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# Regulation of Hypoxia Adaptation: An Overlooked Virulence Attribute of Pathogenic Fungi?

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## Summary

Over the past two decades, the incidence of fungal infections has dramatically increased. This is primarily due to increases in the population of immunocompromised individuals attributed to HIV/AIDS pandemic and immunosuppression therapies associated with organ transplantation, cancer, and other diseases where new immunomodulatory therapies are utilized. Significant advances have been made in understanding how fungi cause disease, but clearly much remains to be learned about the pathophysiology of these often lethal infections.

Fungal pathogens face numerous environmental challenges as they colonize and infect mammalian hosts. Regardless of a pathogen's complexity, its ability to adapt to environmental changes is critical for its survival and ability to cause disease. For example, at sites of fungal infections, the significant influx of immune effector cells and the necrosis of tissue by the invading pathogen generate hypoxic microenvironments to which both the pathogen and host cells must adapt in order to survive. However, our current knowledge of how pathogenic fungi adapt to and survive in hypoxic conditions during fungal pathogenesis is limited. Recent studies have begun to observe that the ability to adapt to various levels of hypoxia is an important component of the virulence arsenal of pathogenic fungi. In this review, we focus on known oxygen sensing mechanisms that non-pathogenic and pathogenic fungi utilize to adapt to hypoxic microenvironments and their possible relation to fungal virulence.

### Keywords

*Aspergillus fumigatus*; *Cryptococcus neoformans*; *Candida albicans*; fungal virulence; hypoxia; sterols

## Introduction/Significance of hypoxia during fungal pathogenesis

Recent advances in medical therapies, organ transplantation, HIV infections, and an increasing geriatric population have generated rising populations of immunocompromised patients. These events have all resulted in a significant increase in life-threatening human fungal infections over the last two decades (1). The limited treatment options and high mortality rates associated with these infections has consequently led to a concerted effort to better understand mechanisms of fungal pathogenesis in mammals. The general rationale behind these studies is that a better understanding of how these organisms cause disease will allow us to develop better technologies for the treatment and prevention of these often lethal infections. One increasing area of fungal pathogenesis research is related to identifying and understanding the basic

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metabolic pathways utilized by these fungi to survive in the harsh and highly variable mammalian host environment.

The three main fungal pathogens that cause human mycoses, *Aspergillus fumigatus*, *Cryptococcus neoformans*, and *Candida albicans*, are typically opportunistic pathogens. These fungi are saprophytic organisms that have evolved a unique combination of attributes to survive in their natural environments. *Aspergillus fumigatus* is typically found in soil and decaying organic material such as compost heaps. *Cryptococcus neoformans* is typically found in pigeon droppings, soil and certain trees. Unlike *Aspergillus* and *Cryptococcus* species, *Candida albicans* is rarely found in the soil or external environment. Instead, it is a normal inhabitant of the human microflora. Thus, *C. albicans* is already highly adapted to the host environment. *C. albicans* possess the ability to survive in disparate host environments, as illustrated by its ability to colonize diverse areas of the host (oral, vaginal, gastrointestinal areas). Coincidentally, many of these attributes, which allow fungal survival in their natural ecological niches, appear to also allow these fungi to cause disease in immunocompromised hosts. One overlooked environmental selection pressure found in natural environments of all three of the most common fungal pathogens of humans is low oxygen tension. Whether in the soil, a compost pile, pigeon guano, or the gut of a mammal, these fungi must deal with low levels of oxygen.

The focus of this review is on the increasing evidence that pathogenic fungi must adapt to rapidly changing oxygen levels during fungal infections. It is well established that oxygen levels vary throughout the mammalian body depending on numerous factors including tissue type and presence or absence of an inflammatory response. For example, oxygen levels in most mammalian tissues are found to be considerably below atmospheric levels (21%) (2-4). Even in the alveoli of healthy lungs, the most oxygen rich organ and site of infection for many fungal pathogens, the oxygen level is around 14%. By the time oxygen reaches the capillaries and diffuses into surrounding tissues its availability is much lower with levels of 2-4% reported (5,6). In addition, it is well established that at sites of inflammation available oxygen is significantly reduced compared to surrounding tissues (7-9). Moreover, in inflamed tissues, the blood supply is often interrupted because the vessels are congested with phagocytes or the pathogen itself (10,11). Thus, it seems highly probable that hypoxic microenvironments are generated during fungal infection.

Indeed, we can look no further than the host response to observe that fungal pathogens are likely exposed to severely low oxygen levels during infection. Immune effector cells, such as neutrophils, function effectively in severely hypoxic microenvironments. These and other cells of the host have evolved distinct mechanisms to deal with hypoxic microenvironments generated during microbial infections. Many of these host response mechanisms are dependent upon the global transcription factor, hypoxia inducible factor (HIF) 1.

HIF 1 is a heterodimeric transcription factor that consists of one of three α-subunits (HIF-1α, HIF-2α, and HIF-3α) and one β-subunit (HIF-1β), and is the central regulator of hypoxic gene expression in mammals (reviewed in (12),(13)). Both the degradation and activity of the HIF-1α subunit are regulated by oxygen-dependent post-translational hydroxyl modifications. Under hypoxic conditions HIF-1α is not hydroxylated, leading to an accumulation of the HIF-1α subunit and expression of hypoxia-responsive genes, including those encoding many glycolytic enzymes, erythropoietin, adrenomedullin, and growth factors (14,15).

In a recent study of **A**cute **R**espiratory **D**istress **S**yndrome (ARDS) and acute inflammatory lung injury, Thiel *et al.* (16) provided evidence for the importance of hypoxic microenvironments in regulation of host immune responses. ARDS patients are normally treated with a life-saving oxygen therapy, but this therapy may have a dangerous side effect in

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patients with uncontrolled pulmonary inflammation. Thiel *et al.* (16) identified a local tissue hypoxia-driven and adenosin A2A receptor (A2AR)-mediated anti-inflammatory mechanism. Their data suggest that oxygenation may lead to elimination of the A2AR-mediated lung tissue-protecting pathway and thereby further exacerbate lung injury. Taken together, the above observations and studies indicate that mammalian immune system responses to microbial infection and inflammation are critically tied to hypoxic microenvironments.

While the role of hypoxia in the immune response to fungal pathogens is relatively unknown, it follows that since immune cells of the host have evolved mechanisms to function in hypoxia, mammalian fungal pathogens like *A. fumigatus*, *C. neoformans* and *C. albicans* are likely exposed to hypoxic conditions during fungal pathogenesis. Indeed, during *A. fumigatus* infection, our laboratory has recently observed significant increases in HIF-1α activity as the fungal infection progresses and inflammation and edema increase in the lung (Grahl and Cramer, unpublished data). In addition, a recent study by Brock *et al.* (17) demonstrated that hypoxia likely occurs *in vivo* in the lung during *A fumigatus* infection. The authors constructed a luciferase-producing bioluminescent *A. fumigatus* strain, which was not attenuated in virulence in a murine model of invasive aspergillosis. Interestingly, luminescence from the lungs decreased after reaching a maximum at one day post infection, despite the high number of fungal hyphae present in histology examinations. The authors hypothesize that this phenomenon might be due to the severe tissue damage in and through the pulmonary lesions, which likely decrease the oxygen concentration in the lung tissue. Oxygen is essential for the light-producing reaction, and thus the lack of luminescence is likely attributable to the hypoxia at the site of infection (17).

Additional evidence that hypoxia may be a key component of the pathophysiology of invasive fungal infections comes from the observation that there are often significant differences in the *in vivo* and *in vitro* test results of antifungal drug efficacies. These differences have recently been postulated to be related to hypoxic conditions found *in vivo* as demonstrated by *in vitro* antifungal drug efficacy tests conducted in hypoxia (6,18). Furthermore, recent studies have identified genes responsible for regulating fungal response to hypoxia, and some of these pathways have been observed to be essential for fungal virulence of mammals. Consequently, it seems probable that pathogenic fungi possess mechanisms to adapt to hypoxic microenvironments found *in vivo* during infection.

The purpose of this review is to summarize recent advances in our understanding of mechanisms that human fungal pathogens use to adapt to hypoxic conditions and to highlight the emerging importance of this area of research in the pathogenic fungi. While studies on hypoxia adaptation in these pathogenic fungi are limited, increasing research attention to this important component of their virulence arsenal has revealed similarities and differences with each other and the model yeast *S. cerevisiae* and *S. pombe*. Importantly, we should clarify the distinction between mechanisms of hypoxia *adaptation* and mechanisms of hypoxic *growth.* It seems clear that these are two distinct biological processes requiring a distinct set of genes and mechanisms. In our literature review, we did not find detailed studies defining these two likely different processes in the fungi. It is likely that periods of adaptation are different for diverse fungi. A close examination of the methodology used in the cited studies indicated that most, if not all, studies are focused on genes allowing the fungi to adapt to hypoxia (i.e. genes expressed/required in the early phase of exposure to hypoxia (within 48 hours of a switch to hypoxia), rather than genes required for actual fungal growth in these conditions. Where possible, we have attempted to highlight these distinctions. Finally, we have divided the manuscript into sections detailing fungi with Upc2p orthologs and fungi with SREBP orthologs given the emerging importance of these pathways in oxygen sensing and hypoxia adaptation in fungi.

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## Fungi with the SREBP analogue Upc2p

**Saccharomyces cerevisiae—***S. cerevisiae* cells adapt to anaerobic conditions by inducing expression of a large number of genes, called “hypoxic genes” (19-25). The hypoxic genes encode oxygen-related functions in respiration, heme, and membrane biosynthesis that are required at higher levels when molecular oxygen is limited (20,26). For the regulation of hypoxic genes, the cell senses oxygen availability through cellular heme levels (27,28), and recent studies suggest that oxygen availability can also be sensed through cellular sterol levels (29).

**Oxygen sensing by heme:** Molecular oxygen is required as a substrate in two consecutive steps of heme biosynthesis catalyzed by the enzymes coproporphyrinogen oxidase and protoporphyrinogen IX oxidase (30). In the presence of oxygen (aerobic growth), heme accumulates, binds to the transcriptional activator Hap1p (**H**eme **A**ctivator **P**rotein) and causes the formation of a Hap1p homodimer specific for DNA binding to the *cis*-element. Hap1p is a protein composed of a zinc-finger DNA binding domain at the N-terminus, a dimerization domain, a heme binding domain within the central region, a heme-responsive motif 7 (HRM7), and a transcriptional activation domain at the C-terminus (31-33). The heme-Hap1p-complex acts as a transcriptional activator of genes containing its recognition site (5′CGG$N_6$CGG) (34-36), such as genes involved in respiration (reviewed in (20)).

In addition, the expression of the *ROX1* (**R**epressor **O**f hypo**X**ic genes) gene is activated by the heme-Hap1p complex and Rox1p accumulates in the cell under aerobic conditions (reviewed in (26)). The Rox1p repressor binds to its recognition site upstream of the hypoxic genes to repress their transcription (37,38). Rox1p binds to the DNA with its HMG domain and recruits the general repression complex, Tup1/Ssn6, which binds to the Rox1p repression domain (reviewed in (26)). The repression through Rox1p varies between hypoxic genes that do not have aerobic counterparts, which are expressed at detectable levels at all oxygen concentrations but their expression is higher when oxygen decreases, like *HEM13*, *OLE1*, *ERG11*, and the autorepressed *ROX1* itself, and hypoxic genes that have an aerobic homologue, like *HMG1/HMG2*, *COX5A/COX5B*, *AAC1*, *AAC2/AAC3*, and *TIF51A/ANBI* (the first gene is aerobic – last and underlined anaerobic). The anaerobic gene is then completely repressed until very low oxygen concentrations are reached (38,39).

For some hypoxic genes, a second DNA binding protein Mot3p enhances Rox1p repression through helping recruit the Tup1p/Ssn6p complex. Two examples are the *ANBI* (**AN**aero**B**ically **I**nduced) gene, encoding a subunit of eukaryotic initiation factor 5 (eIF-5a), an essential translation factor (40), and the *HEM13* gene, encoding the enzyme coproporphyrinogen III oxidase, which catalyzes the rate-limiting step in heme biosynthesis (41). The hypoxic derepression of *HEM13* allows the cell to continue heme biosynthesis under limited available oxygen. The strongly repressed *ANBI* gene has one Mot3p and two Rox1p binding sites in its promoter region, while the promoter of the partially repressed *HEM13* contains one Rox1p and three Mot3p binding sites. The combination of binding sites determines the strength of repression. Multiple Mot3p binding sites plus a single Rox1p binding site are much weaker than multiple Rox1p binding sites plus a single Mot3p binding site (42). Rox1p and Mot3p both interact with Ssn6p of the general repression complex and Rox1p stabilizes Mot3p binding to DNA through interactions with Tup1p/Ssn6p (42).

Under hypoxic or anaerobic growth conditions, heme levels are reduced. Hap1p still binds to its cognate site, but in the absence of heme, Hap1p forms a biochemically distinct **H**igh-**M**olecular-weight **C**omplex, HMC, which contains Hap1p and four other proteins including Hsp28p and Ydj1p. This complex represses transcription (43). Consequently, under hypoxic conditions, *ROX1* and *MOT3* expression is repressed resulting in the activation of hypoxic genes expression. (26).

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During adaptation to anaerobic conditions, a complex program of cell wall remodeling occurs in yeast. Under anaerobic conditions, major aerobic cell wall mannoproteins, encoded by *CWP1* and *CWP2*, are replaced by their anaerobic counterparts, encoded by the *DAN*/*TIR* genes. The *DAN*/*TIR* genes encode a group of eight cell wall mannoproteins that play a significant role in cell wall permeability (23,44). *DAN*/*TIR* genes are regulated by heme, sterol levels, and three DNA binding transcription factors. The heme-dependent repressors Rox1p and Mot3p function synergistically to efficiently repress *DAN*/*TIR* genes under aerobic conditions (45). In addition, the sterol depletion-dependent activator Upc2p acts through a consensus site termed *AR1* to induce the expression of *DAN*/*TIR* genes in anaerobic conditions (46). Sertil *et al.* (47) observed that the histone deacetylase and global repressor Rpd3p is required for the expression of all the *DAN*/*TIR* genes and the hypoxic gene *ANBI*. Moreover, the authors propose that Rpd3p is recruited to the *DAN1* promoter under strict anaerobic conditions. The presence of Rpd3p at the promoter counteracts the function of the repressor Mot3p, which leads to stable binding of the activator Upc2p. Upc2p then recruits the chromatin remodeling complex Swi/Snf to reorganize chromatin, thereby facilitating the binding of the transcriptional machinery that results in the activation of gene expression (47). Upc2p, together with the transcription factor Ecm22p, is also responsible for basal and induced expression of genes encoding enzymes of ergosterol biosynthesis in yeast (*ERG1*, *ERG2*, *ERG3*, *ERG7*, *ERG25*, *ERG26*, and *ERG27*), and it has been implicated in the uptake of sterols under hypoxic conditions (48-52).

**Oxygen sensing by sterols:** While heme has been thought to be the primary oxygen sensor in *S. cerevisiae*, recent studies suggest that sterol levels also play an important role. Upc2p and Ecm22p are functionally related to human sterol regulatory element binding protein (SREBP) with an N-terminal transcription factor domain and a C-terminal transmembrane domain. Although *S. cerevisiae* lacks an ortholog of SREBP, it seems that a potentially analogous oxygen-sensing mechanism exists in budding yeast regulated through Upc2p and Ecm22p. Marie *et al.* (53) have observed that Upc2p and Ecm22p are localized outside of the nucleus in sterol replete conditions, but in conditions of sterol depletion localization shifts toward the nucleus. The authors suggest that the N-terminal transcription factor domain is separated from the C-terminal transmembrane domain by proteolytic cleavage and enters the nucleus to activate gene expression, analogous to SREBP regulation of cholesterol biosynthesis in mammals.

Upc2p and Ecm22p both bind a sequence motif known as the sterol regulatory element (SRE) (48,49,54). Nearly one-third of hypoxically induced genes in *S. cerevisiae* contain at least one potential Upc2p/Ecm22p binding site, suggesting that these transcription factors are major players in the adaptation to hypoxia (55). The activation of target genes by Upc2p occurs in response to low sterol levels, which can be caused by blocks in ergosterol biosynthesis or by hypoxia. Davies and Rine (29) observed that both Upc2p and Ecm22p require a functional version of Hap1p for basal expression of *ERG2*, but when sterols are depleted Upc2p is independent of Hap1p, whereas Ecm22p still depends upon Hap1p for *ERG* gene activation. *ERG2*, *ERG3*, *ERG10*, *DAN2*, and *DAN4* are activated by Upc2p solely in response to sterol depletion whereas *DAN1* and *TIR1* respond to both sterols and heme (46).

**Other oxygen sensing mechanisms:** An additional hypoxic regulatory pathway involving an antagonistic interaction between the Ord1p repressor and the Yap1p factor (a transcriptional activator involved in oxidative stress response) has been discovered in *S. cerevisiae* and regulates both *TIR1* and *SRP1*. The hypoxic response of *TIR1*/*SRP1* (both encode cell wall mannoproteins) depends on the absence of heme but is Rox1p-independent. Under aerobic conditions, Ord1p binds to the *SRP1* promoter and expression is repressed. When conditions change to hypoxia, Yap1p also binds to the *SRP1* promoter, counteracts the Ord1p effect and *SRP1* is expressed (56).

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Multiple pathways involved in regulating hypoxic and anoxic gene expression in yeast may exist. Studies of several other hypoxic/anaerobic genes including *SUT1*, encoding a putative Zn[II]2Cys6-transcription factor that facilitates the uptake and synthesis of sterols under hypoxic conditions (57), *GPD2*, encoding an isoenzyme of NAD-dependent glycerol 3-phosphate dehydrogenase (58), and members of the seripauperine (*PAU*) family, like *TIR1* (59) have demonstrated Rox1p-independent hypoxic/anaerobic induction, but the mechanisms by which this occurs are not yet understood.

Another recently described possible mechanism of hypoxia signaling in yeast involves the mitochondrial respiratory chain, the cytochrome c oxidase and reactive oxygen species (60, 61). It has been shown that mitochondria from yeast, rat liver, and plants are capable of nitrite ($NO_2^-$)-dependent nitric oxide (NO) synthesis (60,62-66). This pathway is induced when cells experience hypoxia, and furthermore, Castello *et al.* (60) suggest that mitochondrially produced NO functions in a signaling pathway to the nucleus by reacting with the superoxide produced by hypoxic mitochondria (67) to form peroxynitrite ($ONOO^-$) that promotes protein tyrosine nitration of specific proteins that may be involved in a signaling pathway to the nucleus. Future research on this mechanism will likely uncover its specific role in hypoxia adaptation.

It seems clear that adaptation to hypoxia is a complex multi-faceted process regulated via the interaction of several different critical metabolic pathways in the cell. The major regulatory pathways discussed above are summarized in Figure 1 and Table 1. We now turn our attention to other fungi and discuss similarities and differences with these hypoxia adaptation mechanisms in *S. cerevisiae*. As many of the pathogenic fungi employ different life-styles than *S. cerevisiae*, it is still unclear which of these pathways involved in regulating responses to hypoxia in baker's yeast are conserved in fungi that invade mammalian hosts.

**Candida albicans—***Candida albicans* is an important human fungal pathogen that causes superficial skin infections as well as deep-seated infections, suggesting that its ability to switch between normoxia and hypoxia is a major determinant of its virulence (68). In *C. albicans* little is known about mechanisms utilized by this yeast to adapt to hypoxic microenvironments. However, our current knowledge suggests that the transcriptional response to hypoxia differs significantly between *C. albicans* and *S. cerevisiae* in important aspects. Although both are generally referred to as facultative anaerobes, genetics studies, conditions required for anaerobic growth, and genome analyses seem to suggest that these hemiascomycota yeast respond differently to changes in oxygen levels.

First, a homologue (Rfg1p) of the *S. cerevisiae* Rox1p has been identified in *C. albicans*, but Rfg1p does not play a role in the regulation of hypoxic genes in this pathogenic yeast as in *S. cerevisiae* (Table 1). Instead, Rfg1p is a transcriptional regulator that controls filamentous growth, and in that role, is critical for *C. albicans* virulence (69).

Second, *S. cerevisiae* genes involved in glycolysis and fermentation are not stimulated by hypoxia (25,70), but hypoxia induces these genes and genes involved in hyphal growth in *C. albicans* while genes of oxidative metabolism are repressed (68). During normoxic conditions the global transcription factor Efg1p regulates the expression of genes involved in glycolysis and respiration, but it has no role in controlling the expression of respiratory genes and is not required to upregulate glycolytic gene expression in hypoxia (68,71). Efg1p also promotes filamentation under normoxic conditions. Recently, it was observed that under hypoxic conditions Efg1p promotes the synthesis of unsaturated fatty acids, the up-regulation of genes involved in the stress response (*HSP12*, *DDR48*, *CTA1*) and represses filamentous growth in *C. albicans*. Thus, the regulatory role of Efg1p in *C. albicans* strongly depends on oxygen (68,72). Transcriptional analyses observed that Efg1p is required to allow hypoxic regulation

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of about half of all genes that are normally regulated by hypoxia in *C. albicans*. In an *efg1* mutant, hypoxic upregulation (e.g. *CTA1*) or downregulation (e.g. *RIP1*) of several genes is abolished, and some genes, like *OLE1* encoding a fatty acid desaturase, are ineffectively expressed in hypoxia. Another major function of Efg1p is to prevent hypoxic regulation of numerous genes that are not normally up- or downregulated under hypoxia (71). Despite the fact that Efg1p is a major regulator of the hypoxic response in *C. albicans*, a homozygous *efg1* mutant shows no severe change in virulence in comparison to the wild-type (73).

Another transcription factor in *C. albicans* affected by hypoxia, Ace2p, is required for filamentation in response to hypoxic conditions. Ace2p also induces fermentative growth and represses respiration, but it is possible that the effect of Ace2p on metabolism is restricted to normal oxygen conditions. This remains to be tested (74). Interestingly, an *ace2* null mutant is almost avirulent in an immunocompetent mouse model, while there is only a low degree of attenuation in a neutropenic mouse model (75,76). This may suggest that different states of the immune system may affect the development of hypoxia *in vivo* i.e. the lack of neutrophils in the neutropenic model minimizes the inflammatory response and hence hypoxic microenvironments encountered by the invading fungus. This remains to be examined and confirmed.

As in *S. cerevisiae* (23,44,46), the cell-wall proteome of *C. albicans* is sensitive to changes in environmental conditions which helps the cell to adjust to harsh environments. For example, iron deprivation and hypoxic conditions affect the expression of cell-wall protein encoding genes, such as iron acquisition and iron-uptake genes i.e. *RBT5* a gene encoding a predicted GPI protein involved in iron acquisition (68,77,78). Numerous oxygen-dependent reactions in the cell are carried out by iron-containing enzymes (79). During hypoxic conditions, there may be competition for iron by iron-containing enzymes, which might lead to an increased expression of cell-wall protein encoding genes involved in iron-acquisition and iron-uptake (80).

**Oxygen sensing in Candida albicans:** In *S. cerevisiae* it has been observed that the cell senses oxygen availability through cellular heme and sterol levels (27-29). In *C. albicans*, no apparent homolog of *ScHAP1* exists, but in recent studies a close ortholog of *S. cerevisiae* Upc2p and Ecm22p, both involved in sensing sterol depletion, has been identified. Upc2p, a transcription factor of the zinc cluster family, is an important regulator of the sterol biosynthesis and azole drug resistance in *C. albicans* (81,82) (Figure 2). Hoot *et al.* (83) showed that transcriptional regulation of *UPC2* expression occurs through Upc2p-dependent as well as a novel Upc2p-independent mechanism. Whether there is also a post-translational control mechanism as described for the mammalian sterol regulator SREBP (discussed in detail below) or as suggested for the *Sc*Upc2p remains to be determined.

Upc2p binds *in vivo* to the promoters of several ergosterol biosynthesis genes and other genes involved or predicted to be involved in lipid metabolism. Znaidi *et al.* (84) observed that up-regulation of *ERG11* during hypoxia is strictly Upc2p dependent. Upc2p also binds the promoters of four genes encoding transcription factors (*INO2*, *ACE2*, *SUT1*, and *UPC2* itself). One of them, Sut1p, controls sterol uptake in *S. cerevisiae* (57,85) suggesting that in *C. albicans* Upc2p and Sut1p may interact in a sterol regulatory network (84).

Interestingly, Upc2p also binds to the promoter of *CBP1*, which was shown to encode a corticosteroid binding protein in *C. albicans* (86). *C. albicans* appears to take up steroids and possibly metabolic precursors from the host, and Upc2p seems to play a role in corticosteroid uptake from mammals and in adaptation of *C. albicans* to hypoxic conditions in the host (84). Thus, an emerging theme with studies in *S. cerevisiae* and *C. albicans* is the role of sterol homeostasis in adaptation to hypoxic microenvironments. This theme will also be expanded

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on in additional fungi discussed below with the discovery of SREBP orthologs. A summary of the known hypoxia regulation mechanisms in *C. albicans* is presented in Figure 1 and Table 1.

## Fungi with SREBP orthologs

**Schizosaccharomyces pombe—**Recently, a novel mechanism of hypoxia adaptation mediated by a highly conserved family of transcription factors, the SREBPs, was characterized in *Schizosaccharomyces pombe* (87). *S. pombe*, also called "fission yeast", is a non-pathogenic yeast that is used as a model organism in molecular and cell biology. SREBPs are a family of endoplasmic reticulum (ER) membrane bound transcription factors first identified in mammals as regulators of cholesterol and fatty acid synthesis (88-92). SREBPs contain two transmembrane segments and are inserted into ER membranes in a hairpin fashion such that the N- and C-terminal ends of the protein are in the cytosol. SREBP is synthesized as an inactive membrane-bound precursor that forms a complex with SCAP (**S**REBP **C**leavage-**A**cting **P**rotein), a multispan membrane protein that is a component of the sterol sensor (90). Under conditions with enough available sterols, the SREBP-SCAP complex is retained in the ER membrane through binding of SCAP to the resident ER protein Insig (93). In sterol-depleted cells, SCAP changes its confirmation, which releases the SREBP-SCAP complex from Insig (94). SCAP then escorts SREBP from the ER to the Golgi apparatus where SREBP is activated by two sequential proteolytic events catalyzed by site-1 and a site-2 proteases that release the N-terminal transcription factor domain from the membrane, allowing the transcription factor to enter the nucleus and direct the transcription of target genes (90,95).

In *S. pombe* apparent orthologs of SREBP (*SRE1*), SCAP (*SCP1*) and Insig (*INS1*) have been identified and characterized. Sre1p is cleaved and activated in response to sterol depletion and hypoxia, and stimulates transcription of genes required for adaptation to hypoxia such as genes involved in heme, sphingolipid, ubiquinone, and ergosterol biosynthesis (Figure 3 and Table 1) (87,96). Thus, in fission yeast, Sre1p and Scp1p appear to monitor-oxygen dependent sterol synthesis as an indirect measure of oxygen supply. Interestingly, there does not appear to be an impact of Ins1p on the SREBP pathway in fission yeast. In addition, Hughes and Espenshade (97) recently identified another component of this pathway, Ofd1p. Ofd1p is a prolyl 4-hydroxylase- like 2-oxoglutarate-Fe(II) dioxygenase that accelerates Sre1p degradation in the presence of oxygen. The N-terminal dioxygenase domain is an oxygen sensor that regulates the activity of the C-terminal degradation domain (97). Altogether, the SREBP pathway functions as an oxygen sensor and is required for adaptation to hypoxia in fission yeast. However, the critical function of Sre1 in allowing hypoxic adaptation and subsequent growth is not clearly defined. It seems likely that Sre1 is playing a pleiotropic role in regulating many different genes required for yeast cells to adapt and grow in hypoxia.

Orthologs of the SREBP pathway were recently identified and characterized in the human fungal pathogens *C. neoformans* and *A. fumigatus*. Yet, the exact components and the mechanism behind SREBP regulation largely remain to be determined in these pathogenic fungi. Moreover, it appears that the SREBP pathway is similar in function to the Upc2p mediated pathway in *S. cerevisiae* and *C. albicans*, but the mechanisms behind the similarities and differences between these two pathways is currently not clear.

**Cryptococcus neoformans—**Unlike the Ascomycete yeast *S. cerevisiae* and *C. albicans*, the Basidiomycete yeas *Cryptococcus neoformans* is generally considered an obligate aerobe. *Cryptococcus* species cause the disease Cryptococcosis in both immunocompromised and apparently healthy hosts and are the most common cause of fungal meningitis (98). *C. neoformans* is primarily found in pigeon droppings and soil contaminated with avian guanos throughout the world (99). One would speculate that these environments are relatively oxygen

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poor suggesting that *C. neoformans* likely has evolved mechanisms to adapt to low oxygen microenvironments. In the laboratory, *C. neoformans* grows optimally under atmospheric oxygen conditions (21%), but oxygen concentrations in the human brain are drastically lower than in the atmosphere and vary significantly among anatomical sites (2). Thus, in order to establish an infection in the brain, it seems likely that *C. neoformans* must adapt to reduced oxygen levels during infection. Therefore, discovering the mechanisms utilized by *C. neoformans* to sense and adapt to low-oxygen conditions is an important area of research aimed towards understanding the pathobiology of this pathogenic yeast. Yet, until recently, the importance of hypoxia adaptation in *C. neoformans* biology and virulence have been largely un-studied.

Recent whole-genome microarray-based transcriptional profiling of *C. neoformans* in a hypoxic microenvironment has started to reveal genes and pathways regulated in response to hypoxia. Among them are genes involved in hexose uptake (sugar transporter and hexose transporter), ethanol production (pyruvate decarboxylase and alcohol dehydrogenases), and sterol metabolism (ergosterol biosynthesis genes) (100). The possibility of fermentation being important for hypoxic growth during infection is supported by another study, where ethanol was found in cerebral tissue of rats infected with *C. neoformans* (101). Yet the importance of fermentation pathways in this obligate aerobe's ability to cause disease and grow in hypoxic microenvironments is unknown. However, as with other yeasts we have discussed, and the filamentous mold *A. fumigatus*, sterol biosynthesis and homeostasis seems to be a common mechanism regulating adaptation to low oxygen environments in fungi.

**The SREBP pathway in Cryptococcus neoformans:** Orthologs of SREBP (*SRE1*), SCAP (*SCP1*) and a Site-2-protease (*STP1*) were identified and characterized in *C. neoformans* (100,102). *C. neoformans* appears to lack an identifiable homologue of Insig, the ER retention-protein that controls ER-to-Golgi transport of SREBP-SCAP complex in mammalian cells. This finding is consistent with *S. pombe* data which suggests that Insig is not required for sterol-dependent regulation of Sre1p and Scp1p (87,102). However, as in fission yeast, the SREBP pathway mediated by Sre1p and Scp1p in *C. neoformans* is crucial for adaptation to hypoxia and sterol biosynthesis (Figure 2). In addition, unlike in *S. pombe*, Sre1p controls low-oxygen expression of genes required for two different pathways of iron uptake (*SIT1* and *FRE7*) (102), which might be crucial for survival under hypoxic conditions. Importantly, *sre1Δ* mutants fail to proliferate in host tissue, fail to cause fatal meningoencephalitis, and display hypersensitivity to the azole class of antifungal drugs (Table 1 and Figure 3) (100,102). It is unclear if the virulence defect is due to deficiencies in iron homeostasis, a known virulence attribute of pathogenic fungi, or the inability of *C. neoformans* to grow in low oxygen microenvironments in the absence of Sre1p. The two phenotypes are likely not mutually exclusive given the importance of iron in ergosterol biosynthesis. However, it is clear that *C. neoformans* needs Sre1p activation to adapt to the host environment. Mechanisms linking ergosterol biosynthesis, iron homeostasis, and fungal virulence in this pathogenic yeast remain unknown. Identification and characterization of additional components of the SREBP pathway are likely to yield important insights into how this pathogenic yeast causes disease.

In order to identify novel Sre1p pathway components in *C. neoformans*, Lee *et al.* (103) observed that responses to cobalt chloride ($CoCl_2$) in *C. neoformans* mimic certain aspects of hypoxia by targeting enzymes in the sterol biosynthesis pathway. $CoCl_2$ has been widely used as a hypoxia-mimicking agent in mammalian systems (104-108), but the mechanisms by which it induces hypoxia-mimicking responses are not fully understood. However, Sre1p is required for adaptation to $CoCl_2$ in *C. neoformans*. Upon $CoCl_2$ treatment, Sre1p is likely activated in response to sterol defects caused by the inhibition of several enzymatic steps in the ergosterol biosynthetic pathway. $CoCl_2$ treatment leads to increased levels of sterol intermediates, including the substrates of Erg25p, 4,4-dimethylfecosterol and 4-methylfecosterol,

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demonstrating that Sre1p regulates sterol homeostasis in response to $CoCl_2$. $CoCl_2$-induced sterol synthesis inhibition and Sre1p activation has also been observed in *S. pombe*, suggesting a conserved role for Sre1p in the adaptation to elevated levels of transition metals (103,109).

Consequently, $CoCl_2$ treatment has been used to screen for pathways involved in oxygen sensing in *C. neoformans.* In this context Ingavale *et al.* (109) observed that $CoCl_2$ sensitivity and/or oxygen sensing and adaptation processes in *C. neoformans* have a complex nature. Importantly they identified several mutants with increased sensitivity to $CoCl_2$ and they observed that most of the $CoCl_2$ sensitive mutants are also sensitive to low oxygen concentrations. Mutants included genes involved in the sterol biosynthesis pathway such as *SCP1*, *SRE1*, *ERG5*, mutants in genes involved in mitochondrial function and energy metabolism such as $H^+$ transporting ATP synthase, NADH:ubiquinone oxidoreductase or ATP:ADP anitporter, and various transporters and enzymes such as hexose transport related protein, seroheme synthase, amino acid transporter and myo-inositol oxygenase. The role of these genes and pathways in fungal virulence has yet to be explored, but the apparent role of the mitochondia in hypoxia adaptation in *C. neoformans* echoes the recent findings in *S. cerevisiae* discussed above.

**The two-component like (Tco) system in Cryptococcus neoformans:** Additional studies observed that the Sre1p pathway acts in parallel with a two-component signal transduction like pathway controlled by Tco1p in hypoxic adaptation of *C. neoformans* (100). Tco1p is a member of a highly conserved family of fungal-specific histidine kinases. Tco1p negatively regulates the expression of melanin formation and, redundantly with Tco2p, positively regulates the HOG MAPK pathway (which is dispensable for virulence) (110). Interestingly, it has been shown that Tco1p is required for growth under hypoxic conditions and for virulence of *C. neoformans* (Figure 2) (100,110). However, it is unclear how this pathway is involved in hypoxic adaptation and fungal virulence (Figure 3). In contrast to mutants in *SRE1*, the *tco1Δ* mutant shows no detectable defects in the regulation of any of the known hypoxic genes ((100), unpublished data). As a result Chun *et al.* (100) hypothesize that the Tco1p pathway might act post-transcriptionally. However, this result may also indicate that novel pathways or altered function of existing known pathways regulated by Tco1p are involved in hypoxia adaptation. Clearly, however, these data suggest that oxygen sensing in *C. neoformans* is highly complex, and likely important for virulence of this organism.

**Aspergillus fumigatus—***A. fumigatus* is a saprophytic, obligate aerobic filamentous fungus commonly found in soil and compost piles. Its primary ecological function is to recycle carbon and nitrogen through the environment (111-113). As with most fungi, it seems self-evident that these microenvironments would place significant oxygen related stress on the mold. While *A. fumigatus* is responsible for a number of clinically relevant diseases, invasive pulmonary aspergillosis (IPA) is the most lethal with mortality rates ranging from 60-90% (114-116). Interestingly, while IPA can be caused by several *Aspergillus* species, the majority of IPA cases are caused by *A. fumigatus.* This may suggest that *A. fumigatus* contains unique attributes that allow it to cause disease (117).

Currently, we have a limited understanding of the *in vivo* growth mechanisms of *A. fumigatus* during IPA (118). Given that the lung is the primary site of infection for this mold, it may be counter-intuitive to think that low oxygen levels would be a critical component of the pathophysiology of IPA. However, during infection, *A. fumigatus* causes significant damage to host tissue through invasive growth by hyphae and subsequent recruitment of immune effector cells (depending on the immune system status of the host). Thus, infection generates significant inflammation and necrosis in lung tissue that can be visualized by histopathology. These pathologic lesions also likely represent areas of poor oxygen availability

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to the pathogen and host. Thus, it is likely that to cause disease *A. fumigatus* must adapt to hypoxic conditions.

In general, mechanisms of hypoxia adaptation in molds have gone largely unstudied. Tarrand *et al.* (119,120) hypothesized that the low rate of *Aspergillus* recovery from clinical material is due to adaptation by the fungus to the physiologic temperature and hypoxic milieu found *in vivo*. However, recent studies with *A. fumigatus* suggest that it cannot grow in anaerobic environments (121). Interestingly, studies with the relatively non-pathogenic model mold *A. nidulans* observed that while the mold could not proliferate without oxygen, ethanol fermentation was required for its long-term survival in anaerobic conditions (122,123). Indeed, analyses of *Aspergillus* genome sequences have revealed numerous potential fermentation pathways in these molds. Yet, as obligate aerobes, it remains unclear what function these pathways may serve in hypoxia adaptation and fungal virulence. Recent studies in our laboratory have identified ethanol fermentation during *A. fumigatus* infection in a murine model of IPA indicating that fermentation may be a component of the virulence arsenal of this mold (Grahl *et al.* unpublished data). However, our knowledge about the mechanisms by which *A. fumigatus*, an obligate aerobe, adapts to hypoxic environments remains extremely limited.

**The SREBP pathway in Aspergillus fumigatus:** Recently, our laboratory identified and characterized an SREBP (Sre1p) ortholog, SrbA, in *A. fumigatus* (124). As in *C. neoformans*, SrbA is crucial for adaptation to hypoxia, mediates resistance to the azole class of antifungal drugs and is involved in sterol biosynthesis in *A. fumigatus* (Figure 2 and Figure 3). In addition, unlike *C. neoformans*, but similar to *S. pombe*, transcriptional profiling of the SrbA null mutant suggested that SrbA does not appear to be involved in iron uptake or homeostasis in *A. fumigatus*. However, these studies may have been limited by the type of media utilized, and direct studies regarding the role of SrbA in iron homeostasis in this pathogenic mold are ongoing.

Importantly, *srbA* null mutants are almost avirulent in two distinct murine models of IPA. While our results strongly suggest that the virulence defect is due to the inability of this mutant to grow in hypoxia due to the loss of hypoxia adaptation mechanisms regulated by SrbA, as with *C. neoformans*, other potential hypotheses may explain the attenuation of virulence (124). For example, SrbA plays an important role in maintenance of cell polarity in *A. fumigatus* (124). We hypothesize that the accumulation of sterol intermediates leads to dysfunction in the formation or localization of sterol microdomains known to be required for maintaining cell polarity. Thus, the alteration of cell polarity may inhibit the ability of the SrbA mutant to cause disease. Yet, the mutant displays a normal growth rate *in vitro* suggesting that the altered cell polarity does not alter growth in these conditions. Altogether, these data promote the hypothesis that hypoxia plays a key role in the pathophysiology of IPA. At the least, it is apparent that SREBPs are critical components of fungal virulence in both pathogenic yeast and molds.

So far the molecular mechanism behind SrbA regulation and activation in molds is unclear. In the yeast *S. pombe* and *C. neoformans*, it seems evident that Sre1p is regulated post-translationally in response to sterol biosynthesis perturbation that occurs in low oxygen environments. Indeed, Hughes *et al.* (125) have identified 4-methyl sterols as the primary activating agent of Sre1p in *S. pombe* and *C. neoformans*. Thus, our finding that the SrbA null mutant in *A. fumigatus* accumulates 4-methyl sterols may also suggest that these sterols are the trigger for SrbA activation in *A. fumigatus* (124).

While many of the phenotypes observed in the *A. fumigatus srbA* mutant may suggest that SrbA is regulated in a similar manner as Sre1p in yeast, our results may also suggest an alternative model in molds. For example, despite intense bioinformatic analyses, we have been

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unable to identify clear homologs of SCAP or the proteases required for Sre1p activation. We have, however, identified a potential Insig1 homolog (*insA*) and we are currently characterizing a possible role for InsA in SREBP signaling in filamentous fungi. Yet, given the conservation of SCAP across many organisms, it is surprising that an ortholog does not appear to be present in the *Aspergilli*. It may be that another protein with a divergent sequence performs a similar function as SCAP, or it may suggest that a novel mechanism of SREBP regulation and activation exists in molds. Studies to examine these potential mechanisms are ongoing in our laboratory.

## Conclusion

In this review we have attempted to survey the known mechanisms utilized by fungi to regulate adaptation to hypoxic microenvironments. It is clear that we are just beginning to understand the mechanisms human fungal pathogens use to survive *in vivo* during infection. With the possible exception of SREBPs, the molecular mechanisms utilized by pathogenic fungi to adapt to hypoxic microenvironments found at sites of infection remain to be elucidated. A master regulator of hypoxia adaptation, such as HIF1 found in mammals, has not been identified in fungi. It remains to be seen whether one exists, or, if as suggested by current data, fungi rely on multiple mechanisms to sense oxygen levels and adapt to low oxygen environments.

In any case, we feel that this area of pathogenic fungal physiology has been ignored for too long. Certainly, some mechanisms of hypoxia adaptation, perhaps such as heme biosynthesis, are likely to be conserved between *S. cerevisiae* and the human pathogenic fungi. However, the different life-styles and selection pressures on nonpathogenic and pathogenic fungi likely have resulted in unique mechanisms of hypoxia adaptation Thus, solely relying on *S. cerevisiae* as a model for how pathogenic regulate adaptation to hypoxic microenvironments is likely not appropriate. Regardless, it seems clear that mechanisms of hypoxia adaptation have important implications for fungal virulence and how we manage and treat invasive fungal infections. Therefore, future studies on discovering the conserved and unique pathways utilized by the major fungal pathogens of humans to adapt to hypoxia are likely to yield important insights into sterol metabolism, fungal growth, mechanisms of drug resistance, and fungal virulence.

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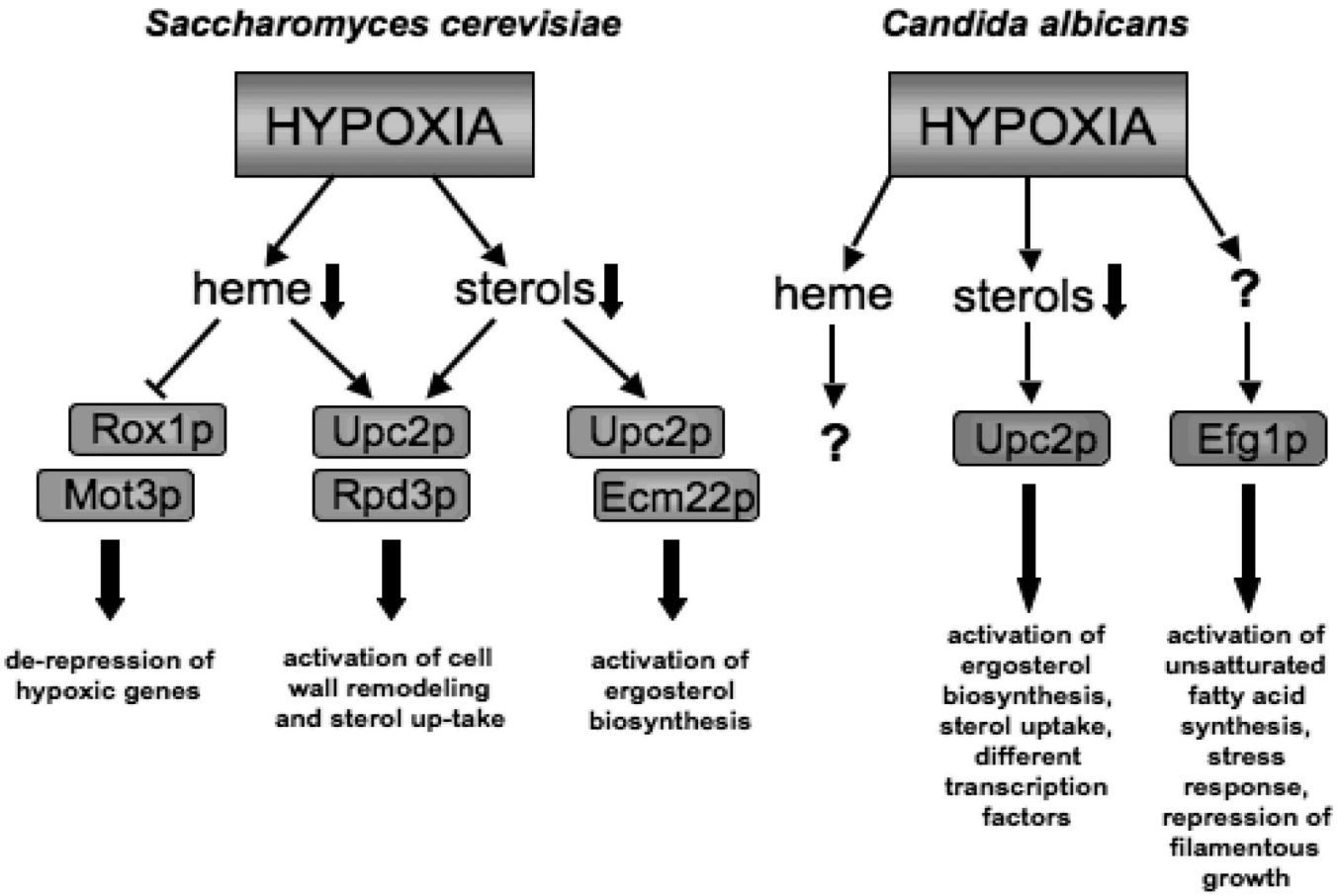


**Fig. 1. Schematic of the oxygen sensing pathways in *Saccharomyces cerevisiae* and *Candida albicans***
The proteins are defined in the text.

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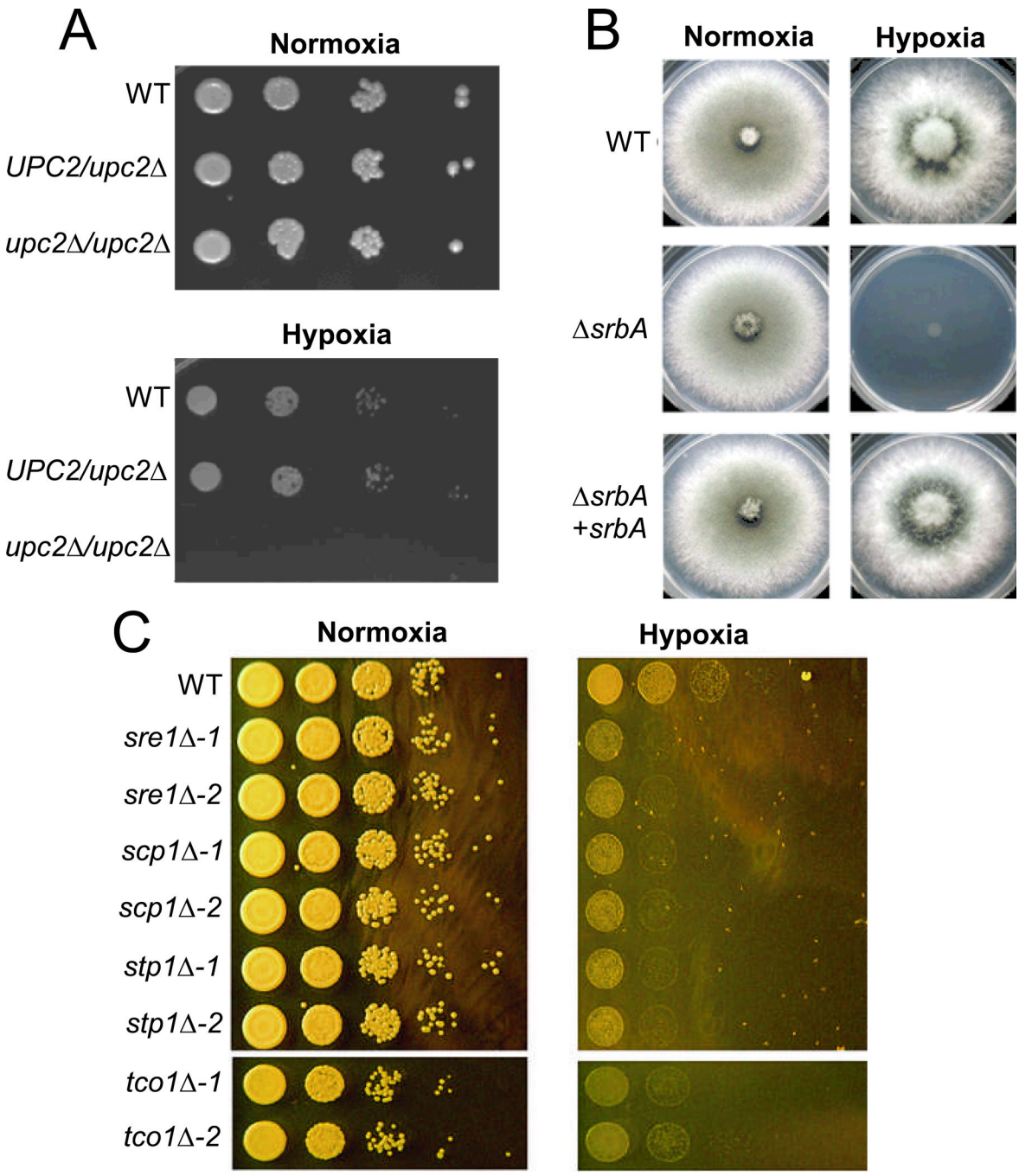


**Fig. 2. Mutants in SREBP Pathway and Tco1 are sensitive to hypoxia**

Growth in normoxic and hypoxic conditions. **A**) *Candida albicans*: A heterozygous *UPC2/upc2Δ* and a homozygous *upc2Δ/upc2Δ C. albicans* strain were serially diluted and spotted on CSM plates and grown at 30°C. The top panel shows growth in aerobic conditions after 48h. The bottom panel shows growth in hypoxic conditions after 96h. Under hypoxic conditions the wild-type (WT) and the heterozygous strain showed comparable growth but the homozygous deletion strain did not demonstrate any detectable growth (Courtesy Chelsea Samaniego and Dr. Theodore C. White); **B**) *Aspergillus fumigatus*: $1\times10^6$ conidia were plated on GMM plates and incubated at 37°C under normoxic and hypoxic conditions for 48h. The wild-type and the reconstituted strain grew comparably under hypoxic conditions while no

growth was detectable for the mutant strain (modified from Willger *et al.* (124)); **C**) *Cryptococcus neoformans*: *C. neoformans* cultures diluted to $OD_{600nm}$ = 0.6 were diluted serially in 10-fold increments prior to being spotted onto YPD plates. The plates were incubated in normoxic or hypoxic conditions in the dark at 37°C. Under hypoxic conditions all mutants in the SREBP pathway and the *tco1Δ* mutants showed reduced growth compared to the wild-type (modified from Chun *et al.* (100)).

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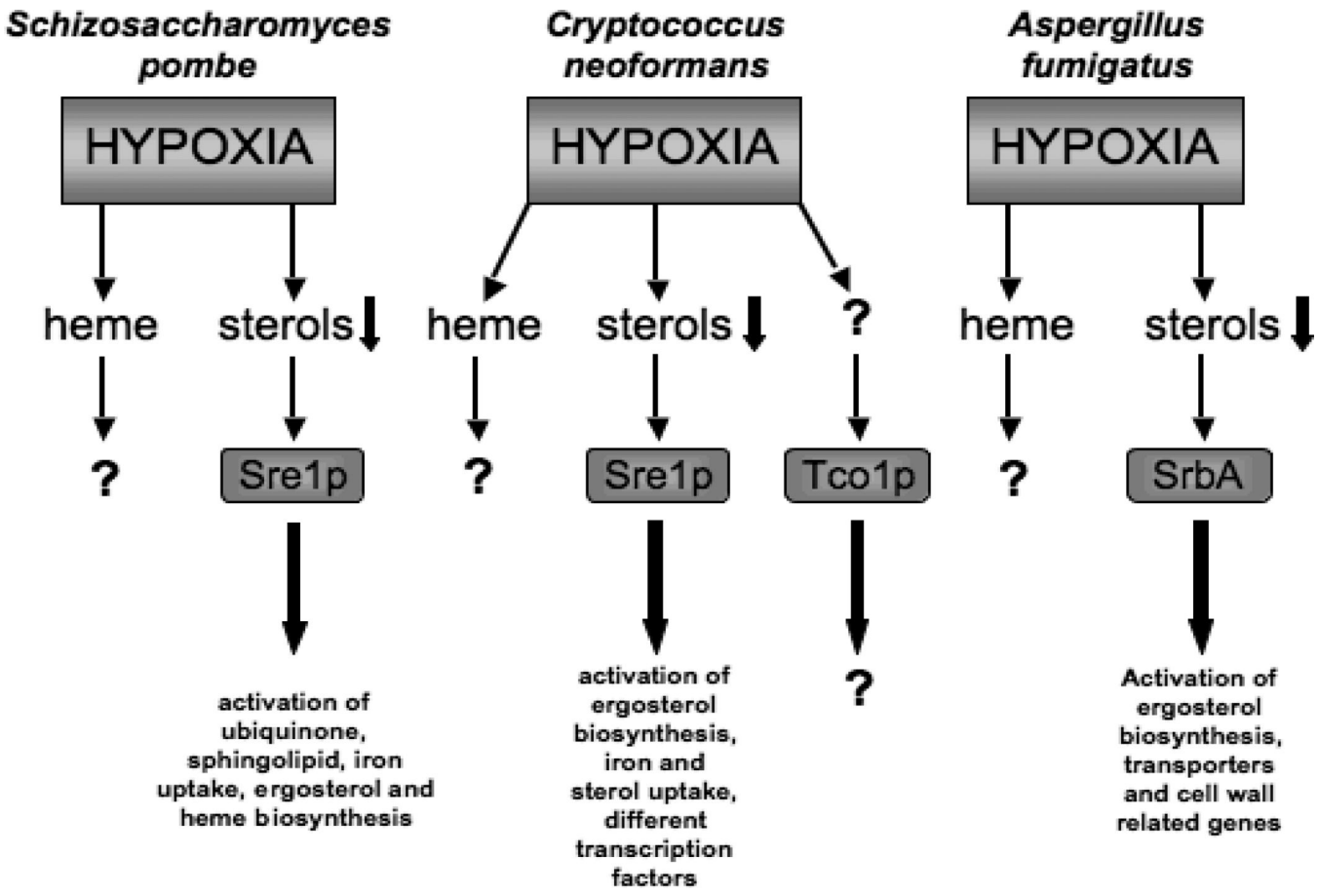


**Fig. 3. Schematic of the oxygen sensing pathways in *Schizosaccharomyces pombe*, *Cryptococcus neoformans* and *Aspergillus fumigatus***

The proteins are defined in the text.

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**Table 1**

Hypoxia sensing mechanisms and pathways.

| Hypoxia sensing pathways | mammals | *S. cerevisiae* | *C. albicans* | *S. pombe* | *C. neoformans* | *A. fumigatus* |
|---|---|---|---|---|---|---|
| **HIF pathway** | **a)** HIF-la, HIF-2a, HIF-3a; HIF-lb | No homolog found | No homolog found | No homolog found | No homolog found | No homolog found |
| **Heme level sensing pathways** | No homolog found | **a)** Hap1p/Rox1p/Mot3p | Rfg1p* (Rox1p homolog) | No homolog found | No homolog found | No homolog found |
| | No homolog found | **b)** Yap1p/Ord1p | Cap1p$^{x}$ | Pap1p$^{x}$ | No homolog found | AfYap1$^{x}$ |
| **Sterol sensing pathways** | **b)** SREBP-1(a/c)*, SREBP-2*, SCAP*, INSIG-1*, INSIG-2*, S1P*, S2P* | **c)** Upc2p$^{y}$/Ecm22p | Upc2p$^{y}$ | Sre1p, Scp1p, Ins1p* | **a)** Sre1p, Scp1p, Stp1p* | SrbA, InsA* |
| **Tco1 hypoxia sensing pathways** | no known | no known | Nik1p$^{x}$ | no known | **b)** Tco1p | Bos1$^{x}$ |
| **Regulated pathways in response to activation of hypoxia sensing pathways** | **regulated by a):** erythropoiesis and iron metabolism (e.g. erythropoietin and transferrin); oxygen transport (e.g. adrenomedullin) glucose uptake and glycolysis (e.g. glucose transporter 1, Lactate dehydrogenase A) | **regulated by a):** ***e.g.*** heme biosynthesis (*HEM13*), cell wall remodeling (*DAN/TIR* genes); fatty acid synthesis (*OLE1*); eIF-5a factor subunit AnbIp; respiration and ATP exchange (*COX5b; ACC3*) | **regulated by Upc2p:** azole drug resistance and sterol biosynthesis (*ERG11*); several transcription factors (*INO2, ACE2, SUT1, UPC2*); corticosteroid uptatke (*CBP1*); | **regulated by Sre1p:** heme biosynthesis (*HEM13, HEM14, HEM15*); sphingolipid biosynthesis (e.g. *SPBC887.15c*); fatty acid biosynthesis (e.g. *CUT6*); ergosterol biosynthesis (e.g. *ERG1, ERG5, ERG6, ERG11, ERG25*) | **regulated by a):** iron uptake (e.g. *SIT1* and *FRE7*); azol drug resistance and sterol biosynthesis (e.g. *ERG3, ERG11, ERG25*); **crucial for virulence** | **regulated by SrbA:** resistance to azole drugs and sterol biosynthesis (*erg25*, erg24, and *erg3*); transporters and cell wall related genes; cell polarity; **crucial for virulence** |
| | **regulated by b):** lipid synthesis (cholesterol biosynthesis and uptake) | **regulated by b):** cell wall mannoproteins (*TIR1/SRP1*) | | | **regulated by b):** no impact on expression of hypoxic genes - may be post-transcriptional regulation?; **required for virulence** | |
| | | **regulated by c):** cell wall remodeling (*DAN/TIR* genes); ergosterol biosynthesis and sterol uptake (e.g. *ERG2, ERG3, ERG10, DAN2, DAN4*) | | | | |

* no role in hypoxia response

$^{x}$ role in hypoxia unknown

$^{y}$ SREBP analog