the majority of human fungal pathogens do not normally inhabit the human body, at least as far as we currently understand, and are often associated with infection only in immunocompromised patients, a key question is how these fungi evolved and maintained their ability to adapt to hypoxia. The mold *A. fumigatus* is typically found in soil and decaying organic material such as compost heaps. These environments are relatively oxygen poor, as oxygen concentrations in compost piles rapidly change with the metabolic activity of the microflora and range from atmospheric (21%) to hypoxic (1.5% and lower) (122). This indicates that organisms that thrive in such environments have evolved hypoxia adaptation mechanisms. Moreover, the soil itself can become hypoxic after heavy rains or due to increased CO<sub>2</sub> levels, and thus soilborne organisms have evolved mechanisms to tolerate low and rapidly changing oxygen levels (26, 74). Although most molds are traditionally considered obligate aerobes, *A. fumigatus* has been observed to tolerate oxygen levels as low as 0.1%, and several older studies even suggest that, under the right nutrient conditions, *A. fumigatus* can survive and grow anaerobically (47, 86, 117). In addition, *Fusarium* species seem particularly adept at tolerating hypoxic and even anoxic conditions, which is consistent with their resident ecological niche, soil (45, 51). Thus, these studies strongly suggest that molds like *A. fumigatus* and *F. oxysporum*, which cause human disease, may not be typical obligate aerobes but rather are likely to be facultative anaerobes. More research on the

metabolic physiology of these important pathogens is needed to further understand their ability to deal with low oxygen levels.

With regard to human-pathogenic yeasts, *C. albicans* is capable of low levels of growth under anaerobic conditions and can also ferment glucose to ethanol predominantly under low-oxygen conditions, suggesting a Pasteur effect typically associated with other facultative fermentative yeasts (99). In contrast, *C. neoformans* seems to be a true obligate aerobe, though the precise effects of low-oxygen conditions on *C. neoformans* or *Cryptococcus gattii* growth and metabolism have not been elucidated to the degree that they have in *C. albicans*. In RPMI 1640, a medium commonly used for Clinical and Laboratory Standards Institute (CLSI) anti-fungal drug susceptibility screening, oxygen is a limiting factor for *C. neoformans* growth and cell cycle progression also seems dependent on oxygen availability (82, 83, 118). Taken together, these data suggest that human-pathogenic fungi have evolved mechanisms to adapt to low-oxygen environments that occur in their natural environments as well as during fungal pathogenesis. It is unclear whether hypoxia adaptation mechanisms directly contribute to the distinction between pathogenic and nonpathogenic fungi, but data suggest that responses are similar in nonpathogens such as *Schizosaccharomyces pombe* (119). A further detailed analysis of pathogenic and nonpathogenic fungal hypoxia adaptation mechanisms is needed to determine whether human-pathogenic fungi contain unique mechanisms of hypoxia adaptation that allows them to thrive in mammalian hosts.

#### FUNGAL ADAPTATION TO HYPOXIA—A GENOMIC APPROACH

In order to understand how human-pathogenic fungi adapt to oxygen limitation and whether this is important for fungal pathogenesis, the molecular mechanisms associated with the ability to adapt to hypoxia have been investigated by global fungal transcriptome and proteome profiling studies. Perhaps not surprisingly, hypoxia causes significant changes in both mRNA levels and protein abundance in human-pathogenic fungi. While certain conserved themes have emerged from these studies, an important consideration for their interpretation is the methodology used to generate the RNA or protein samples examined for differential changes in response to hypoxia. Oxygen concentrations are often variable between studies (typically between 0.2% and 1% or not defined when flasks were flushed with nitrogen), fungal material was harvested after different periods of hypoxic exposure ranging from 2 h to around 8 days, and carbon, nitrogen, and micronutrient sources in the growth media are also variable (21, 71, 105, 116, 121). Despite these variables, there are general themes that have emerged as to how human-pathogenic fungi adapt to hypoxia that are discussed below organized via the respective human fungal pathogens.

#### CANDIDA ALBICANS

Three major transcriptome analyses of the *C. albicans* response to hypoxia have been published and generally have concordant results despite differences in experimental design, particularly with respect to how oxygen levels were manipulated and the fungal strain used (2, 105, 116). Major themes of the *C. albicans* hypoxia response emerging from these studies include transcript level increases of genes involved in iron metabolism, heme biosynthesis, fatty acid metabolism, ergosterol biosynthesis, glycolysis and fermentation, cell wall and membrane structure, and hypha-specific

transcripts (2, 105, 116). In contrast, transcripts involved in oxidative respiration, such as those from the tricarboxylic acid cycle and mitochondrial respiration chain along with general ATP synthesis, were decreased in response to hypoxia (2, 105, 116). The increase in transcripts associated with ergosterol biosynthesis, heme, and unsaturated fatty acids is perhaps not surprising, given the importance of oxygen in the biosynthesis of these critical molecules. Ergosterol biosynthesis transcript increases due to hypoxia are highly dependent on the transcription factor *UPC2* (75, 108, 116), while the transcriptional regulator *EFG1* is critical for positively regulating fatty acid biosynthesis in hypoxia (105). Recently, the hypoxic induction of glycolytic genes in *C. albicans* was shown to be highly dependent on the transcription factors *TYE7* and *GAL4* (2). Hypoxia is associated with the induction of hyphal formation in *C. albicans*, particularly under conditions in embedded agar, and the transcript profile of hypoxia-grown yeast in liquid culture is partially similar to a hyphal gene pattern of expression (6, 16, 105). This may have particular relevance to fungal pathogenesis as the yeast-to-hypha transition is thought to be important for *C. albicans* virulence (reviewed in reference 114). *EFG1* regulates about half of all hypoxia-regulated genes in *C. albicans* and plays a unique role in hypoxia signaling as it also prevents the expression of genes not required for the hypoxia response. *C. albicans* contains an *EFG1* homolog, *EFH1*, that is also involved in morphogenetic and hypoxic signaling (30). Deletion of both *EFG1* and *EFH1* derepresses an alternative pathway of hypha formation that is dependent upon oxygen. In contrast, null mutants of the transcriptional regulator *ACE2* required for the transcription of genes involved in cell separation are unable to form filaments under hypoxic conditions (79). Though the exact mechanism of the filamentation defect of the *ACE2* null mutant under hypoxia is unknown, it may be due to differences in the activity of the respiration chain and/or defects in oxygen-dependent steps in the ergosterol biosynthesis pathway.

#### CRYPTOCOCCUS NEOFORMANS

Compared to *C. albicans*, less is known about the effects of hypoxia on *C. neoformans*. A transcriptome analysis of the *C. neoformans* response to hypoxia revealed that, similar to the *C. albicans* hypoxia response, heme biosynthesis, fatty acid metabolism, ergosterol biosynthesis, and stress response transcripts were increased (21). However, in stark contrast to *C. albicans*, respiration-related transcripts were also increased in response to hypoxia while cell wall and capsule biosynthesis genes were reduced (21). Given the different lifestyles of *C. neoformans* and *C. albicans*, it may not be surprising that these two organisms have different transcriptional responses to hypoxia. In support of the key role of respiration in *C. neoformans* hypoxia adaptation, an *Agrobacterium tumefaciens*-mediated forward genetic screening identified several mutants defective in hypoxic growth that were associated with mitochondrial function (56).

To date, hypoxia responses in *C. neoformans* have been shown to be dependent on the transcriptional regulator *SRE1*, a member of the sterol regulatory element binding protein (SREBP) family, and *TCO1*, a member of a highly conserved family of fungus-specific histidine kinases (19, 21). *SRE1* was shown to be a key regulator of genes involved in ergosterol biosynthesis and metal uptake in response to hypoxia and the *SRE1* null mutant is significantly impaired in hypoxic growth (19, 21). Surprisingly, transcripts induced in hypoxia appear to not be affected in terms of



quantity in the absence of *TCO1*, and the authors hypothesize that *TCO1* acts posttranscriptionally to mediate its impact on hypoxic growth of *C. neoformans* (21). Thus, many exciting areas for investigation of the *C. neoformans* hypoxic response remain to be elucidated, and it will be important to determine whether these mechanisms are conserved in the related human pathogen *C. gattii*.

### ASPERGILLUS FUMIGATUS

In addition to *C. albicans* and *C. neoformans*, recent studies assessing transcriptome and proteome responses to hypoxia in the mold *A. fumigatus* have been conducted (5, 121). These studies differ in design, as one study focused on early time points of hypoxia adaptation (2 to 24 h) in batch culture (5) while the other study focused on long-term hypoxic exposure with an incubation time of around 8 days in chemostat cultures with glucose limitation (121). In the *A. fumigatus* early hypoxia adaptation study, the authors observed that transcripts involved in glycolysis, fermentation, ergosterol biosynthesis, the  $\gamma$ -aminobutyrate (GABA) shunt, and iron uptake were induced in hypoxia (5). The induction of the GABA shunt has not been previously observed to be induced in response to hypoxia in either *C. albicans* or *C. neoformans* but has in the related filamentous fungus *A. nidulans* (76, 107). The GABA shunt bypasses the tricarboxylic acid (TCA) cycle, contributes to glutamate formation, and is hypothesized to be involved in the prevention of NADH accumulation in the absence of a terminal electron acceptor such as oxygen (35). This indicates that *Aspergillus* species not only use fermentation to replenish sources of NAD<sup>+</sup> for continued glycolytic flux but also appear to use the GABA shunt to prevent NADH accumulation. Under the culture conditions examined, TCA cycle- and mitochondrial respiration chain-associated transcripts were found to be largely decreased in *A. fumigatus* during the early hypoxia response, similar to *C. albicans* (5, 105, 116). However, in the *A. nidulans* hypoxia transcriptome analysis, several TCA cycle-associated transcripts were increased in response to hypoxia, though other respiration-associated transcripts were largely decreased (76). An *A. nidulans* hypoxia proteomic study in the same laboratory found that respiration and TCA cycle protein abundance were not altered in hypoxia (107). Thus, it is unclear whether the observed TCA hypoxia responses are due to differences between the culture conditions used in the studies. However, this question warrants further investigation as the reductive TCA cycle has been observed to be important for *Mycobacterium tuberculosis* adaptation to oxygen-limiting environments through increased fumarate reductase activity (124). *A. fumigatus* contains a putative fumarate reductase ortholog, OsmA, which to date is uncharacterized.

In contrast to the transcriptomic and proteomic studies conducted at early hypoxia adaptation time points, Vödisch et al. found that after long-term exposure to hypoxia protein levels of glycolysis-, TCA cycle-, and respiration-, but not fermentation-associated enzymes are increased in *A. fumigatus* (121). This suggests that oxidative respiration might be important for long-term growth under hypoxic conditions, while fermentation seems to be used for early hypoxia adaptation. However, the lack of fermentation in this study is likely a result of the extreme glucose-restricted conditions and constant turnover of the medium used in the chemostat (121).

Like *C. neoformans*, *A. fumigatus* has a member of the SREBP family of transcriptional regulators named SrbA. Loss of SrbA in

*A. fumigatus* as in *C. neoformans* significantly impairs growth in hypoxia and many of the SREBP-dependent transcripts in *C. neoformans* were also found to be SREBP dependent in *A. fumigatus*, including transcripts involved in ergosterol biosynthesis and metal uptake (10, 128). Taken together, transcriptome and proteome profiling experiments have revealed common themes among the human-pathogenic fungi with regard to mechanisms of hypoxia adaptation that have facilitated mechanistic analyses through gene replacement approaches based on molecular genetics. The impact of these studies on our understanding of human fungal pathogenesis is addressed in the following section.

### FUNGAL ADAPTATION TO HYPOXIA—LINKS TO FUNGAL PATHOGENESIS

Taken together, null mutants of key hypoxia-regulated genes or regulators of fungal hypoxia adaptation generally display attenuated virulence in murine models of fungal infection (Table 1). A common theme of many of the genes listed in Table 1 is that they are key regulatory proteins that mediate a significant number of biological responses in fungi not exclusive to hypoxia adaptation. Thus, defects in the regulation of basic fungal metabolism often results in significant growth attenuation in low-oxygen environments when metabolic control becomes essential for survival in the absence of oxygen (particularly with regard to the biosynthesis of sterols, fatty acids, heme, and iron homeostasis mechanisms). In theory, many of the molecules whose biosynthesis is reduced or inhibited in hypoxia may be available to the invading fungus from host cells. However, whether fungi have appropriate uptake systems to utilize these molecules in the absence of oxygen is unclear. What does seem clear is that these molecules that require oxygen for their biosynthesis are likely indirect sensors of oxygen levels that stimulate changes in overall fungal metabolism to allow the organisms to adapt to hypoxia. How pathogenic fungi actually sense and respond to changing oxygen levels is still an area of ongoing investigation (reviewed in reference 42).

A common theme in fungal hypoxia responses that appears to be directly related to fungal pathogenesis is the ability of fungi to sense changes in sterol levels. In the human fungal pathogen *C. albicans*, a *UPC2* ortholog has been identified and shown to be activated under hypoxic conditions and in response to lowered sterol levels (52, 108, 116, 127). *UPC2* null mutants have significant growth reductions in hypoxia and no longer form filaments, but *UPC2*'s link with pathogenesis is unknown (Table 1). However, a main line of evidence for a direct link between fungal hypoxia adaptation and the ability to cause lethal disease comes from studies on SREBPs in the yeast *C. neoformans* and the mold *A. fumigatus* (8, 19–21, 128). SREBPs are conserved in a wide range of eukaryotes and are membrane-bound transcription factors that are activated by proteolytic cleavage. The SREBP pathway in fungi was first identified and studied in the nonpathogenic yeast *S. pombe*, where it was found to be a major regulator of the hypoxic response and essential for growth under these conditions (9, 54). In both *S. pombe* and *C. neoformans*, *SRE1* has been shown to be activated by proteolytic cleavage in response to hypoxia (which stimulates a depletion of total cellular ergosterol levels) and direct ergosterol depletion (as mediated by various antifungal agents that target ergosterol biosynthesis such as the triazoles) (9, 19, 54, 90, 119). In both *C. neoformans* and *A. fumigatus*, the respective SREBP ortholog (Sre1 for *C. neoformans* and SrbA for *A. fumigatus*) is required for fungal growth in hypoxia and responses to

TABLE 1 List of genes involved in hypoxia adaptation in human-pathogenic fungi and their role in virulence<sup>a</sup>

Gene	Function	Organism	Hypoxia null mutant phenotype (reference[s])	Role in virulence (reference[s])
<i>UPC2</i>	Transcription factor, zinc cluster family	<i>Candida albicans</i>	Significant growth attenuation (75, 116)	Not reported
<i>CZF1</i>	Transcription factor, zinc finger domain	<i>C. albicans</i>	Required for filamentous response to hypoxia (16)	Not reported
<i>EFG1</i>	Transcription factor, APSES family	<i>C. albicans</i>	Hyperfilamentous response to hypoxia (105)	Increased <i>in vivo</i> proliferation (66), virulent but kinetics of mortality altered (73)
<i>EFH1</i>	Transcription factor, APSES family	<i>C. albicans</i>	None	Increased intestinal tract persistence (126)
<i>TYE7</i>	Transcription factor	<i>C. albicans</i>	Moderate growth defect (2)	Significant attenuation <sup>b</sup> (2)
<i>ACE2</i>	Transcription factor, Swi5 family	<i>C. albicans</i>	Required for filamentous response to hypoxia (79)	Significant attenuation (65)
<i>RAS1</i>	Small GTPase	<i>C. albicans</i>	Required for filamentous response to hypoxia (116)	Significant attenuation (69)
<i>CDC35</i> ( <i>CYR1</i> )	Adenylate cyclase	<i>C. albicans</i>	Required for filamentous response to hypoxia (116)	Significant attenuation (95)
<i>SCH9</i>	AGC protein kinase	<i>C. albicans</i>	Required for filamentous response to hypoxia (113)	Significant attenuation (72)
<i>TCO1</i>	Histidine kinase part of two-component regulatory system	<i>Cryptococcus neoformans</i>	Significant growth attenuation (21)	Significant attenuation (21)
<i>SRE1</i>	Transcription factor, SREBP family, bHLH <sup>c</sup> DNA binding	<i>C. neoformans</i>	Significant growth attenuation (19, 21)	Significant attenuation (19, 21)
<i>SCP1</i>	Sterol cleavage-activating protein, senses sterols and is involved in Sre1 signaling	<i>C. neoformans</i>	Significant growth attenuation (19, 21)	Significant attenuation (21)
<i>STP1</i>	Protease involved in proteolytic cleavage of Sre1	<i>C. neoformans</i>	Significant growth attenuation (8, 21)	Significant attenuation (8, 21)
<i>KAP123</i>	Nuclear transport, involved in <i>SRE1</i> pathway	<i>C. neoformans</i>	Significant growth attenuation (20)	No attenuation (20)
<i>GSK3</i>	Glycogen synthase kinase, involved in <i>Sre1</i> turnover/phosphorylation	<i>C. neoformans</i>	Significant growth attenuation (20)	Significant attenuation (20)
<i>srbA</i>	Transcription factor, SREBP family, bHLH DNA binding	<i>Aspergillus fumigatus</i>	Significant growth attenuation (128)	Significant attenuation (128)
<i>ireA</i>	Type 1 membrane protein, protein kinase domain and endoribonuclease domain	<i>A. fumigatus</i>	Moderate growth attenuation (37)	Significant attenuation (37)
<i>cycA</i>	Cytochrome <i>c</i>	<i>A. fumigatus</i>	Moderate growth attenuation	Significant attenuation
<i>ADH1</i>	Alcohol dehydrogenase	<i>Fusarium oxysporum</i>	Moderate growth attenuation (22)	Moderate attenuation (22)

<sup>a</sup> Does not include the cobalt chloride-sensitive *C. neoformans* mutants isolated from a T-DNA insertion mutagenesis library that are also sensitive to hypoxic growth. A list of these genes can be found in Table 1 of the original publication (56).

<sup>b</sup> Significant attenuation in *Galleria mellonella* model. Single *Tye7* mutant virulence not reported in mice, but a *gal4/tye7* strain is highly attenuated in A/J mice (4).

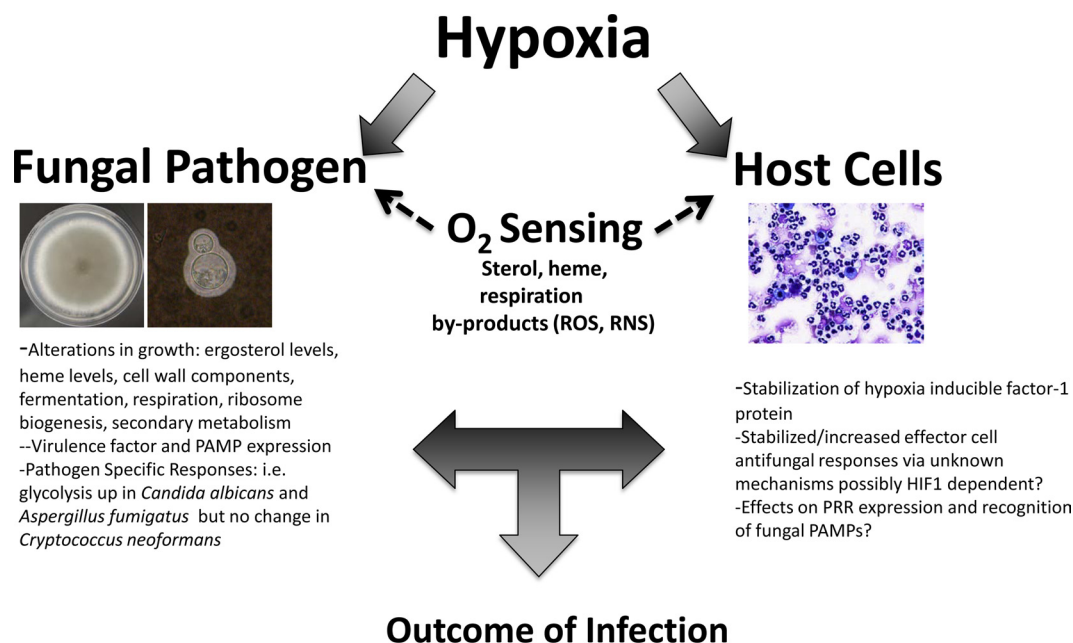
<sup>c</sup> bHLH, basic helix-loop-helix.

triazole antifungal drugs. Subsequently, *sre1* and *srbA* null mutants are strongly attenuated in virulence in murine models of cryptococcosis and IPA (19, 21, 128). Deletion of other regulatory components of the SREBP pathway in *C. neoformans* also results in hypoxia growth defects and attenuated virulence (8, 20, 21). A more in-depth review of the mechanisms of SREBP regulation in yeast was recently published (9).

An apparently SREBP-independent pathway of sterol homeostasis has also been found to be critical for *A. fumigatus* hypoxia adaptation and virulence, the fungal unfolded-protein response, which in mammals is known to be critical for hypoxia responses (96). An *A. fumigatus* endoplasmic reticulum stress sensor IreA null mutant was found to be moderately attenuated in hypoxic growth compared to normoxic growth and was essentially avirulent in a murine model of IPA (37). Like SREBP null mutants, the IreA null mutant displays sensitivity to triazole antifungal drugs, alterations in transcript levels of key ergosterol biosynthesis genes,

and a reduction in total cellular ergosterol content (35). Thus, perturbations in ergosterol biosynthesis are a common theme among some of the isolated fungal null mutants with defects in hypoxic growth.

Another group of molecules that may be critical for hypoxia responses in fungi are reactive oxygen and nitrogen species (ROS and RNS, respectively). In the model yeast *Saccharomyces cerevisiae*, it has been proposed that the fungal respiratory chain is involved in oxygen sensing, growth in hypoxia, and hypoxic gene regulation through production of ROS and RNS (17, 27, 46, 56, 68, 91, 92). Furthermore, several studies have suggested that increased oxidative stress observed in hypoxia may act as a putative second messenger that activates redox-sensitive transcription factors to enable hypoxia adaptation (18, 29, 46). Recently, loss of cytochrome *c* (*CycA*) in *A. fumigatus* was shown to decrease hypoxic growth and concomitantly increase ROS resistance with a significant attenuation in virulence (43). While the mechanism is



**FIG 2** Confirmed and potential effects of hypoxia on fungal pathogenesis. Molecular oxygen is critical for numerous cellular processes, including ATP production and the biosynthesis of key molecules such as sterols, fatty acids, and NAD. Thus, loss of oxygen at sites of fungal infections influences the physiology of both the fungal pathogen and immune cells of the host that affect the final outcome of the fungus-host interaction. Images, from left to right: *A. fumigatus* colony on nutrient agar; *C. neoformans* yeast; and monocytes, neutrophils, and macrophages from lung bronchoalveolar lavage fluid. PAMP, pathogen-associated molecular pattern, PRR, pattern recognition receptor. Suggested impacts on the host side are based on data largely from bacterial pathosystems and have not been confirmed to be active in fungus-host interactions to date.

speculative at this point, it may be that alteration of ROS homeostasis and RNS production from complex IV of the mitochondrial electron transport chain interferes with the ability of *A. fumigatus* to adapt to hypoxia and pulmonary microenvironments. Thus, the exact role of the respiratory chain in hypoxic signaling in human fungal pathogens is still undefined but promises to be an exciting area of further investigation.

An intriguing finding with the *A. fumigatus* cytochrome *c* mutant is its inability to produce ethanol in response to hypoxia (43). While ethanol production *per se* was found to not be essential for *A. fumigatus* growth in hypoxia and *in vivo*, ethanol fermentation mutants stimulated increases in interleukin-8 homolog levels and neutrophil numbers in the lung in a corticosteroid model of IPA that correlated with decreased fungal burden (44). These results suggest that fungal responses to hypoxia *in vivo* may not only affect fungal growth directly but also affect the production of fungal metabolites that can subsequently alter host immune responses.

Further evidence for this hypothesis, which remains to be further examined, are the significant changes in cell wall biosynthesis transcripts in response to hypoxia in *C. albicans* and *A. fumigatus* that could alter pathogen-associated molecular pattern expression and/or exposure (Fig. 2). It has been shown that cell wall protein expression is altered in response to hypoxia in *C. albicans* (111). Thus, the effects of hypoxia on fungal pathogenesis are likely to be more complex than simply altering rates of *in vivo* fungal growth. Consequently, in future studies, it will be intriguing to elucidate how different fungal metabolites are produced in response to changing oxygen levels and how this affects fungal pathogenesis.

In addition to fungal transcription factors that respond to changes in sterol levels, other transcriptional regulators associated

with hypoxia responses have been investigated for their roles in fungal pathogenesis. In *C. albicans*, these include *EFG1*, *EFH1*, *ACE2*, and *TYE7* (2, 30, 79, 105). *EFG1* null mutants are hyperfilamentous under low-oxygen conditions, possibly due to down-regulation of the filamentous repressor *NRG1* under these conditions, and thus, *EFG1* negatively regulates hypoxia-induced filamentation in *C. albicans* (105). Consequently, in an *in vivo* infection model, an *efg1* null mutant grew more than a wild-type strain (66). Thus, this *in vivo* result partially supports a role for hypoxia in *C. albicans* pathogenesis, as the yeast-to-hypha switch is generally thought to be a major virulence attribute of this organism. In general, the *in vivo* phenotype of the *EFG1* null mutant is surprising given the importance of this transcription factor in regulating gene expression in *C. albicans*. Furthermore, a recent study observed that under hypoxic conditions *EFG1* induces all major classes of genes known to be associated with biofilm formation, a major cause of persistence and antifungal resistance in *C. albicans* infections, and that *EFG1* is required for biofilm formation (112). As biofilms contain significant layers of hypoxia, it is tempting to speculate that regulators of biofilm formation may also have roles in fungal hypoxia adaptation that have been largely unexamined (39, 81, 97). Another important role for the *Efg1*/*Efh1* gene expression network, and thus hypoxia, is in *Candida* commensalism. *EFH1/EFH1* null mutants of *C. albicans* are able to persist in the gastrointestinal (GI) tract at higher levels than wild-type strains, while cells that overexpress *EFH1* are significantly reduced in GI tract colonization (126). Thus, given the hypoxic environment in the GI tract and the propensity of *Candida* to colonize this location, it is tempting to speculate that *Candida* responses to hypoxia are critical for evasion of the host immune system under these conditions to allow commensalism.

With regard to *ACE2* null mutants that display a significantly attenuated ability to form filaments under hypoxia, they are attenuated in virulence in a murine model of systemic candidiasis (Table 1) (65, 79). The exact molecules that stimulate *ACE2* activity are unknown. In contrast, a *TYE7* null mutant displays a slight reduction in hypoxic growth on solid agar and a corresponding moderate virulence attenuation in a *Galleria* model of infection. Additional deletion of *GAL4*, leading to a *gal4/gal4/tye7/tye7* double mutant, significantly attenuates hypoxic growth and virulence in a murine model of systemic candidiasis (2). Both transcription factors are important for controlling glycolysis, a pathway important in fungal responses to hypoxia. Thus, it is intriguing to speculate that glycolytic intermediates may be involved in regulating hypoxia responses in *C. albicans*. In addition to the transcriptional regulators discussed above, hypoxia-induced filamentation in *C. albicans* has been observed to be dependent on both Ras1 and Cdc35 (Cyr1), which are important in virulence, but the exact mechanisms remain unclear (116). In the future, it will be intriguing to monitor the development of hypoxia at sites of *Candida* infection in available animal models and observe whether the occurrence of hypoxia correlates with filamentation and virulence *in vivo*.

An additional transcriptional regulator involved in hypoxia responses has been examined in *C. neoformans*, TCO1. TCO1 is a fungus-specific hybrid histidine kinase family member involved in two-component signal transduction (4, 21). TCO1 null mutants have an attenuated ability to grow in hypoxia and in a murine model of cryptococcosis. To date, the mechanisms behind TCO1-mediated hypoxic adaptation and fungal virulence are still largely undefined and remain to be determined. Importantly, TCO1 null mutant data strongly suggest that other SREBP-independent pathways of hypoxia adaptation exist in pathogenic fungi and remain to be elucidated.

Not all fungal mutants with attenuated hypoxic growth display a reduction in virulence. An intriguing *C. neoformans* mutant that is defective in hypoxic growth is one that lacks the nuclear transport protein Kap123. While this mutant is highly attenuated in hypoxic growth, it remains fully virulent in a murine model of cryptococcosis, suggesting that not all fungal mutants defective in low-oxygen growth are attenuated in virulence (20). However, the *in vivo* proliferation of the *Kap123* null mutant was not measured, and thus, it is unclear if the lethality observed was due to fungal growth or an enhanced host inflammatory response due to other unstudied defects in the mutant. Regardless, the *Kap123* mutant remains an interesting tool for further understanding the link between fungal hypoxia adaptation and virulence.

Pleiotropic phenotypes of many fungal hypoxia mutants with attenuated virulence need to be taken into consideration when assessing the link between fungal hypoxia adaptation and virulence. Good examples of this important observation are studies with the fungal SREBPs that have demonstrated that in both *C. neoformans* and *A. fumigatus* regulation of iron acquisition is altered in SREBP null mutants (10, 19). As both *A. fumigatus* (e.g.,  $\Delta hapX$ ) and *C. neoformans* ( $\Delta cir1$  and  $\Delta hapX$ ) iron acquisition/homeostasis mutants are attenuated in virulence (59–62, 102, 103), these studies suggest a potential role for SrbA-mediated iron acquisition in fungal virulence and complicate the direct link between the hypoxia growth defect of SREBP null mutants and fungal virulence (10, 19). Given the importance of iron in some ergosterol biosynthesis enzymes, coregulation of the expression of

the genes in these two important metabolic pathways is logical and is a promising area of investigation.

However, taken together, our current knowledge suggests that the ability of human-pathogenic fungi to cause lethal disease is, in part, mediated by their ability to adapt to hypoxic microenvironments that occur during fungal pathogenesis. To definitively confirm that *in vivo* hypoxia, fungal hypoxia adaptation, and fungal virulence are really “cause and effect,” studies on spatial and temporal aspects of hypoxia development during invasive fungal infections and additional knowledge of fungal hypoxia adaptation mechanisms are needed. Thus, the identification and discovery of a gene required solely for fungal hypoxia adaptation and growth remain a “holy grail” that may or may not exist.

One clear area for further investigation is the expansion of fungal hypoxia adaptation mechanism research into other clinically relevant fungi. Mechanisms of hypoxia adaptation have not been extensively explored in many important human fungal pathogens, particularly the dimorphic systemic fungi such as *Coccidioides immitis*, *Histoplasma capsulatum*, and *Blastomyces dermatitidis*. It also will be intriguing to determine whether hypoxia adaptation is important for Mucorales (some require low oxygen for dimorphism [85]) pathogenesis and for other pathogens such as *Penicillium marneffei* and *Fusarium* species (which grow well anaerobically through novel fermentation systems). Along these lines of investigation, whether plant fungal pathogens also face *in vivo* oxygen limitation and whether this is critical for virulence in fungus-plant pathosystems should be further studied. Some support for an affirmative answer in these plant-based pathosystems was recently observed with alcohol dehydrogenase (*Adh1*) mutants of the root pathogen *Fusarium oxysporum* that were attenuated in virulence (22). Thus, many areas of investigation remain to be explored with regard to fungal pathogenesis and its relationship to hypoxia adaptation mechanisms.

## HYPOXIA AND HOST IMMUNE RESPONSES TO FUNGI

Outcomes of invasive fungal infections are two-way streets depending on both fungal and host factors that are dynamic in response to the interaction. Consequently, we would be remiss not to discuss the implications of *in vivo* hypoxia for host antifungal responses. It should be noted that this is an almost completely unexplored area of fungal pathogenesis, but data from other pathosystems strongly suggest that it could be a significant area for investigation with regard to human fungal infections. It has been shown that HIF-1, a key host transcription factor mediating mammalian hypoxia responses, plays an important role in the immune response and host defense to microbial pathogens (reviewed in reference 80). More recently, with the establishment of conditional knockouts, the ability to study the role of HIF-1 $\alpha$  during infection has been made possible. For example, the deletion of HIF-1 $\alpha$ , the oxygen tension-regulated  $\alpha$  subunit of HIF, from the myeloid lineage has demonstrated the importance of HIF-1 $\alpha$  in bacterial pathogenesis. The use of macrophages from HIF-1 $\alpha$  conditional knockout mice increased the survival of group B *Streptococcus*, in contrast to the functional bactericidal activity of wild-type macrophages (25). Additionally, HIF-1 $\alpha$  is critical for macrophage effector functions against *Staphylococcus aureus* and group A *Streptococcus* in normoxia and hypoxia (24, 25, 89, 129). The importance of HIF-1 $\alpha$  in innate responses is also supported by pathogens such as *Chlamydia pneumoniae*, which have been shown to block the actions of HIF-1 $\alpha$  in order to survive within



the host (100). HIF-1 $\alpha$  is also required for the control of *Yersinia enterocolitica* in the intestine, suggesting the importance of HIF-1 $\alpha$  in mucosal defense (48). Recently, it was found that *C. albicans*, along with other microbial pathogens, can stabilize HIF-1 $\alpha$  in the presence of oxygen, supporting the idea that HIF-1 $\alpha$  may be important for phagocytic cell responses to fungi (125). A mechanistic understanding of how hypoxia affects the killing and clearance of fungal infections by cells of the innate immune system is a fruitful area for further investigation (Fig. 2). This holds potential for an increased understanding of fungal pathogenesis, improvement of existing treatment strategies, and development of new therapeutic options.

## TO AIR OR NOT TO AIR: TRANSLATIONAL IMPLICATIONS OF FUNGAL HYPOXIA ADAPTATION

In summary, the occurrence of hypoxia during human fungal infections and the apparent need for fungal adaptation to oxygen limitation for virulence suggest that further exploration of these mechanisms may prove to be clinically beneficial. While “to air or not to air” may be abuse of a famous literary quote, it does reflect the idea that manipulation of oxygen (and/or CO<sub>2</sub>) levels at sites of human fungal infection may have promise as a therapeutic approach. The effects of oxygen on fungus-host interactions are likely to be multifaceted, and manipulation of oxygen levels and/or oxygen-mediated signaling pathways *in vivo* may have both positive and negative effects on the outcome of these infections. For example, while controversial and undefined, the potential use of hyperbaric oxygen to perfuse tissue to increase host cell antifungal activities and possibly thwart pathogen growth should not be overlooked (23, 38, 40, 58, 78, 93, 104). It is unclear whether increased tissue oxygen perfusion would inhibit or promote fungal growth and how it would affect the antifungal immune response in an immunocompromised patient.

In addition to the potential for manipulating oxygen levels at the site of infection, direct fungus-centric approaches to turn off key fungal hypoxia adaptation pathways that are critical for virulence is another area for therapeutic exploration. As discussed in this minireview, hypoxia has significant impacts on ergosterol biosynthesis, a target for two classes of antifungal drugs (triazoles and polyenes). In general, hypoxia induces an increase in mRNA levels of the triazole target Erg11A, which is coincidentally regulated by the *A. fumigatus* SREBP SrbA (11). *In vitro*, these antifungal agents are often highly efficacious, but *in vivo*, fungal eradication is difficult, often necessitating very long therapeutic regimens of antifungal treatment. It is thus tempting to speculate that *in vivo* hypoxic microenvironments adversely affect antifungal drug delivery to sites of infection and their efficacy due to changes in target gene expression. *In vitro* analyses of oxygen effects on antifungal drug activity provide some support for this idea, but further experimentation is needed (87, 123).

With regard to fungal SREBPs, low-oxygen conditions induce the activation of this pathway, likely in response to sterol depletion upon hypoxia exposure, and are required for virulence. Thus, it has been proposed that fungal SREBPs may be excellent drug targets due to their absolute requirement for virulence in *C. neoformans* and *A. fumigatus* (9). With regard to SREBPs, amino acid sequence similarity suggests substantial differences between human and fungal orthologs that indicate it may be possible to design an effective therapeutic targeting this protein. However, whether those specific differences can result in a functionally at-

tenuated protein or fungal SREBP signaling pathway is unclear. A similar strategy can certainly be pursued with regard to other fungal proteins essential for hypoxia adaptation and virulence, especially if they are conserved in function across the different fungal pathogens. However, the use of oxygen to “turn off” these pathways may stimulate other pathways that exacerbate diseases caused by fungi. Only experimental validation of oxygen use and targeting of these pathways in models of mammalian fungal infection can begin to answer these questions.

Finally, besides direct fungus-centric targeted approaches, the idea that hypoxia can alter key innate immune system antifungal responses is worthy of further investigation. Immunomodulation strategies targeting host hypoxia responses, either agonistically or antagonistically, could have great therapeutic benefits in many fungal disease settings.

In conclusion, much remains to be learned about fungal hypoxia adaptation and how it relates to outcomes of fungal pathogenesis. Many areas for further investigation remain, as outlined in this minireview, but our knowledge has progressed to the point where hypotheses can be proposed and experiments designed to answer the question of whether “to air or not to air” a valid approach for improving treatment outcomes of these often lethal and increasingly common infections.

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