SRBA-REGULATION OF ERGOSTEROL BIOSYNTHESIS IN *ASPERGILLUS FUMIGATUS*: GATEWAY TO AZOLE RESISTANCE & HYPOXIA ADAPTATION

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Immunology & Infectious Diseases

MONTANA STATE UNIVERSITY
Bozeman, Montana

May 2013

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DEDICATION

For all of you that have taught, guided, and inspired me over the years. Thank you.

This thesis is dedicated to my family:
Dad, Mom, Jamie, Adam, and especially Luke.

Thanks for the love and laughter.

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ABSTRACT

Aspergillus fumigatus is a human fungal pathogen and the primary cause of Invasive Aspergillosis (IA). A rise in susceptible patient populations has dramatically increased the incidence of IA, and led to the emergence of triazole antifungal drug resistance. Triazoles target Erg11, an enzyme involved in ergosterol biosynthesis. Ergosterol biosynthesis has been widely targeted for antifungal drug development, but little is known about this pathway in *A. fumigatus*. We have identified a transcription factor, SrbA, which mediates triazole susceptibility, growth in hypoxia and low iron, and virulence during IA. Transcriptional studies identify ergosterol biosynthesis as one of the major genetic targets of SrbA, including *erg11* and *erg25*. In this study, we examined the mechanism of Δ*srbA* triazole susceptibility. Construction of an *erg11A* conditional expression strain in the Δ*srbA* background restored *erg11A* transcript levels and, consequently, wild-type sensitivity to fluconazole and voriconazole. However, *pniiA-erg11A-*Δ*srbA* did not restore hypoxia growth or the total ergosterol defect of Δ*srbA*. Increased accumulation of C4-methyl sterols indicates that the Erg25-step of ergosterol biosynthesis is defective in these strains. *A. fumigatus* encodes for two C4-demethylases, *erg25A* and *erg25B*. Erg25A serves in a primary role over Erg25B, as Δ*erg25A* accumulates more C4-methyl sterol intermediates than Δ*erg25B*. That both *erg25* genes retain function, and are not limited to a singular substrate is unique in the eukaryotic kingdom. Genetic deletion of both *erg25* genes is lethal, and single deletion of these genes revealed alterations in ergosterol biosynthesis. Δ*erg25A* displayed moderate sensitivity to hypoxia, reactive oxygen species (ROS), and dithiothreitol, but was not required for virulence in a murine model of IA. Erg25 assists in the ability of *A. fumigatus* to grow in hypoxia, as construction of a strain that constitutively expresses *erg25A* in the Δ*srbA* background restored the hypoxia growth defect of Δ*srbA*. This restoration revealed substantial insufficiencies in *pflavA-erg25A-*Δ*srbA* when adapting to hypoxia, as this strain was hypersensitive to cell wall perturbation and ROS. Additionally, restoration of *erg25A* impacted triazole antifungal susceptibility of Δ*srbA*, demonstrating a complex feedback system involved in ergosterol biosynthesis. These results demonstrate SrbA's involvement in a dynamic stress adaptation program mediated in part through ergosterol biosynthesis.

CHAPTER ONE

INTRODUCTION

Invasive Fungal Infections – New Players in Human Infectious Diseases

Prior to the second half of the twentieth century, invasive fungal infections were considered relatively rare (267). The implementation of newer broad-spectrum antibiotics in the 1930s, ‘40s, and ‘50s greatly improved patient morbidity and mortality, allowing very sick patients to live longer than historically expected. This longevity did provide a niche for a limited number of documented opportunistic invasive fungal infections that would not have previously been noted, but did not contribute to an overwhelming increase in incidence (89). This is chiefly because most fungi do not cause overt disease in immune sufficient mammals, with the exception of some of the endemic fungi, such as *Blastomyces dermatitidis, Histoplasma capsulatum, Coccidiodes spp., Paracoccidioties brasiliensis, Sporothrix schenckii*, and the pathogenic yeast, *Candida albicans* (267). However, the rising number of invasive fungal infections documented each year caused by non-endemic fungi suggests that mammalian fungal pathogens may not be as unusual as we once considered. As modern medicine continually finds ways to overcome life-threatening disorders, it redefines the immune status and longevity of the human race. As these parameters change, so too must our definition of what constitutes a pathogen.

Invasive Aspergillosis: A Brief Introduction

The fungal genus *Aspergillus* consists of over 185 distinct species with 15 genomes sequenced to date. This genus belongs to the phylum Ascomycota, and many members of this phylum, including the *Aspergilli*, are molds. These organisms are important medically and commercially: commercially, for the production of fermented products (*A. oryzae*), and citric acid (*A. niger*), and medically, as the causative agents for the production of the carcinogen and food contaminant aflatoxin (*A. flavus*), allergic disease (*A. clavatus, A. fumigatus*), and invasive disease (*A. fumigatus, A. nidulans, A. terreus*). *Aspergillus fumigatus* is the most common species of *Aspergillus* cultured from patients with invasive disease, and thus a majority of biomedical research focuses on this pathogenic organism (69).

Aspergillus fumigatus is a filamentous saprophytic fungus first characterized in 1729 by Pier Antonio Micheli, a Catholic priest and botanist (215). Micheli assigned this genus name based on the *Aspergillus* macroscopic colony morphology, which looks like the aspergillum utilized by the Roman Catholic church (215). *Aspergillus* is commonly found in compost piles, where it encounters a vast array of obstacles to its growth and viability including high temperature, macro- and micronutrient availability, pH variation, oxidative stress, and oxygen availability (17, 32, 57, 203, 359).

Fungi are heterotrophs, meaning they absorb their nutrition from the surrounding environment. Fungi secrete enzymes into their local environment to degrade complex substrates and then take up these simpler substrates for metabolic use. Unlike bacteria,

which have evolved specific virulence factors to promote disease, it has been hypothesized that the characteristics *A. fumigatus* has developed as a saprobe are its primary means of causing disease in the mammalian host.

Casadevall and Pirofski have defined virulence as the relative capacity of a microbe to cause damage (52). Utilizing this definition, *A. fumigatus* has been classified as a Class 4 pathogen, as it can cause disease during immune deficiencies (chemotherapy, immunmodulation, genetic disease) as well as during immune system hyperactivation (allergy, asthma, allergic bronchopulmonary aspergillosis) (52). The specific attributes of *A. fumigatus* that allow it to cause host damage in distinct host settings remain an active area of investigation.

The most severe form of *Aspergillus* infection is Invasive Aspergillosis (IA). With mortality rates ranging from 30-95%, and an estimated 200,000 cases per year, continued research into host risk factors, antifungal therapies, and pathogen susceptibility is desperately needed (44). Classically, this invasive form of disease affects patients with a severe underlying defect in their immune system, such as prolonged neutropenia, transplantation, intensive corticosteroid therapy, hematological malignancy, chemotherapy, advanced AIDS, and chronic granulomatous disease (169). More recently, however, reports of IA in patients with COPD, those with increased bone marrow iron stores, cirrhosis, and use of anti-TNF therapies, such as infliximab, have also been reported as risk factors for IA (168, 169, 208, 327).

Major Milesones in Medical History that Increase Invasive Fungal Disease

Over the past century three major medical milestones have resulted in increased disease caused by fungi. Although not all of these medical milestones have uniquely predisposed patients to IA, all of them have greatly increased the incidence and frequency of invasive fungal infections.

Chemotherapy & Corticosteroids

The first milestone was the development of chemotherapy for organ transplantation and cancer treatments. In 1980, the first patient utilizing cyclosporine, a fungal-derived immunosuppressive compound, successfully underwent liver transplantation (308). Although cyclosporine and other immunosuppressive drugs have revolutionized transplant medicine, they have also inadvertently increased the number of individuals with susceptibility to an invasive fungal infection (62, 171).

Transplant-center studies indicate a ~3.1% incidence of IFIs (invasive fungal infections) in the 12-months following solid organ transplantation (SOT), with heart or lung transplants accounting for ~14% of conducted transplants. Invasive Aspergillosis (IA) accounted for the #1 and #2 diagnosed fungal infections in lung and heart transplant patients, respectively. Overall, the average incidence of IA following heart or lung transplant ranges from 0-35% and is highly variable depending on the transplant center, underlying immunocompromised state, and presence of a secondary SOT (ex. heart-lung) (242, 291, 295). Critically, patients with IA had the lowest 12-month survival rate after infection. Thus, *A. fumigatus* is an important barrier to successful heart and lung

transplant outcomes.

The combined efforts of effective cancer chemotherapy greatly increased the incidence of invasive fungal infections in the twentieth century (309). Simply put, chemotherapy is the use of drugs to kill cancer cells, and some types of chemotherapy also kill host cells that are essential to warding off opportunistic mycoses. According to the World Health Organization, rates of cancer incidence are on the rise, and with this increased incidence will coincide an increased susceptible patient base to invasive fungal infections.

IA incidence in cancer patients is due to several combined risk factors, including prolonged neutropenia, hematological malignancy, possible hematopoietic-stem cell transplant, and receipt of corticosteroids or other immunosuppressive therapies (208). The primary affected cancer groups are those with acute myeloid leukemia (AML), with a 10% incidence of IA (49, 129, 240). Currently, mortality in AML patients with IA is approximately 33%, a significant decrease from 48% in the 1990s, and 38.5% in the early 2000s. This decrease in mortality is attributed to increased awareness of the potential for IA, the development of the triazole voriconazole, and possibly combination drug use, though the latter is still somewhat controversial (240).

In addition to cyclosporine, corticosteroids, particularly glucocorticoids, are widely used for their immunosuppressive properties. Patients employing corticosteroids include those suffering from autoimmune disorders, severe allergic disease, and other various lymphocyte disorders (185). Glucocorticoids function by blocking the glucocorticoid receptor, inhibiting cytokine and eicosanoid production, cell adhesion, chemotaxis,

antigen presentation, antigen recognition, phagocytosis, immune cell survival and proliferation (185). Glucocorticoids decrease the ability for innate effector cells to respond appropriately to fungal pathogens. In a murine model of *A. fumigatus*, corticosteroid treatment is accompanied by a large influx of immune cells, facilitating a decrease in oxygen, and causing an abundance of hypoxia lesions (114). Corticosteroid treatment also decreases the host's ability to repair damaged tissue, and reduce the ability of neutrophils to cause damage to *Aspergillus* hyphae (185).

Although IA is observed in patients under current corticosteroid therapy, clinical data suggest that patients having ceased corticosteroid therapy are also at risk for IA. Patients who have been on corticosteroids and are experiencing graft-versus-host disease have more extensive pathology owing to the complex immunobiology of this disorder. Therefore, treatment options for IA in the context of corticosteroid usage are increasingly complex (185).

In the fall of 2012, the unfortunate contamination of steroids used for joint injection was the cause of invasive fungal meningitis. Although not the major contaminant, *A. fumigatus* was attributed to at least one of these deaths. Although these patients were considered immunocompetent, direct introduction of the fungal spores into a joint such as knee, shoulder, ankle, or spine, resulted in germination and ultimately fungal dissemination. Much still is unknown about why these immunocompetent patients were susceptible to infection, and if any underlying etiology differentiated patients who succumbed to the infection versus those who had full recovery.

HIV/AIDs and CMV

The second risk milestone for invasive fungal infections is HIV/AIDS, first identified in the early 1980s, and has since grown to epidemic proportions throughout the world. Physicians first identified patients with AIDS based on their presentation with *Pneumocystis carnii* pneumonia, a rare fungal infection that only occurs in patients with a compromised immune system (111). AIDS patients have highly depleted CD4+ T cell numbers. The average individual has approximately 500-1500 CD4+ cells/microliter, but an individual with HIV is generally considered to have AIDS when their CD4+ count dips below 200-350 cells/microliter (1, 274). Having a CD4+ density below this value greatly increases susceptibility to infections from fungi such as *Pneumocystis* or *Cryptococcus,* where this cell type is critical to pathogen clearance (283). Although *Aspergillus* is not frequently observed in patients with AIDS, this immunosuppressive virus has highly impacted human susceptibility to other invasive fungal infections around the globe.

Another viral infection that predisposes for invasive fungal infections is Cytomegalovirus (CMV). CMV is a member of the herpesvirus family, and infection is asymptomatic in healthy adults. However, in the immunocompromised patient, CMV can cause significant pulmonary disease, and predispose patients to IFIs (108). CMV affects similar patient populations as those described for *Aspergillus,* including solid organ transplant and cancer patients. Importantly, concomitant infection with CMV and fungi is known to cause “superinfection” in transplant patients (35, 369), and transplant patients infected with CMV and have a significantly higher risk of developing IA (142, 331).

Interestingly, prophylactic treatment of CMV with immunoglobulin also decreases the risk of invasive fungal infection after bone marrow transplant (112, 246, 305), strongly suggesting a mechanistic rationale for predisposition of CMV patients to *Aspergillus spp.* infection.

Immunomodulatory Therapy

The third major medical milestone that predisposes patients to IFIs is immunomodulatory therapy. As mentioned previously, some patients undergoing chemotherapy are at a higher risk for IA. The use of immunomodulatory drugs such as alemtuzumab, infliximab, or fludarabine immunosuppression, in conjunction with neutropenia in chemotherapy patients, increase the risk factors for IA (240).

More recently, infliximab treatment alone has been linked with IA (350), adding immunomodulatory therapy, even without additional comorbidities, to the growing list of factors that may predispose patients to IA. Infliximab is used to treat autoimmune diseases such as multiple sclerosis, Chron's disease, rheumatoid arthritis, and ulcerative colitis. Two other TNF-blockers, etanercept and adalimumab, also appear to predispose patients to IA, but at a lesser extent than infliximab (95, 343, 344). These differences in susceptibility have been attributed to the diverse mechanism of action of these TNF-inhibitors (95), and have the potential to illustrate key immunological components that are critical for prevention of IA.

Underlying Genetic Disease

Along with the three major medical milestones, a fourth risk factor for IFIs and IA is underlying genetic disease, such as Chronic Granulamotous (CGD), Cystic Fibrosis (CF), asthma, or COPD. CGD is a genetic disorder, either X-linked or autosomal recessive, which causes a defect in the subunits of the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase. This disorder affects one child in every 200,000-250,000 live births, and renders the individual susceptible to fungal and bacterial infection. Patients with CGD are especially susceptible to *Aspergillus* infections, possibly due to the lack of neutrophil extracellular trap (NET) formation, which is a mechanism for killing extracellular pathogens whereby the neutrophils ejects its chromatin onto the invading pathogen, binding up the pathogen, and allowing for efficient dispersal of antimicrobial proteins (46, 203). The overall incidence of fungal infections with CGD patients is around 20%, with *Aspergillus spp*. contributing to 78% of the infectious genera (26, 83). IA accounts for 50-75% of the mortality associated with CGD (26, 83).

Patients with COPD are uniquely at risk for IA. Although some patients with COPD are under treatment with corticosteroids, those that are not are still at risk due to changes in lung architecture, frequent hospitalizations, lesions in the mucosa or impairment of mucociliary clearance, prolonged antibiotic treatment, or prior long-term use of corticosteroids (169). These patients may also have underlying comorbidities or genetic alterations making them more susceptible to developing IA, but these factors are unknown at present.

Although IA is still quite rare in COPD patients, 1% of IA patients also have COPD and this combination lends itself to an especially high mortality rate (91%). Diagnostics of COPD patients with IA is especially confounding, as radiological imaging provides nonspecific or inconclusive results, requiring the isolation of a positive specimen for accurate diagnostics (169).

Aspergillus spp. also contribute to the development and severity of asthma, both in asthma with fungal sensitization, and in the specific disease called Allergic Bronchopulmonary Aspergillosis (ABPA) (44). ABPA is characterized by severe asthma, sputum production, elevated levels of IgE, and hypersensitivity to *Aspergillus* (93, 162, 166). The alterations in lung architecture associated with Cystic Fibrosis also predispose to ABPA.

A. fumigatus produces allergenic compounds (44). With its small size, and ubiquitous presence, approximately 12 million people with chronic rhinitis are sensitized to fungal allergens like those produced by *A. fumigatus*. Asthma with fungal sensitization is thought to affect between 3.25 and 13 million people worldwide, and may contribute to mortality associated with severe asthma. ABPA may affect upwards of 4 million people worldwide, and is found especially in patients with asthma or cystic fibrosis (44), where approximately 1-2% of asthmatics, and 7-9% of those with Cystic Fibrosis have all the essential diagnostic criteria of ABPA (87, 117, 118, 165, 177).

Aspergillus is routinely cultured from the sputum of CF patients, with some geographical areas reporting a prevalence of up to 58% (251, 296, 307). The lungs of a CF patient are especially vulnerable to *Aspergillus* infection, as their ability to remove

conidia is impaired, leading to increased rates of colonization (58). Short-term treatment with antifungals is generally positive in patients with CF and ABPA, but the benefit of longer-term treatment is currently understudied (245, 307, 312). Additionally, treatment of *Aspergillus* in the CF lung may be complicated by the presence of a bacterial co-infection, such as *Pseudomonas aeruginosa* or *Staphylococcus aureus* (13, 18, 179), making this form of *Aspergillus* infection especially difficult to diagnose and treat (179).

One of the major challenges facing patients with *Aspergillus* infections is the accurate and timely diagnosis of infection. Of the worldwide public health agencies, only the U.S. Centers for Disease Control (CDC) currently reports and tracks fungal infections (44). As numbers of susceptible patients continue to rise, so too must awareness of these infections in the medical community.

Diagnosis of IA

As highlighted above, diagnosis of IA is often complex. Although a halo-sign on a radiological image is a classic approach to diagnosing IA, it has a sensitivity of 25%, and can also indicate a bacterial pneumonia (45). Definitive diagnosis of an *Aspergillus spp*. infection involves either demonstration of tissue invasion in a biopsy, or obtaining a purified culture from the biopsy specimen. As the patients who become infected with *Aspergillus* are generally very ill, an invasive biopsy is generally not well tolerated and prohibited. In the absence of a pure culture from a biopsy specimen, an attempt is made to culture *Aspergillus* from the sputum or bronchioalveolar lavage (BAL).

Two problems arise with culturing from the BAL: first, isolation from BAL is only suggestive of an invasive form of disease, and second, BALs have a demonstrated false-positive rate. This rate depends on the study, but ranges from 0-75% (261).

Enzyme immunoassay (EIA) of the cell wall polysaccharide galactomannan on BAL fluid is a more robust detection method for IA, and may be performed as a routine screen for patients at risk for developing IA. Alternative diagnostic tools also include PCR amplification of *Aspergillus* DNA.

Galactomannan EIA may be confounded if the patient has been treated with beta-lactam antibiotics, or been on intravenous hydration or nutrition, and may cross-react with similar cell wall components from other fungi, including *Penicillium marneffei, Histoplasma capsulatum*, and several bacterial species (354). Likewise, PCR of *Aspergillus* DNA can be problematic, especially as the PCR is generally performed on blood. Sensitivity for *Aspergillus* via PCR ranges from 36-98%, and specificity from 72-100%. These ranges likely reflect differences in specimen quality, DNA extraction method utilized, and primer specificity in different diagnostic settings (155, 249, 354, 355). To date, galactomannan assay of BAL is our best tool for the detection of IA.

Treatment of IA

Once a putative or probable *Aspergillus* diagnosis is given, a treatment protocol must be established and executed. Three main classes of antifungals have been historically utilized in the treatment of aspergillosis: polyenes (amphotericin B), echinocandins (capsofungin, micafungin, anidulafungin), and triazoles (itraconazole, posaconazole,

voriconazole) (346). Fluconazole, a triazole used frequently in the treatment of *C. neoformans* and *C. albicans*, is ineffective against *Aspergillus spp* (91). Newer triazoles, such as voriconazole, have increased the margin of success in the treatment of IA (10.4-32.9%) above traditional amphotericin B (AmB) therapy, and slightly increased survival rate (12.9% higher at 12 weeks). Voriconazole treatment does not exhibit the damaging renal side-effects common to AmB ($p<0.001$), although transitory visual disturbances were noted in 44.8% of patients in one study (128). Current primary therapeutic treatment of IA is now primarily voriconazole with AmB as an alternative. For salvage therapy, itraconazole, posaconazole, capsofungin, micafungin, or a lipid form of AmB may be utilized. Additionally, in the case of infection of native or artificial heart valves, surgical intervention accompanies a robust and aggressive antifungal regimen (346).

As described above, treatment options for IA are quite limited, highlighting the need for new antifungal drug development. This need has never been so apparent as with the identification of triazole-resistant *A. fumigatus* isolates (76). The mechanisms involved in antifungal drug targeting and resistance will be discussed in a later section.

Invasive Aspergillosis is a growing problem. As the highlighted patient populations continue to increase, and more immunomodulatory therapies are employed, we will continue to increase the incidence of Invasive Aspergillosis. With over 200,000 world-wide cases of IA per year, and a mortality rate of nearly 50%, understanding the mechanisms of how *Aspergillus* causes disease, characterizing the essential immunological entities required to clear *Aspergillus*, developing more sensitive diagnostic tools, and establishing a deeper arsenal of antifungal drugs are absolutely

imperative (44). As *A. fumigatus* is ubiquitous in the environment, eliminating disease is not simply a case of eradication, but instead a complex puzzle that requires the combined efforts of physicians, pharmaceutical companies, and researchers to solve.

Aspergillus fumigatus: Biology of a Killer

Environmental Niche & Morphology

Aspergillus spp. are ubiquitous in the environment, where they function primarily as composters or saprobes (44). Their basic morphology consists of a mass of vegetative mycelium. On individual mycelia or hyphae, asexual reproductive structures, called conidiophores assemble, apical to a blunted hyphal cell called a vesicle. Conidiophores resemble a child's drawing of the rising sun, where the vesicle is the body of the sun, and the rays radiating from the vesicle are the phialides, which pinch off little droplets of sunshine called conidia. Conidia are the asexual propogule of *Aspergilli,* and are also the infectious particle for patients.

Conidia are excellent disease vectors; at 2-3μM these small particles are easily aerosolized and can be distributed into the alveolar sacs of the lung when inhaled. The average individual inhales hundreds of *Aspergillus* conidia per day (136), but these are efficiently cleared by the innate immune system, particularly the resident alveolar macrophages in the lung (143). Disease occurs when the innate immune system is unable to clear the invading conidia, which is able to begin its growth into a hyphal or filamentous fungus.

Conidia are similar to the seeds of plants in that they are resistant to a variety of

stresses and exist in a dormant state. A portion of the stress resistance of conidia is because they are hydrophobic in nature and immunologically inert due to being coated by a rodlet layer of hydrophobins (3). Conidia are also melanized, giving the conidia of *A. fumigatus* a green-ish gray hue. Melanin protects conidia from monocyte/macrophage attack *in vitro*, and mutants lacking melanin have decreased virulence in animal models of invasive Aspergillosis (146, 176, 325, 326). Melanization is also a notable virulence factor in the human pathogenic fungus *Cryptococcus neoformans* (360, 361).

Conidia contain all the genetic information required for formation of a new organism, some of which is pre-packaged for rapid use and growth as mRNA during condiogenesis (174). Lack of removal via engulfment by a macrophage in the warmer temperatures of the lung allows the conidia to germinate. Germination can be separated into four distinct stages: breaking of dormancy, swelling, establishing cell polarity, and formation of a germ tube (22, 67, 97, 124).

Fungal Cell Wall: Providing Physiological Stability & Immune Recognition

The fungal cell wall is an important entity as its biochemical composition both protects it from lethal environmental forces (ex. ultraviolet light, reactive oxygen species, etc.), and also prevents fungal cells from being recognized and eliminated by host cells. The cell wall is comprised of two distinct layers; the first or external layer is comprised of hydrophobic rodlets and melanin, which protect the fungus from desiccation and UV light (97). The second layer is a polysaccharide lattice structure that provides stability to the cell membrane, and is comprised of α- and β-glucans, chitin, and galactomannan.

Although mannans are recognized by the immune system, β-glucan is the cell wall component with the most immunostimulatory properties, as it is recognized as a pathogen associated molecular pattern (PAMP) by the receptor Dectin-1. Dectin-1 is found on many host cell types, including monocytes, macrophages, neutrophils, and some T cells, and has been characterized for its role in clearance of *A. fumigatus* and other fungal pathogens (43).

β-glucans are relatively well masked by rodlets during the conidial stage, but become vulnerable to immune recognition in the stages of germination subsequent to swelling (43). This mask, possibly provided by melanin or α-glucan (54), is not as effective in the swelling or germ tube stage of growth, when the fungal wall is being reorganized. Unmasking of β-glucans by the echinocandin Caspofungin has a clear effect on immunostimulation via the C-type lectin Dectin 1, enhancing phagocytosis and clearance of *Aspergillus* conidia via neutrophils (173).

Germination & Hyphal Growth

As the conidia swells, it grows isotropically, and rapidly undergoes a change in its surface adhesion (238). Between swelling and the elongation of the germ tube, actin, microtubules, septins, vesicles, and many other components must be organized in order to facilitate the rapid alterations the cell wall and membrane must undergo in order to produce a germ tube (222, 287). This process is highly dependent on the continuous transport of secretory vesicles (123). During this time, the germ tube emerges, requiring precise allocation of cell wall and membrane building materials to assemble at the apical tip of the emerging hyphae. This process, however, is not efficiently organized until after

the germ tube emerges, as the tip elongation machinery has not yet fully assembled (317). The assembly of a fungal organelle called the Spitzenkörper to the elongating germ indicates that the machinery for hyphal tip elongation is complete. The Spitzenkörper is a vesicle cluster that localizes to the apical center of the germ tube and serves as the central hub for shuttling necessary materials from the beginning of the germ tube into the rapidly rearranging cell wall and membrane (124).

Adaptation to Stress

Several factors have been proposed to contribute to the virulence of *A. fumigatus*, including perturbation of the mucociliary system, interference with phagocyte function, interference with opsonization, adhesion to host tissues, thermotolerance, and toxin production (324). Stress adaptation, however, has been suggested to be one of the main features that allows *A. fumigatus* to be such a successful pathogen (125). *A. fumigatus* encounters, and adapts to, a variety of cellular stresses within the mammalian host. These stresses include: temperature (28, 270), alkaline pH (34), starvation (206), host factors, reactive oxygen intermediates (259, 282, 370), antifungal drugs, iron restriction (352), and low oxygen (114).

Hypoxia Adaptation

Adaptation to hypoxic microenvironments may be an inherent property required for the saprophytic life-style for *A. fumigatus* outside the host (321). Evidence also suggests that hypoxic microenvironments exist at the site of *A. fumigatus* infection in the lung (41, 114), although this environment has been understudied in the *Aspergillus* field.

That hypoxia may be an important mechanism for the pathogenicity of *Aspergillus* was first suggested by Hall and Denning in 1994 (121), where they demonstrated efficient growth at very low oxygen concentrations. Tarrand *et al.* suggested that the ability to culture *Aspergillus* from clinical specimens may be affected by hypoxia diagnosis, and that culturing *Aspergillus* samples at a more hypoxic oxygen concentration would be reminiscent of human physiology, and thus more likely to grow. Brock, M. *et al*. demonstrated that a luciferase-expressing strain of *A. fumigatus* was unable to luminescence after 24 hours of infection. This finding was contrary to the premise of this experiment: to quantify fungal burden throughout infection. They postulated that quenching of luminescence started after 24 hours of invasive growth due to the lack of oxygen available to cause bioluminescence. *Grahl, et al*. utilized technology pioneered in the tumor detection field to correlate sites of <1.5% oxygen, with foci of *A. fumigatus* infection (114), definitively demonstrating that hypoxia occurs in during *A. fumigatus* infection of the murine lung.

Many components of *A. fumigatus* biology require oxygen, and it is considered an obligate aerobe. Exposure to hypoxia elicits an induction of glycolysis, iron uptake, and ergosterol biosynthesis, while simultaneously repressing the TCA cycle and oxidative phosphorylation (23). In addition, hypoxia alters the interaction between *A. fumigatus* and the mammalian immune system. Hypoxia alters the cell wall thickness of *A. fumigatus* hyphae, and augments the total β-glucan exposed to host antigen presentation cells. Hypoxia may also give advantage to macrophages and neutrophils, two cell types critical to the detection and removal of *A. fumigatus* conidia and germlings (288). In conclusion,

hypoxia is a biologically relevant environmental condition that has recently been identified as a critical component of both host and pathogen biology and interaction.

Our understanding of fungal biology in general, and that of *A. fumigatus* in particular, is constantly evolving. Recent evidence from our laboratory suggests that hypoxia adaptation may also be a crucial component of *A. fumigatus* virulence. Hypoxic microenvironments have been characterized in association with a variety of infectious pathogens including *Mycobacterium tuberculosis, C. albicans, C. neoformans, Streptococcus pyogenes, Staphylococcus aureus,* and *Pseudomonas aeruginosa, Leismania amazonensis* (109, 269, 374), during tumorgenesis (16), after vascular injury (293), and in the course of skin infection (374). The widespread identification of hypoxia or hypoxia-inducible factors in these varied biological settings suggests that adaptation to low oxygen or hypoxia environments is a key factor for many biological processes.

Although hypoxic environments are hypothesized to occur during the course of infection for several organisms, the physical verification and characterization of this phenomenon has only been established in the settings of *Mycobacterium tuberculosis*, *Pseudomonas aeruginosia*, and *A. fumigatus* infection (109, 114, 207, 333, 363). Although the presence of hypoxic lesions in the murine lung during *A. fumigatus* infection has been established, very little is known about the critical components of *A. fumigatus* biology that are required for disease progression in hypoxia.

Discovery of SrbA

Ethanol discovered in the BAL of an infected mouse lung indicated that *A. fumigatus* may be encountering hypoxia during the course of infection (114). Further investigation has revealed the substantial presence of hypoxia lesions, defined as areas of local oxygen concentration less than 1.5% O2 (114), were established in all three of the major murine models of IA utilized in the *Aspergillus* literature. We therefore determined that *A. fumigatus* may encounter hypoxia during the course of invasive infection, and hypothesized that the ability to adapt to hypoxia is one mechanism that allows *A. fumigatus* to be a successful pathogen.

Microarray analysis of *in vitro A. fumigatus* cultures revealed genes whose transcript levels were altered in the presence of hypoxia (359). Among these genes was a transcription factor with suggested effect on adaptation to low oxygen environments. This transcriptional factor, SrbA, is a member of the sterol element binding protein (SREBP) family, and has proven to be a crucial component in hypoxia and low iron adaptation, polarized growth, virulence, and triazole drug resistance (36, 359). Thus, this transcription factor appears to regulate not only *A. fumigatus'* ability to cause disease, but also in how it responds to antifungal agents.

SREBPs in *H. sapiens* and *S. pombe*

In *Homo sapiens*, sterol regulatory element binding proteins (SREBPs) are crucial for cellular synthesis and regulation of fatty acids, triglycerides, and sterols. The *H. sapiens* SREBP can be found in three isoforms arising from two genes. Two isoforms are formed

from SREBP-1, owing to alternative open reading frames of the *srebp* gene. These three isoforms are SREBP-1a, SREBP-1c, and SREBP-2. Each serves a distinct set of functions: SREBP-1a activates fatty acid and cholesterol biosynthesis, SREBP-1c activates fatty acid synthesis, and SREBP-2 activates cholesterol biosynthesis and uptake (286).

Human SREBPs are uniquely regulated helix-loop-helix transcription factors, as the SREBP protein is synthesized and then stably bound in the ER. SREBPs are maintained in the ER via interaction with a sterol sensing protein called Scap. In low sterol environments, the SREBP-Scap complex is transported to the Golgi via COPII vesicles. In the Golgi, the N-terminal portion of SREBP is proteolytically cleaved via site-1 (S1P) and site-2 proteases (S2P), freeing the transcription factor portion of the protein. Once freed, the N-terminal SREBP can translocate to the nucleus, whereby it activates genes with a sterol-response element (SRE) (286).

SREBPS are regulated on many levels, by autoregulation, microRNAs, oxysterol production, and through regulation of supporting proteins critical to SREBPs cleavage and activation. Overall, human SREBPs have important functions in cholesterol homeostasis, insulin regulation, and fatty acid biosynthesis. These critical components of mammalian biology solidly link SREBPs as key factors in a variety of pathologies, including hypercholesteremia, tumorigenesis, activation of the inflammasome, autophagy, and neuronal physiology (286).

In *Schizosaccharomyces pombe*, much of the SREBP-machinery is conserved, and functions in a similar fashion, with slight alterations in how SREBP cleavage occurs

(313, 314). Fission yeast utilize members of the Golgi E3 ligase family to mediate cleavage of SREBP via the proteosome. This is a significantly different mechanism than in humans, as fission yeast lack a S2P. This divergent mechanism appears to be partially conserved in *A. fumigatus* (358), but much of the mechanism of SREBP activation and regulation in this organism is currently unknown.

Initial Characterization of SrbA

Characterization of Δ*srbA* revealed a substantial sensitivity to the triazole antifungal drugs fluconazole and voriconazole (359). This finding was especially interesting, as Δ*srbA* became highly susceptible to fluconazole, an azole with no activity against wild-type *A. fumigatus*. This clinically significant result led to the first specific aim of my proposed research, and lays the foundation for the remaining studies in my dissertation project.

In addition to the azole susceptibility phenotype observed in Δ*srbA*, the mutant demonstrates altered hyphal development and the inability to growth in hypoxia (1% O_2) (359). Due to these dramatic phenotypes, we hypothesized that this mutant would have altered virulence in a corticosteroid murine model of IA. Substantially, Δ*srbA* is completely avirulent in this model, indicating that the processes regulated by SrbA are critical for fungal dissemination and growth.

As a transcription factor, SrbA influences the transcription of many genes, including many involved in ergosterol biosynthesis (36, 37, 359). Interestingly, one of the genes that appeared to be highly SrbA-dependent was *erg11*, which is the target for the triazole antifungal drugs. Further transcriptional studies have determined that Δ*srbA* has

decreased mRNA accumulation of many of the ergosterol biosynthesis genes, especially in the oxygen-dependent portions of the ergosterol biosynthesis pathway (36).

Since many of the ergosterol biosynthesis enzymes require oxygen as a cofactor, the inability of Δ*srbA* to grow in hypoxia, coupled with the azole sensitivity phenotype, make the regulation of ergosterol biosynthesis by SrbA of interest in understanding the role of hypoxia adaptation in *A. fumigatus*. These findings suggest a possible mechanism by which azole resistance is regulated in this pathogenic fungus.

These preliminary findings laid the groundwork for my thesis, which examines the role of SrbA in the regulation of key steps in the ergosterol biosynthesis pathway as a mechanism for the phenotypic changes observed in Δ*srbA*.

Biological Roles of Ergosterol

Ergosterol is the main sterol component of the cell membrane in most Ascomycetes (351), and is homologous to cholesterol, the main sterol found in humans. Ergosterol is synthesized in the endoplasmic reticulum (ER), then transported to the plasma membrane via both vesicular and non-vesicular transport (27, 328), wherein it is incorporated into the plasma membrane. Ergosterol serves important functions in plasma membrane rigidity, fluidity, and permeability (244)

Ergosterol was first identified as a fungal-specific sterol, but also can be found in parasitic protozoa, such as trypanosomes (74, 92). It differs from its mammalian counterpart, cholesterol, in the addition of a 7,8-double bond in the second sterol ring, a 22,23-double bond in the sterol side chain, and an additional methyl at C24 of the sterol

side chain (Figure 1.1).

Ergosterol biosynthesis requires the cooperative action and coordination of approximately 20 genes. The pathway can be broken into two general classifications: the oxygen independent, and the oxygen dependent portions of the pathway. For this thesis, we will focus on the oxygen-dependent portion of ergosterol biosynthesis, which involves the transformation of squalene to ergosterol (Figure 1.2).

Oxygen-dependent ergosterol biosynthesis requires 12 molecules of oxygen (55, 180). With the exception of Erg1, all of the oxygen requiring steps also require an iron cofactor: Erg11 and Erg5 are heme-binding, wheras Erg25 and Erg3 are non-heme iron binding enzymes (180). In *A. fumigatus*, many of these enzymatic steps are encoded by multiple genes, which provide genus-specific modifications to the synthesis of ergosterol, or perhaps serve as a backup system for this critical biosynthetic pathway (Table 1.1).

Membrane Polarity and Integrity

Sterols play an important role during germination and hyphal elongation as major components of the cell membrane. Sterols are widely diffused throughout the growing hyphae, but are particularly aggregated in the hyphal tip and at the septa, which compartmentalizes individual hyphal cells from one another (188). The hyphal tip or apex is crucial for elongation as it is the site where vesicles containing cell wall materials are exocytosed (332). Sterol-rich domains similar to lipid rafts are also hypothesized to accumulate at the hyphal tip. Lipid rafts are comprised of sterols and sphingolipids, in a unique environment that may allow for the incorporation or exclusion of proteins, such as GPI-anchored proteins; as well as for the exo- and endo-cytosis of vesicles. Thus sterols

play important roles in both cell membrane stability and integrity, as well as in the highly complex process of vesicle transport (332).

The plasma membrane has been well studied in the filamentous fungus *Aspergillus nidulans*, a close relative to *A. fumigatus.* In *A. nidulans*, the plasma membrane is comprised of several domains, or functional areas. The major domain that contains sterols is a domain called a lipid raft, this domain is comprised of sterols and sphingolipids clustered with glycerophospholipds. These domains are thought to contribute to protein localization of GPI-anchored and lipid-associated proteins, and to play a role in cell signaling and polarity (280, 294, 318).

Although not much is known about lipid rafts in fungi, a subset of these sterol-sphingolipid-glycerophospholipd domains has been characterized, called sterol rich domains (12). These sterol rich domains (SRDs) are unique to fungi, contain high levels of the sterol ergosterol, and can be visualized via staining with the fluorescent dye filipin, a relative of the polyene class of antifungals (318, 332). At several micrometers in size, SRDs are much larger than their lipid raft cousins, which range from 10-200 nanometers in *A. nidulans* (252). Preliminary evidence suggests that SRDs play an important role in the establishment of polarity in *A. nidulans*, but, as is the case for Spitzenkorper assembly, the formation of well-organized SRDs occurs at some point after germ tube emergence, and prior to the development of mature hyphae. Importantly, the integrity of SRDs is critical to the localization of hyphal-elongation proteins to the apical portion of the hyphal tip (318). Genetic deletion of microdomain scaffolding proteins essential to SRD localization results in hyphal deformity, where hyphae were uneven, and had lost an

organized sense of polarity, which was evidenced by abnormal branching (318). Overall we see that SRDs are critical for the efficient germination and hyphal elongation of *Aspergilli*.

Ergosterol in the plasma membrane serves important functions in membrane rigidity, fluidity, and permeability (244). Much of our knowledge of the functions of ergosterol in the membrane has come from identification and characterization of ergosterol biosynthesis mutants in the model organism *Saccharomyces cerevisiae*. In *S. cerevisiae*, mutants in enzymes essential for the last five steps of ergosterol biosynthesis are viable, and produce altered ergosterol intermediates that have profound effects on membrane function. Viable *erg* mutants have been characterized that have hypersensitivities to LiCl (353), NaCl (353), ethanol (233), cyclohexamide (88), and brefeldin A (340). These sensitivities have been attributed to the increased permeability of the membrane when various intermediates are substituted in the place of ergosterol (2). Overall, *erg* mutant membrane analysis demonstrates the importance of cell membrane integrity in basic resistance to a variety of cellular disturbances.

In addition to providing general resistance to deleterious compounds, sterols in the membrane are highly ordered, and perturbations of this rigidity compromise the organism when placed under various cellular stresses. This is most drastically seen with sterol auxotrophs, where membranes have a complete loss of rigidity (194). Incorporation of altered intermediates is likely to cause voids in the membrane, as the insertion of lipid moieties with non-ergosterol intermediates fails to allow for correct packing of the membrane components (2). Additionally, cholesterol cannot fully substitute for ergosterol

in *S. cerevisiae*; validating that the basic structure of ergosterol is crucial for integrity and fluidity of fungal membranes.

Target of Antifungal Drugs

In addition to ergosterol being crucial for membrane integrity and polarity in many Ascomycete fungi, the ergosterol synthesis pathway and its resulting product is the target of both the polyene and triazole antifungal drugs. Three main classes of antifungals have been historically utilized in the treatment of Aspergillosis: polyenes (amphotericin B), echinocandins (capsofungin, micafungin, anidulafungin), and triazoles (itraconazole, posaconazole, voriconazole) (346). Two of these three classes of antifungal drugs (polyenes and triazoles) target ergosterol or ergosterol biosynthesis, making the study of this sterol and its transcriptional regulation critical to both current and future patient care. The gravity of these studies is especially appreciated with increasing documentation of triazole resistance in the clinic.

Polyenes: Polyenes were originally derived from bacteria in the genus *Streptomyces*, and have been in use treating fungal infections for more than fifty years (214). However, due to extreme toxicity issues, polyene antifungals have extremely limited usage in the clinic. This toxicity is related to its primary mechanism of action, whereby the polyene binds ergosterol in the fungal membrane (115). A second mechanism of polyene antifungal action is also theorized, whereby AmB permeabilizes the fungal membrane via channel formation, causing leakage of cellular components and cell death. In humans, polyenes may mistakenly bind to cholesterol, thus inhibiting this critical membrane

components and causing toxicity (115).

One strategy to minimizing this AmB-associated toxicity was to formulate the drug into liposomes. Liposome-associated Amphotericin B (L-AmB) treated mice infected with *C. neoformans*, survive longer and have lower fungal burden than mice treated with the non-liposomal variety of intravenous AmB. This liposomal derivative has allowed physicians to administer higher dosages of AmB for longer periods of time than was previously possible (116, 229).

Triazoles: The triazole class of antifungal drugs function by targeting the lanosterol 14α-demethylase (Erg11A/B or Cyp51A/B) of the ergosterol biosynthesis pathway. Thus, azoles inhibit the conversion of lanosterol to ergosterol, which is a vital component of fungal membrane integrity. Fungistatic action of this compound is thought to occur through accumulation of toxic sterol intermediates that contain a methyl group at C14 (159).

Although azoles target the cytochrome P450 family, azoles do not preferentially bind to mammalian P450, providing an advantageous mechanism to target pathogenic fungi such as *A. fumigatus*. Current azoles with FDA approval include: fluconazole, itraconazole (ITZ), posaconazole, ketoconazole, and voriconazole.

Resistance to FDA-Approved Triazole Antifungal Drugs: Currently, resistance to azole drugs is thought to evolve from prolonged exposure (14, 300). Azoles are fungistatic, not fungicidal; therefore the population of cells that survive immune clearance provide excellent directional selection in favor of resistance (14). Since fungi

do not regularly exchange plasmids, or other vectors of DNA transmission, horizontal exchange of a resistance mutation is not generally passed from colony-to-colony. This leads to the alarming conclusion that resistant populations may be generated from simultaneous single-colony mutations. Additionally, resistant isolates are not thought to be transmitted from patient-to-patient, as this occurrence is rare (156, 247). However, it has also been observed that some environmental isolates also contain the "classical" point mutation in the L98H mutation, and resistance is conferred in these isolates as well (300).

Clinical isolates of *A. fumigatus* resistant to itraconazole began to be documented a few years after its FDA approval, with at least two distinct resistance mechanisms being identified (76). A study in a Netherlands hospital demonstrated an annual prevalence of itraconazole (ITZ)-resistant A. fumigatus between 1.7-6% (211). Cultures from isolates from other locations in The Netherlands exhibited 14.5% itraconazole resistance, and similar annual trends were seen in Belgium, United Kingdom, Greece, France, Sweden, and Norway. Alarmingly, 94% of the resistant isolates exhibited identical point mutations at T364A of the cyp51A gene, which substitutes a leucine 98 for a histidine (L98H), which is the "classical" *A. fumigatus* resistance mechanism to ITZ (304). A 34-base tandem repeat in the promoter region of *erg11A/cyp51A* may also accompany the L98H mutation, and is thought to contribute to the azole cross-resistance observed in these strains (211, 212).

The L98H resistance mechanism has been linked to use of agricultural azole fungicides in The Netherlands. Five fungicides employed between the years of 1970 and 2005 induce *A. fumigatus* cross-resistance to medical triazoles. Interestingly, the usage of

these five fungicides directly precedes the first isolate cultured with the L98H resistance mechanism. Additionally, a tandem repeat in the promoter region of *erg11A* associated with L98H resistant isolates was experimentally induced with treatment of one of these five suspect fungicides. These results suggest that susceptible patients undergoing transplant, chemotherapy, or corticosteroid treatment either acquired a resistant isolate or were carriers of resistant fungal spores (299).

Almost identical to the rapid spread of itraconazole, voriconazole (VCZ) resistant isolates have surfaced in the clinic setting. According to our knowledge, the first documented case of a significant decrease in voriconazole sensitivity occurred just ten years after its FDA approval (137). The first published case of voriconazole resistance (MIC >16ug/mL), occurred just one year later, and can be confirmed in nine separate cases as of 2007 (336). Although the occurrence of VCZ-resistant isolates is still rare, an increasing trend towards multiple-azole resistant strains is cause for grave concern. Another Netherlands' study documents no multiple-azole resistance cases of IA between 1945-1998, but a 12.3% rate of incidence from 2002-2007 (59, 336). Clearly, azole drug resistance is a major emerging problem related to treating IA.

Even with a predominant resistance-mechanism, other mechanisms of azole resistance have been documented. These mechanisms primarily have included target modification (*erg11A*) and upregulation of both the MDR-and ABC-class of drug efflux pumps (59, 227). Recently, a structural model of *erg11A* has allowed researchers to better evaluate the contribution of target modifications to triazole resistance (301). Alterations in *erg11B* may also contribute to triazole resistance, as *erg11B* can functionally replace

erg11A (47). Although alterations in *erg11B* do not appear to occur at nearly the same rate as in *erg11A*, there have been mention of two such isolates that conferred increased *erg11B* mRNA abundance, and thus resistance to triazole antifungal drugs (47).

In addition to the characterization of target modification and efflux pump expression in resistant isolates, three mutations in the promoter region of *erg11A/*cyp51A have been identified that increase *erg11A/cyp51A* transcript abundance, and confer partial resistance to triazole antifungal drugs (5). These results suggest that alteration in transcription factor binding may be an alternative mechanism for triazole drug resistance, but binding partners have not yet been identified that coincide with these mutated regions.

In addition, a number of strains exhibiting unknown resistance mechanisms have been documented in the literature (50, 300), indicating that these organisms are not limited to the resistance mechanisms characterized to date. Overall, understanding the mechanisms of resistant mutations is vital in order to increase the effectiveness of currently available azoles.

The *Aspergillus* research community has made significant strides in the last decade to understand the molecular mechanisms of triazole antifungal resistance. There are, however, still many unanswered questions pertaining to the mechanisms of resistance, and how best to circumvent resistance in the clinic. Overexpression of both *erg11A* and *erg11B* has been linked with triazole antifungal resistance, and alterations in the promoter regions of *erg11A* have been documented (5, 47). These findings strongly suggest that transcription factor alteration is a possible mechanism for triazole resistance in *A. fumigatus*. This mechanism has been demonstrated for *C. albicans*, for both the

transcription factor(s) that transcriptionally activate *erg11*, and also for the transcription factors that mediate efflux pump overexpression (51, 77, 85, 86, 221, 279, 292, 356, 375).

Recently, a resistant isolate with a mutation in HapE, a member of the CCAAT-binding complex, was isolated, suggesting that iron regulatory mechanisms may be deeply involved in triazole resistance mechanisms (50). This finding broadens our knowledge of transcriptional regulation of ergosterol biosynthesis, and illustrates some of the complexity surrounding regulation of triazole resistance in *A. fumigatus*. Although the mechanism behind HapE-induced resistance is currently unknown, its identity strongly suggests that transcription factor alteration can induce resistance in *A. fumigatus*.

There are several aspects to triazole resistance that are currently undefined. These areas include identifying mechanisms of unknown resistance, further determining the contribution of drug efflux pumps to triazole antifungal drug resistance in *A. fumigatus,* and the identification of transcription factors that regulate *erg11A* and *erg11B* and their physical binding sites within the promoter regions of these genes. The last area, identification of transcription factors that regulate *erg11A* and *erg11B*, is especially of interest to us, due to the transcriptional relationship we have observed between SrbA and *erg11A* (36). As the mechanisms involving regulation of *erg11/cyp51* have primarily been characterized in yeasts (134, 198, 292, 356), studies describing this process in *A. fumigatus* must be conducted. In particular, defining how regulation differs in *A. fumigatus* from *S. cerevisiae* should be a top priority for the furtherance of triazole antifungal drug development.

Iron, Siderophores & Ergosterol Biosynthesis

Iron is a crucial cofactor for ergosterol biosynthesis. As mentioned previously, several of the ergosterol biosynthesis genes require iron as a cofactor, and SrbA appears to be involved in the coordination of iron regulation and uptake (36). In addition, disorders involving accumulation of circulating or bone marrow iron stores are one risk factor for developing an invasive *Aspergillus* infection (277).

Iron is taken up from its extracellular environment by three mechanisms: low affinity ferrous iron acquisition, high affinity reductive iron assimilation, and siderophore-assisted iron uptake. Reductive iron assimilation occurs when ferrous iron is reduced extracellularly, and then taken into the cell via active transport through iron permeases. Siderophores are ferric iron chelators that are secreted into the extracellular environment, take up iron, and then reenter the fungal cell through siderophore-iron transporters, which are highly conserved throughout bacteria, plants, and fungi (25). Once the siderophore is inside the fungal cell, iron is released through hydrolysis. Interestingly, even organisms that cannot produce their own siderophores, such as *C. neoformans* and *C. albicans*, possess siderophore-iron transporters, suggesting that they may be able to steal siderophores from other microbial organisms (15, 145, 320). In addition, *A. fumigatus* possesses intracellular siderophores, which are unique among fungi (276). These intracellular siderophores are hypothesized to be one of the iron-storage mechanisms employed by *A. fumigatus* (276, 277).

Elimination of *A. fumigatus* siderophore biosynthesis decreases growth, conidiation and resistance to oxidative stress in iron-limited conditions, highlighting the importance

of siderophore-mediated iron uptake (276, 345). In addition, elimination of both intra- and extra-cellular siderophore biosynthesis renders *A. fumigatus* completely avirulent in a murine model of Aspergillosis (276, 277).

In low oxygen, the transcription factor HapX mediates repression of iron-requiring pathways (275). HapX is a member of the CCAAT-binding complex and interacts with the CCAAT-binding core complex that is comprised of HapB, HapC, HapE and HapX (135). Pathways repressed by HapX include heme biosynthesis, cellular respiration, ribosome biogenesis, and ergosterol biosynthesis. HapX also activates ribotoxin AspF1 and siderophore biosynthesis. A HapX-null mutant is attenuated in virulence in *A. fumigatus* (36, 275, 277). Interestingly, transcriptional profiling suggests that *hapX* may be regulated by SrbA (36).

Identification of a triazole resistant, clinical isolate that overexpresses HapE suggests that iron regulatory mechanisms may be involved in triazole resistance mechanisms (50). Ergosterol biosynthesis and iron acquisition are additionally linked via siderophore biosynthesis (366). Mevalonate is a limiting prerequisite for TAFC-biosynthesis, one of the extracellular siderophores *A. fumigatus* produces (366). This supply-and-demand appears to be regulated by 3-hydroxyl-3-methyl-glutaryl (HMG)-CoA reductase, which is thought to be the main sterol sensor in *A. fumigatus*, and is a key sterol biosynthesis checkpoint for most eukaryotic organisms (366). Therefore, ergosterol biosynthesis and iron acquisition are two delicately intertwined pathways, both through feedback networks, cofactor requirements and transcriptional regulation.

Ergosterol Biosynthesis and Hypoxia

Ergosterol biosynthesis is also significantly affected by oxygen. In fact, so intimate is the relationship between oxygen sensing and sterol biosynthesis, that evolutionary biologists have hypothesized that sterols evolved, at least in part, as a response to the rise in atmospheric O_2 (104). Ergosterol biosynthesis produces ergosterol, the main sterol of the fungal cell membrane. Ergosterol biosynthesis requires twelve molecules of oxygen to transform lanosterol into ergosterol (55). Even fungal species capable of anaerobic growth, such as *C. albicans*, or *S. cerevisiae* require molecular oxygen for ergosterol biosynthesis (60, 284). Importantly, hypoxic adaptation and ergosterol biosynthesis have been conclusively linked to regulation by a sterol regulatory-element binding protein (SREBP) in the fungal organism *Schizosaccharomyces pombe* (141, 323).

The influence of oxygen in ergosterol biosynthesis is a well-documented characteristic of *S. cerevisiae*, *C. albicans*, *C. neoformans*, and *A. fumigatus* biology (55, 181, 195). The oxygen requirements of each biological step in this process vary, with a majority of the oxygenation requirements surrounding the demethylation of C4 (*erg25*), although oxygen is required by *erg1*, *erg11*, *erg24*, *erg25*, *erg3* and *erg5* (180). Two C4-demethylation steps are required for the production of ergosterol, consuming six of the twelve oxygen molecules required for synthesis. *Erg25* mutants are sterol auxotrophs in *S. cerevisiae* (103), demonstrating their importance to ergosterol biosynthesis. Additionally, high copy numbers of *erg25* rescued growth of a hypoxia-deficient mutant in *C. neoformans*, suggesting that *erg25* plays a large role in fungal ergosterol biosynthesis under oxygen-limiting conditions (180).

Erg24 is another crucial, oxygen-requiring step of ergosterol biosynthesis. *S. cerevisiae*, a facultative anaerobe, requires *erg24* for survival under aerobic conditions (21). An *erg24* mutant in *C. albicans* demonstrated slower growth and attenuated virulence in a murine model of systemic *Candidiasis* (150). Additionally, *erg24* has been shown to be important for hypoxia adaptation in *S. cerevisiae* (290), and to be regulated by the *C. albicans* hypoxic transcription factor *upc2* (376). Upc2 is likely a functional analogue of the SREBPs in other fungal organisms.

Link Between SREBP-Regulation of Ergosterol Biosynthesis & Hypoxia Adaptation

SREBPs are membrane-bound transcription factors that sense sterol levels in many eukaryotic organisms (33). Low sterol or oxygen concentrations activate this ER-bound transcription factor, and the protein complex dissociates. Upon dissociation, SREBP is cleaved into a DNA-binding portion (amino-terminal), and a putative protein-protein interaction domain (carboxy-terminal). Once cleaved, the amino-terminal portion is translocated to the ER/Golgi, processed, and transported to the nucleus where it acts as a global transcription factor for ergosterol biosynthesis. SREBP not only regulates ergosterol biosynthesis, but also is transcriptionally activated in hypoxia, as an important transcription factor of hypoxia-inducible proteins (33). As this cascade of events is stimulated by the availability of sterols in the lipid membrane, many of the biological processes of *S. pombe* are tied to requirements of both oxygen and ergosterol biosynthesis.

Several null mutants have been constructed in *C. albicans* and *C. neoformans* that

exhibit a growth defect in hypoxia. These mutants also display altered virulence in murine models of infection. Most notably, the SREBP-homologue in *C. neoformans* controls ergosterol biosynthesis, and is avirulent in a murine model of *Cryptococcosis* (55).

Other factors are also involved in the regulation of ergosterol biosynthesis, including oxysterols and heme (29, 131, 255). However, work by the laboratory of Dr. Peter Espenshade, Johns Hopkins School of Medicine, has demonstrated that the major regulatory factor for SREBP-dependent ergosterol biosynthesis is oxygen (255).

Function of UPC2 in Regulation of Ergosterol Biosynthesis & Adaptation to Hypoxia in *S. cerevisiae*/*C. albicans*

Sterol biosynthesis in *S. cerevisiae* is controlled by two transcription factors, Upc2 and Ecm22 (337). Upc2 is also involved in the hypoxic response, including the uptake of sterols under hypoxic conditions (65, 72, 186, 357). This function of Upc2 in regulation of ergosterol biosynthesis appears to be conserved in *C. albicans*, and *Ca*Upc2 has been implicated in triazole resistance mechanisms (86, 134, 195, 292, 356). Although Upc2 is not a homologue of SREBPs, these two classes of transcription factors have analogous functions, similar localization and activation patterns, and are proposed to be an example of convergent evolution in the fungal kingdom (33, 134, 198, 292, 356).

The inactive protein of both classes is tethered to a cytoplasmic membrane, and both are released via proteolytic cleavage to function as transcription factors (134, 292, 356). Also, genetic deletion of either Upc2 or SREBPs results in decreased accumulation of ergosterol, increased susceptibility to antifungal drugs, and an inability to induce

expression of *erg11* under antifungal drug pressure (134, 195, 292, 356). Unlike SREBPs, Upc2 seems to have no effect on the basal regulation of *erg11*, as levels of *erg11* are unchanged in the *upc2*Δ strain without the addition of antifungal drugs (134, 292).

Presence of SREBP-Like Protein in *C. albicans*

Upc2 and Emc22 are the main sterol regulatory transcription factors in *C. albicans*, and have been well characterized. There are, however, SREBP-like proteins in *C. albicans*, and they serve slightly overlapping, but diverse functions from the SREBPs characterized in *S. pombe* and *C. neoformans.* Similar to SREBPs, Cph2 is a member of the helix-loop-helix transcription factor family (175), and shares homology with *S. cerevisiae* proteins Tye7 and Hms1. It was originally characterized due to its roles in promoting psuedohyphal growth. Additionally, the promoter sequences of Cph2 reveal a dual E-box motif and sterol response element (SRE), suggesting that Cph2 is regulated in a similar fashion as SREBPs, and may form heterodimers (175). A null mutant of Cph2 dampens the ability of the fungus to undergo hyphal growth, which can be partially restored by ectopic expression of Tec1, a regulator of hyphal development (175). Overexpression of Cph2 also caused *C. albicans* to undergo filimentation in yeast-favorable conditions, suggesting that Cph2 directly regulates key machinery involved in hyphal development (175). In addition to hyphal development, Cph2 may regulate key virulence attributes in *C. albicans*, such as secreted aspartyl proteinase genes SAPs4-6, which have been shown to promote virulence in both systemic and mucosal Candidiasis (175), and may be involved in biofilm development via Tec1-dependent regulation of

biofilm regulator Bcr1 (84, 232). Although Cph2 appears to be regulated in a similar manner to other fungal SREBPs, the lack of overlapping gene targets and dissimilar null-mutant phenotypes suggest that SREBPs serve divergent functions in *S. pombe* and *C. neoformans*. It is especially interesting to note that although *C. albicans* and *A. fumigatus* are more closely related to each other than to *S. pombe* or *C. neoformans*, that *A. fumigatus* appears to utilize SREBP as its main sterol regulatory transcription factor, not UPC2.

Ergosterol Biosynthesis Genes as Direct Targets of SREBPs

The link between transcriptional regulation of ergosterol biosynthesis genes and SREBP has been strongly suggested in the *S. pombe* and *C. neoformans* literature, but is mostly correlative, as direct transcription factor-gene interactions have not been verified for many *erg* genes. The best characterized direct interactions occur between both *C. neoformans*' Sre1 and *erg3* and *S. pombe*' Sre1 and *erg3*, where direct interaction was verified via CHiP-PCR (323).

Interaction with remaining members of ergosterol biosynthesis has been suggested, in particular those in the oxygen-requiring portion of the ergosterol biosynthesis pathway, downstream of lanosterol. Direct evidence that SREBPs interact with *erg* genes has been limited thus far to a depletion in total ergosterol in SREBP-null mutants in the fungi *S. pombe* and *C. neoformans*, and marked sensitivity to C14-demethylase inhibitors.

Sterol Intermediates:
Plausible Role in Signaling

Sterol intermediates have been implicated in feedback and regulation within a variety of biological processes, including ergosterol biosynthesis and immune regulation. In humans, cholesterol intermediates act as ligands for the liver X receptor (LXR), a member of the nuclear hormone receptor family and is critical for the expression of genes regulating cholesterol metabolism, sterol transport, and fatty acid biosynthesis (365). LXR has high affinity for the oxysterol form of certain sterol intermediates, especially those with a hydroxyl at C22, C24, or C27 (365). Stigmasterol is another sterol intermediate with affinity for LXR, but it is not an oxysterol. Stigmasterol is characterized by a double bond at C22, and an ethyl group at C24. Stigmasterol also may interfere with processing of SREBP-2 by promoting the formation of SCAP-INSIG complexes in the ER (365).

C4-methylated sterol intermediates play an important role in the activation of meiosis. Meiosis-activating sterols (MAS) were originally discovered through a screen for naturally occurring compounds that triggered mouse oocyte meiosis *in vitro*. Two different types of MAS were identified and named for the location of their identification: FF-MAS, found in the follicular fluid, and T-MAS, first isolated from the testis of an adult bull. FF-MAS, or 4,4-dimethyl-cholesta-8,14,24-trien-3β-ol, and T-MAS, or 4,4-dimethyl-cholesta-8,24-dien-3β-ol, have been primarily implicated in the maturation of the testis and spermatogenesis (157).

Sterol intermediates are also involved in immune regulation. In the human hair follicle, treatment with 7-dehyrocholesterol results in a dramatic increase in the

expression of inflammatory genes (241), including TLR-4, IFN-α, IFN-α7 and NF-κB. These findings have implications for treatment of patients with Cicatricial Allopecia (241). Additionally, individuals with mutations in the *erg25* homologue, SC4MOL, demonstrate hyper accumulation of C4-methyl sterols, and increased CD25 and TLR-2 expression on granulocytes (130). Similar phenotypes are observed in patients with severe psoriasis, and an accumulation of methyl sterols is reported in these patients as well. These defects may be linked to LXR function.

Finally, accumulation of C14- and C4-methylated sterols is a known SREBP-independent mechanism of sterol feedback. HMG-CoA reductase degradation is regulated in part by INSIG-dependent ubiquitination. INSIG, in turn, responds to levels of C14- and C4- methylated sterols. Increased accumulation of these sterol intermediates quickens the half-life of HMG-CoA reductase, effectively slowing down sterol biosynthesis (230).

A role for sterol intermediates in other biological processes in fungi has not yet been elucidated. Intermediates are known to serve as a biofeedback mechanism for key checkpoints in the ergosterol biosynthesis pathway, and this has been initially characterized in *S. cerevisiae* (189). Although nuclear receptors, and thus nuclear receptor hormones, do not exist *per se* in fungi (225), members of the zinc family of transcription factors may perform a similar role in fungi. It is intriguing to postulate the variety of roles fungal sterol intermediates may have in their biology and disease capacity.

Characterization of Ergosterol Biosynthesis Genes in *Aspergillus fumigatus*

In *A. fumigatus*, ergosterol biosynthesis progresses in a slightly different fashion than in the yeasts *S. cerevisiae* and *C. albicans* (9). Although each organism retains proteins of similar enzymatic function, the accumulated sterol intermediates in *A. fumigatus* are structurally distinct from those accumulated in *C. albicans* or *S. cerevisiae*. In these yeasts, lanosterol is converted first to zymosterol and then fecosterol in its transition to the end product ergosterol (Figure 1.3). *A. fumigatus*, however, does not accumulate zymosterol, and instead accumulates the intermediates eburicol, 4,4-dimethyl fecosterol and 4-methyl fecosterol, suggesting a different order of ergosterol biosynthesis enzymatic action (9). This difference suggests a fundamental rearrangement of sterol intermediates in *A. fumigatus*, which is not unusual in the fungal kingdom (100, 228, 243).

A majority of our current knowledge about ergosterol biosynthesis in *A. fumigatus* has come from research into triazole resistance mechanisms. Erg11/Cyp51 is a 14α-demethylase, and the target of the triazole antifungal drugs. Erg11 is encoded for by two genes, *erg11A* and *erg11B*. Both of these isoforms have 40 to 70% homology with *erg11* genes from other fungal species and ~60% homology to each other (210). Single gene replacement mutants of either *erg11A* or *erg11B* are viable (210). As observed in other fungi, complete elimination of *erg11* in *A. fumigatus* by way of double gene replacement is lethal (138). Recently, fluconazole was shown to physically bind Erg11B with much greater efficiency than Erg11A, whereas voriconazole was shown to physically bind both homologs with high affinity (349). Together with the increased susceptibility of *erg11A*

null mutants to fluconazole, these data suggest that the inherent fluconazole resistance by *A. fumigatus* is due largely to the presence of two functional Erg11 enzymes (212).

As alterations in C5-sterol desaturase or Erg3 are a known triazole resistance mechanism in *C. albicans*, *Candida dubliniensis*, *Candida lusitaniae,* and *S. cerevisiae*, (21, 106, 144, 160, 161, 253, 272, 297, 367), examiniation of the function of *erg3* in *A. fumigtatus*, and the impact of *erg3* on triazole antifungal resistance has also been examined (8). *A. fumigatus* contains three genes that putatively encode for a C5-sterol desaturase or Erg3. Although *erg3A*, *erg3B*, and *erg3C* all contain the classic architecture for a C5-sterol desaturase, *erg3A* and *erg3B* are much more similar in their amino acid sequence to other fungal C5-desaturases than *erg3C* (8). It appears that *erg3C* may be a viable C5-desaturase, however, as genetic deletion of both *erg3A* and *erg3B* did not eliminate the production of ergosterol (8). Interestingly, genetic deletion of *erg3B* but not *erg3A* caused accumulation of intermediates lacking a C5-double bond, and a slight sensitivity to triazole antifungals. These results suggest that Erg3B is the primary C5-desaturase in *A. fumigatus* (8).

To date, the *Aspergillus* community has focused on characterizing the *erg11* and *erg3* steps of ergosterol biosynthesis, primarily due to their implications in the mechanisms of triazole antifungals and as a mode of resistance. These studies have provided critical understanding of the synthesis of ergosterol in *A. fumigatus*, and have laid the foundation for my studies in this dissertation.

Whys and Wherefores: The Effects of Sterol Biosynthesis on Invasive Disease

Aspergillus fumigatus is a filamentous fungus that has become an emerging and increasingly important pathogen. IA can be clinically challenging to treat because the arsenal of antifungal drugs effective against *Aspergillus spp.* is limited. This is largely due to the genetic similarity between fungi and man, which limits the available drug targets to combat fungal pathogens. Added to the limited variety of drug targets are typical problems associated with anti-microbials: pharmaceutical research and development, side effects of prescribed drugs, effectiveness of therapy, and development of resistance mechanisms in the organism. Each of these challenges applies to antifungals targeting *A. fumigatus*. Currently, the drug of choice for treating IA is the triazole voriconazole, which displays less severe side effects than the previous standby amphotericin B (128).

Recently, the emergence of azole drug resistant strains of *A. fumigatus* has been reported in the clinic (304, 335). As there are few drugs available to combat IA, to increase the effectiveness of currently available pharmaceuticals, it is important to understand the mechanisms behind anti-fungal drug resistance. We have discovered a transcription factor in *A. fumigatus*, SrbA, that mediates azole drug resistance, adaptation to hypoxia and low iron, and fungal virulence (359). Yet, the mechanism behind how this gene mediates these phenotypes is unknown. Through transcriptional studies, we have determined that SrbA regulates several ergosterol biosynthesis genes. This finding is of interest as ergosterol biosynthesis is targeted by two of the three main classes of

antifungal drugs, the triazoles and the polyenes. Additionally, ergosterol biosynthesis regulators in *S. cerevisiae* and *C. albicans* are important in both triazole antifungal resistance and adaptation to hypoxia. As Δ*srbA* is sensitive to the triazole antifungals and unable to grow in hypoxia, these parallels are of great interest. As SrbA-homologues in *S. pombe* and *H. sapiens* regulate sterol synthesis, we hypothesize that the phenotypes observed in Δ*srbA* are due to dysregulation of ergosterol biosynthesis, mediated by the transcriptional activition of SrbA.

Results from these studies are likely to yield a better understanding of how *A. fumigatus* responds to azole drugs and adapts to the harsh environments in the host lung. Ultimately, these aims seek to understand mechanisms related to the invasive growth of *A. fumigatus* in the host. Additionally, information garnered in this study may lead to future azole drug efficacy through modulation of SrbA activity.

Global Hypothesis

These significant preliminary findings lead to the development of my dissertation hypothesis, and are the subject of this body of work. The global hypothesis of my research is that the transcription factor SrbA is involved in the transcriptional regulation of ergosterol biosynthesis in the fungal pathogen, *Aspergillus fumigatus.* Investigation into the molecular makeup of this formidable pathogen has led me to develop two additional hypotheses: That SrbA transcriptional regulation is involved in susceptibility of *A. fumigatus* to triazole antifungal drugs through direct regulation of C14-demethylases, encoded by *erg11*. Secondly, that SrbA transcriptional regulation is

involved in the adaptation to hypoxia encountered in the host via the direct regulation of C4-sterol methyl oxidases, encoded by *erg25*. These hypotheses, their experimental investigations, and evidences will be discussed in the chapters to follow.

Figures

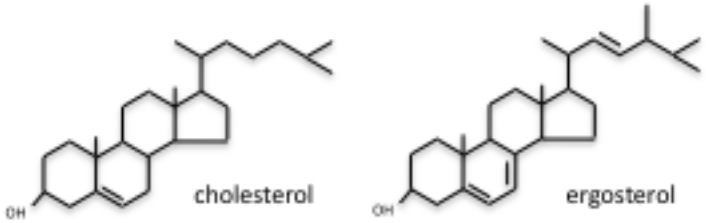


Figure 1.1 Structural differences in Cholesterol and Ergosterol.

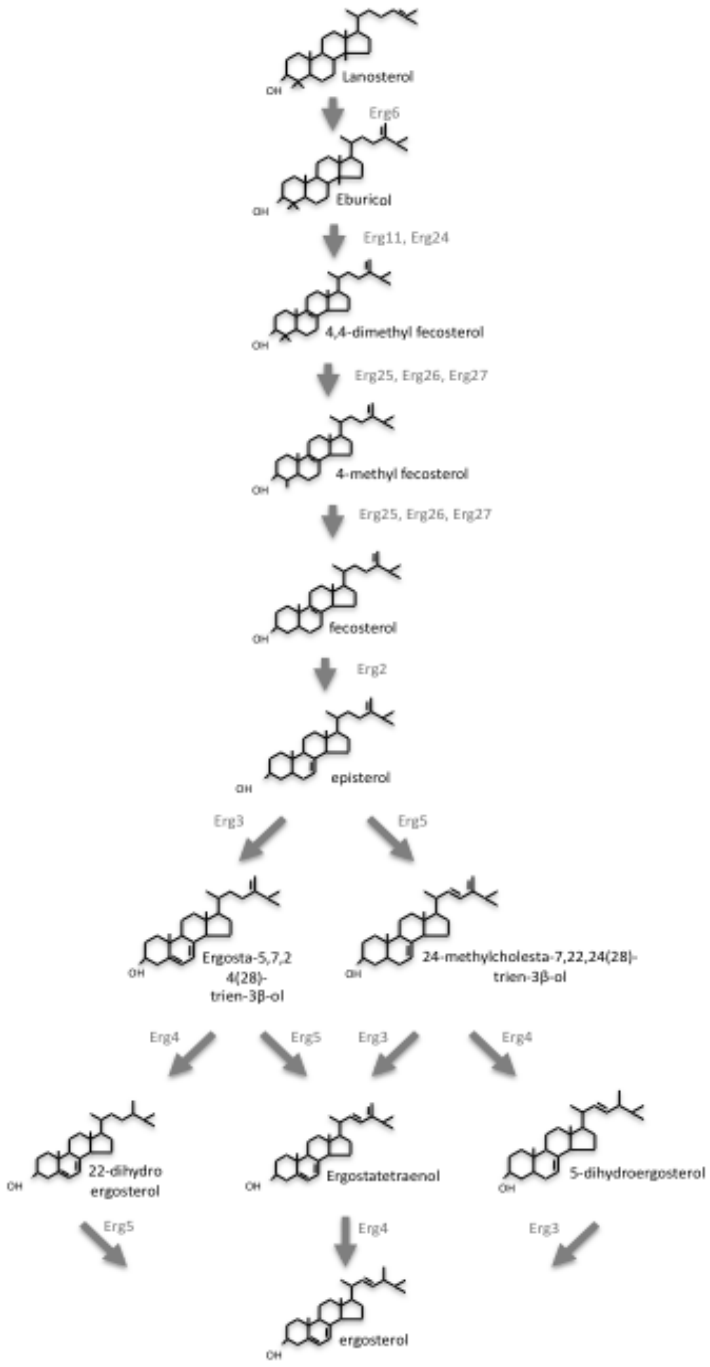


Figure 1.2 Ergosterol Biosynthesis in *A. fumigatus*. Modified from (9).

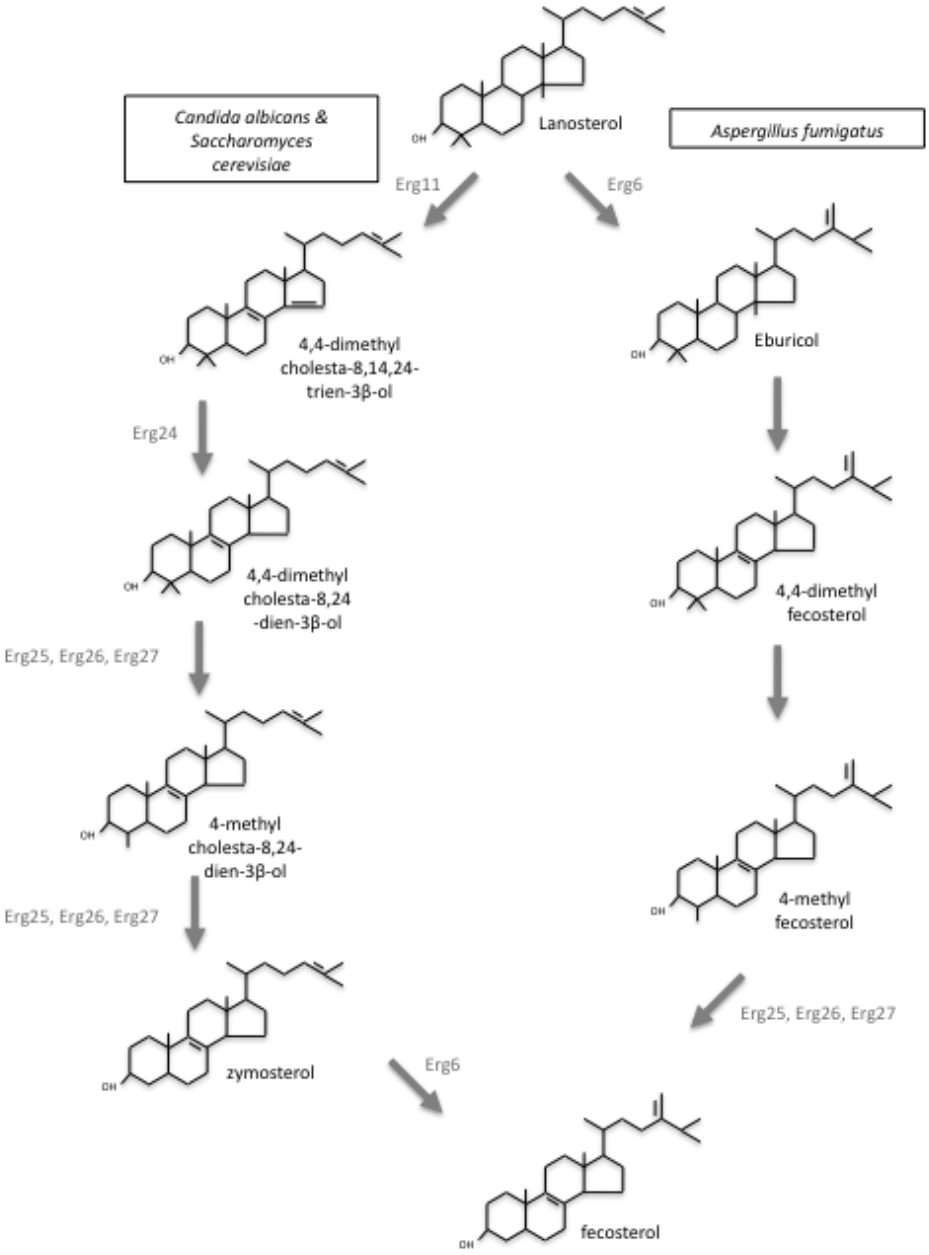


Figure 1.3 Comparing the Lanosterol-to-Fecosterol transition in *C. albicans* & *S. cerevisiae* with *A. fumigatus*. Figure modified from (9).

Tables

	# Copies	Names in *A. fumigatus*	Ascession Numbers
erg1	1	*erg1*	Afub_055310
erg2	1	*erg2*	Afub_005060
erg3	3	*erg3A* *erg3B* *erg3C*	Afub_017380 Afub_093140 Afub_085530
erg4	2	*erg4A* *erg4B*	Afub_062080 Afub_007490
erg5	1	*erg5*	Afub_004350
erg6	1	*erg6*	Afub_099400
erg7	3	*erg7A* *erg7B* *erg7C*	Afub_052590 Afub_069030 Afub_072030
erg11	2	*erg11A* *erg11B*	Afub_063960 Afub_089270
erg24	2	*erg24A* *erg24B*	Afub_006090 Afub_003560
erg25	2	*erg25A* *erg25B*	Afub_084150 Afub_098170
erg26	2	*erg26A* *erg26B*	Afub_033070 Afub_030680
erg27	1	*erg27*	Afub_010930

Table 1.1 Ergosterol Biosynthesis Genes in *A. fumigatus*

CHAPTER TWO

SREBP-DEPENDENT TRIAZOLE SUSCEPTIBILITY IN *ASPERGILLUS FUMIGATUS* IS MEDIATED THROUGH DIRECT TRANSCRIPTIONAL REGULATION OF *ERG11A* (*CYP51A*)

Contribution of Authors and Co-Authors

Manuscript in Chapter 2

Author: Sara J. Blosser

Contributions: Designed the study, conducted the experiments, collected and analyzed the data, wrote the manuscript.

Co-Author: Robert A. Cramer

Contributions: Obtained funding for the study, assisted with study design, discussed results of the experiments and implications for this study, edited the manuscript.

Manuscript Information Page

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Journal Name: Antimicrobial Agents and Chemotherapy

Status of Manuscript:

_____ Prepared for submission to a peer-reviewed journal
_____ Officially submitted to a peer-reviewed journal
_____ Accepted by a peer-reviewed journal
__x__ Published in a peer-reviewed journal

Antimicrobial Agents and Chemotherapy, 2012; 56(1):248-257.

Abstract

As triazole antifungal drug resistance during invasive *Aspergillus fumigatus* infection has become more prevalent, the need to understand mechanisms of resistance in *A. fumigatus* has increased. The presence of two *erg11* (*cyp51*) genes in *Aspergillus* spp. is hypothesized to account for the inherent resistance of this mold to the triazole fluconazole (FLC). Recently, an *A. fumigatus* null mutant of a transcriptional regulator in the sterol regulatory element binding protein (SREBP) family, the Δ*srbA* strain, was found to have increased susceptibility to FLC and voriconazole (VCZ). In this study, we examined the mechanism engendering the observed increase in *A. fumigatus* triazole susceptibility in the absence of SrbA. We observed a significant reduction in the *erg11A* transcript in the Δ*srbA* strain in response to FLC and VCZ. Transcript levels of *erg11B* were also reduced but not to the extent of *erg11A*. Interestingly, *erg11A* transcript levels increased upon extended VCZ, but not FLC, exposure. Construction of an *erg11A* conditional expression strain in the Δ*srbA* strain was able to restore *erg11A* transcript levels and, consequently, wild-type MICs to the triazole FLC. The VCZ MIC was also partially restored upon increased *erg11A* transcript levels; however, total ergosterol levels remained significantly reduced compared to those of the wild type. Induction of the *erg11A* conditional strain did not restore the hypoxia growth defect of the Δ*srbA* strain. Taken together, our results demonstrate a critical role for SrbA-mediated regulation of ergosterol biosynthesis and triazole drug interactions in *A. fumigatus* that may have clinical importance.

Introduction

Aspergillus fumigatus is a human fungal pathogen that must adapt to a variety of *in vivo* obstacles in order to cause disease. Some of these significant obstacles include high temperature, macro- and micronutrient availability, pH variation, oxidative stress, and oxygen availability (17, 32, 57, 205, 359). Understanding the mechanisms employed by *A. fumigatus* to overcome these challenges may lead to the design of improved antifungal drugs and therapeutic options for patients. Current treatment for invasive aspergillosis (IA) typically involves utilization of the triazole class of antifungal drugs. While this class of drugs is not associated with the significant toxicity issues of amphotericin B, some serious side effects have been reported and long-term usage is not ideal (139, 216, 224). In addition, resistance to the triazoles has recently been documented and appears to be becoming more prevalent in some areas of the world (76, 137, 335). Thus, understanding potential resistance mechanisms to these drugs is important for maintaining and improving our current standard of care.

Aspergillus spp. are inherently resistant to the triazole fluconazole (FLC), an antifungal drug commonly used in superficial and systemic *Candida* spp. and other fungal infections or prophylactically used prior to transplantation or cancer therapies (63, 201, 210, 235). This resistance precludes the use of this antifungal drug in the treatment of IA, though it is sometimes given prophylactically to patients at risk (362). The mechanism behind the inherent FLC resistance in *Aspergillus* spp. is thought to be due to the existence of multiple paralogs for the FLC target in the genome. *Aspergillus* spp. contain two genes encoding 14α-demethylase (*erg11*, also called *cyp51*), which is the

target of the triazoles (210). Both of these isoforms have 40 to 70% homology with *erg11* genes from other fungal species and ~60% homology to each other (210). Single gene replacement mutants of either *erg11A* or *erg11B* are viable (210). As observed in other fungi, complete elimination of *erg11* in *A. fumigatus* by way of double gene replacement is lethal (138).

Recently, FLC was shown to physically bind Erg11B with much greater efficiency than Erg11A, whereas VCZ was shown to physically bind both paralogs with high affinity (349). Together with the increased susceptibility of *erg11A* null mutants to FLC, these data suggest that the inherent FLC resistance by *A. fumigatus* is due largely to the two functional Erg11 enzymes (212). Along these lines, resistance to antimicrobial agents, including antifungal drugs, generally occurs via one of three mechanisms: target modification, target overexpression, or increased efflux of drug pumps (10). A fourth mechanism is observed in bacteria, namely, microbial degradation of the antimicrobial agent, but this mechanism has not been observed in fungi to date (10, 322). Transcription factor modification may be another understudied antifungal resistance mechanism and has been studied in the human fungal pathogen *Candida albicans* (51, 77).

In *C. albicans*, gain-of-function mutations in four transcription factors, Tac1, Mrr1, Cap1, and Upc2, have been linked to increased antifungal drug resistance (85, 86, 221, 279, 375). Gain-of- function mutations in Tac1, Mrr1, and Cap1 are associated with increased drug efflux and have been well characterized. Conversely, gain-of-function mutations in Upc2 do not confer increased expression of drug efflux pumps (279), and this resistance is thought to be due to an increase in the *erg11* transcript or other Upc2

target genes (134, 279, 292, 356). Upc2 regulates sterol biosynthesis and *erg11* expression, demonstrating another mechanism whereby drug target overexpression can occur (86). However, whether such mutations occur in transcription factors or their binding sites in *A. fumigatus* is unknown. Recently, Albarrag *et al*. attributed the insertion of the *Aft1* transposon into the *erg11A/ cyp51A* promoter region of an *A. fumigatus* patient isolate to the azole resistance of that strain (5). However, the connection between this transposon insertion and the increased *erg11A/cyp51A* expression and azole drug resistance is currently unknown.

We have characterized a transcriptional regulator in the sterol regulatory element binding protein (SREBP) family, SrbA, in *A. fumigatus* that has an important role in hypoxia adaptation, polarized growth, virulence, and triazole drug interactions (359). The role of SREBPs in the regulation of ergosterol biosynthesis and hypoxia adaptation was originally observed in *Schizosaccharomyces pombe* and *Cryptococcus neoformans* (55, 60, 141, 323). In these fungi, SREBPs sense sterol levels as an indirect measure of oxygen availability, and the corresponding reduction of sterol levels in low oxygen conditions triggers N-terminal proteolytic cleavage of the endoplasmic reticulum (ER)-bound SREBP (141, 255). As the N terminus contains the transcription factor portion of this protein, translocation of the cleaved form triggers transcription of oxygen and sterol biosynthesis-related genes (90). Although direct binding between SREBPs and ergosterol biosynthesis genes has been demonstrated for *erg3* (323), transcriptional profiling of SREBP mutants suggests that additional ergosterol biosynthesis genes are likely directly or indirectly regulated by SREBPs in fungi. These results indicate an important role for

SREBPs in the regulation of ergosterol biosynthesis, much like the observed relationship between Upc2 and ergosterol biosynthesis in *C. albicans* (134, 279, 292, 356).

As ergosterol is the main sterol component of the cell membrane in most ascomycetes (351), and as this synthesis pathway and its resulting product is the target of both the polyene and triazole antifungal drugs, a better understanding of the transcriptional regulatory mechanisms of this pathway is clinically relevant. Importantly, transcriptome profiling of Δ*srbA* in *A. fumigatus* has demonstrated differential regulation of *erg3*, *erg25*, and *erg24*, suggesting that regulation of sterol biosynthesis is also conserved in *A. fumigatus* (36, 359). In this study, we observe that *erg11* genes are downstream effectors of SrbA in *A. fumigatus*. Our results suggest that SrbA is a primary, direct transcriptional regulator of *erg11A* and, to a lesser extent, *erg11B*. We observe that this differential regulation of *erg11* transcripts by SrbA is the likely underlying mechanism for the increased susceptibility of the Δ*srbA* strain to FLC and VCZ. Therefore, our results add further support to the hypothesis that inherent FLC resistance in *Aspergillus* spp. is due to duplication of *erg11* function. While it is currently unknown if increased expression of *erg11A/cyp51A* is a clinically relevant azole drug resistance mechanism in *A. fumigatus*, our results suggest that this may be possible and worthy of further investigation in clinical azole-resistant isolates.

Materials and Methods

Strains and Media

Aspergillus fumigatus strain CEA17 was used to construct the *srbA* null mutant strain

(359). The *erg11A* inducible strain was generated in a Δ*srbA* strain in which the *pyrG* selectable marker was recycled. *pyrG* was recycled by excising a central 1.1-kb fragment of the *pyrG* open reading frame (ORF) from the *srbA* gene replacement plasmid and then transformed into the Δ*srbA* background on media containing 5-fluoroorotic acid (Invitrogen). Confirmation of a single integration at the *srbA* locus was done with PCR and Southern blot analyses. For generation of the p*niiA-erg11A*-Δ*srbA* strain, genomic DNA was extracted from the p*niiA-erg11A*-Δ*erg11B* strain (138) (gift of Terry Roemer, Merck) and the p*niiA-erg11A* construct was amplified via PCR, transformed into the *pyrG*-Δ*srbA* strain, and screened as previously described (359). This strain was verified via Southern blot analysis for single, locus-specific integration of the p*niiA-erg11A* construct. The wild-type strain referred to in this article is strain CBS 144.89 (gift of Jean-Paul Latgé, Institut Pasteur), which is the background strain for CEA17. All strains were routinely grown at 37°C on glucose minimal medium (GMM) that contains 1% glucose (289). For induction/repression experiments, strains were grown in liquid GMM with 20 mM sodium nitrate (NO_3) or 20 mM ammonium tartrate (NH_4) as the sole nitrogen source (138).

Quantitative Real-Time PCR

A. fumigatus strains were cultured in liquid media (GMM or induction/repression minimal media) for 12 h following germination of conidia, with the exception of the FLC and VCZ treatment experiments. Mycelia were harvested via vacuum filtration and lyophilized overnight prior to tissue disruption with 0.1 mm glass beads. Total RNA was extracted using TRIsure (Bioline) according to the manufacturer's instructions and

purified via RNeasy columns (Qiagen). Genomic DNA removal was completed with Turbo DNase I (Ambion). A secondary genomic DNA removal step was done with the Qiagen QuantiTect reverse transcription kit (Qiagen). cDNA was synthesized via oligo(dT)-primed cDNA synthesis. This quantitative real-time PCR (qRT-PCR) was conducted on a Bio-Rad MyiQ5 real-time PCR detection system and analyzed using the iQ5 optical system software package, which utilizes the method pioneered by Livak and Schmittgen for quantitative analysis (192).

qRT-PCR was conducted in technical triplicates except where noted. The normalized fold expression graphed in each figure represents the means and standard deviations of three biological replicates as normalized to two housekeeping genes, such as *tefA*, *GAPDH*, or *actin*. An mRNA control was used as a no-template control in each analysis.

FLC or VCZ treatment for qRT-PCR analysis was conducted by using the following steps. (i) *A. fumigatus* strains were grown in liquid GMM at 37°C until germinated. (ii) Germlings were treated with a 50% MIC of FLC (CBS 144.89, 128 μg/ml; Δ*srbA* strain, 0.50 μg/ml) or VCZ (CBS 144.89, 0.0625 μg/ml; Δ*srbA* strain, 0.006 μg/ml) for each strain and solubilized in water or dimethyl sulfoxide (DMSO) for 6 and 12 h. (iii) Samples were then harvested, frozen, and lyophilized, and RNA was extracted as described above.

Antifungal Susceptibility Testing

Susceptibility to FLC and VCZ were evaluated with Etest strips (AB Biodisk). Strips were placed on an induction/repression agar plate containing a lawn of 10^5 conidia. Growth inhibition was evaluated at 48 h by visualization of growth inhibition when

grown at 37°C.

The standard RPMI-MOPS (where MOPS is morpholinepropanesulfonic acid) (Sigma-Aldrich) plates were not utilized due to the nitrogen source requirements of the strains utilized in this study.

EMSA

For SrbA protein preparation, cDNA encoding the first 275 amino acids of *A. fumigatus* SrbA were cloned into the plasmid pPAL7 to utilize the Profinity eXact tag (Bio-Rad). The resulting plasmid pPAL7_SrbA1-275 was transformed into *Escherichia coli* strain BL21(DE3). The recombinant protein was purified from *E. coli* using Profinity eXact purification resin columns according to the manufacturer's instructions. (Bio-Rad). An affinity-purified rabbit polyclonal antibody was generated utilizing this recombinant protein by Pacific Immunology using standard protocols. The *erg11A* probe was amplified via PCR, and then biotin-labeled probes were allowed to anneal to the PCR template for 5 min at 95°C, followed by a 15-min incubation step at 37°C. Labeled probes were primers (IDT DNA) labeled with a 3' biotin end biotinylation kit (Thermo Scientific) according to manufacturer's instructions. A total of 40 fmol of the probe was coincubated with 1 μg of recombinant protein for 20 min at room temperature according to the LightShift chemiluminescent electrophoretic mobility shift assay (EMSA) kit instructions (Thermo Scientific). This reaction mixture was run on a 6% native gel, transferred to a positively charged nylon membrane (Roche), and developed according to the chemiluminescent nucleic acid detection module instructions (Thermo Scientific). For the supershift assay, ~0.25 μg recombinant polyclonal SrbA antibody was coincubated

with the above EMSA reaction mixture and developed as described previously.

Total Ergosterol Extraction

Total ergosterol from *A. fumigatus* strains was extracted 12 h postgermination in liquid GMM with 20 mM NO_3 (induced, Δ*srbA* mutant and CBS 144.89) or liquid GMM with 20 mM NH_4 (repressed). Mycelia were harvested via vacuum filtration, dried, and weighed. Two hundred milligrams of dry fungal tissue was extracted as described previously (9). Briefly, tissues were treated with 3 ml of 25% alcoholic potassium hydroxide (3/2, methanol to ethanol) and then vortexed vigorously for 1 min. Samples were incubated at 85°C for 1 h. Sterols were extracted with 1 ml of distilled water and 3 ml of pentane and vortexed for 3 min. The upper aqueous layer was allowed to separate from the cellular debris, and 1 ml of this extract was transferred to a clean glass tube and evaporated in a fume hood at room temperature. Upon preparation for injection, samples were resolved in 1 ml of methanol and syringe filtered through a 0.2 μm-pore-size filter (Acrodisc; Waters). Total ergosterol was measured using a Shimadzu CLASS-VP high-performance liquid chromatograph (HPLC) and detected via an SPD-M10AVP photo diode array at 280 nm on a μBondapack C_{18} column (300 mm by 3.9 mm, 10 μm). The quantity of ergosterol per strain was calculated from a standard curve of ergosterol (Acros Organics, catalog no. 117810250).

Sterol Profile

Mycelia were grown and harvested as described above for total ergosterol extraction. Extraction was done as described previously (9) and exactly as in the total ergosterol

extraction, with the exception that the neutral lipids were extracted twice with 1.5 ml hexane. The upper aqueous layer was separated from the cellular debris, and 1 ml of this extract was evaporated via nitrogen. Vials were stored at -20°C until ready for injection. Samples were resolved in 200 μl toluene and derivatized with 200 μl BFSTA [*N*,*O*-*bis*(trimethylsilyl)trifluoroacetamide] for 1 h at 70°C. Sterols were measured via gas chromatography-mass spectrometry (GC-MS), using a Shimadzu QP-2010 GC with a quadrupole mass ionizer. Peaks were identified via electron ionization (EI) fragmentation patterns. All data are relative to ergosterol content.

Statistical Analysis

All results presented with statistical significance were analyzed with an unpaired two-tailed Student's *t* test. A P of <0.05 was considered significant.

Results

Inherent Resistance to FLC in *A. fumigatus* is Linked to an Increase in *erg11A* Transcript Levels

The triazole FLC is one of the antifungals of choice for treatment of invasive fungal infections (IFIs) caused by *C. albicans* (154). However, in treatment of invasive aspergillosis (IA), FLC is ineffective due to the inherent inactivity of this triazole against *Aspergillus* spp. (197). Because FLC preferentially binds Erg11B over Erg11A, while VCZ binds both paralogs with high affinity (349), we investigated if *erg11A* and *erg11B* transcripts were differentially regulated in *A. fumigatus* during exposure to FLC or VCZ. Wild-type *A. fumigatus* was allowed to germinate in liquid culture and then dosed with a

50% MIC of FLC or VCZ for 6 and 12h. FLC or VCZ exposure elicited increased levels of both *erg11A* and *erg11B* mRNA abundance (Fig. 2.1). However, the magnitude of *erg11A* mRNA abundance was much larger than the magnitude of *erg11B* mRNA abundance. VCZ treatment resulted in a 3-fold increase in *erg11B* mRNA, consistent with the ability of VCZ to target both *erg11* paralogs in wild-type *A. fumigatus* (Fig. 2.1B).

Inherent Resistance to FLC in *A. fumigatus* is Abolished in the Δ*srbA* Strain and Linked to *erg11A* Transcript Abundance

In *A. fumigatus*, triazole drug resistance has primarily been observed in strains containing mutations in Erg11A that result in altered target drug affinity (7, 99, 298). However, in *C. albicans*, increases in target transcript levels also contribute to triazole resistance. A similar mechanism of the *erg11A/cyp51A* transcript increase has recently been described in clinical samples of *A. fumigatus* (5). In the described strain, transposon integration upstream of the *erg11A* start codon resulted in increased expression of *erg11A* and drug resistance (5). Thus, increases in *erg11A* transcript levels may also be a clinically relevant mechanism of triazole resistance in *A. fumigatus*, although this remains to be experimentally confirmed.

We previously identified a transcription factor in *A. fumigatus*, SrbA, whose activity is critical for wild-type levels of triazole drug susceptibility (359). Yet the mechanism for the increased FLC and VCZ MICs of the Δ*srbA* strain is unknown. Based on previous studies, and our initial analyses of *erg11A* and *erg11B* mRNA abundance in response to

FLC and VCZ, we next examined the mRNA abundance of these transcripts in the Δ*srbA* strain. A statistically significant decrease in mRNA abundance was observed for *erg11A* mRNA in the Δ*srbA* strain compared to that in the wild type (Fig. 2.2) ($P < 0.03$). *erg11B* mRNA abundance was consistently higher in the Δ*srbA* strain than *erg11A* but also less than in the wild type. Thus, these results suggest that a fundamental decrease in the *erg11A* transcript in the Δ*srbA* strain may confer the increased susceptibility of this strain to FLC and VCZ and that SrbA may directly regulate *erg11A* transcript abundance.

To experimentally test this hypothesis, we asked if the *erg11A* and *erg11B* pattern of transcript induction with FLC or VCZ was altered in the Δ*srbA* strain. Because the Δ*srbA* strain is susceptible to FLC at a clinically relevant MIC (1.0 μg/ml (359)) and to VCZ at a MIC approximately 8-fold below the wild type, we treated mutant and wild-type germlings for 6 and 12 h postgermination at 50% of the strain MIC of each drug. When treated with FLC, *erg11A* mRNA abundance in the Δ*srbA* strain was significantly reduced compared to that in the untreated wild-type strain (Fig. 2.3A). *erg11B* transcript levels were also reduced in the Δ*srbA* strain compared to those of the wild type but not to the magnitude of *erg11A*. This finding is consistent with the inability of this strain to produce wild-type levels of *erg11A* mRNA in untreated media (Fig. 2.2). As with FLC, treatment with VCZ initially elicited a decrease in *erg11A* mRNA abundance in the Δ*srbA* strain (Fig. 2.3B); however, prolonged exposure to VCZ, unlike FLC, was able to stimulate an increase in the *erg11A* transcript in the Δ*srbA* strain (Fig. 2.3B). The subsequent increase in the *erg11A* transcript in response to VCZ in the Δ*srbA* strain may suggest the presence of an additional transcriptional regulator of *erg11A* that can partially

compensate for loss of SrbA function in response to VCZ. *erg11B* transcripts remained relatively unchanged in response to VCZ in the Δ*srbA* strain. Taken together, these results suggest that SrbA may be a direct regulator of *erg11A* transcript levels and that decreased *erg11A* mRNA abundance may be responsible for the increased susceptibility of the Δ*srbA* strain to FLC and VCZ.

Construction of an *erg11A* Conditional Mutant in the Δ*srbA* Background

In order to test our hypothesis, an *erg11A* conditional expression strain was constructed in the Δ*srbA* background. This strain was generated by replacement of the native *erg11A* promoter with the nitrogen-inducible *niiA* promoter (138). The resulting p*niiA*-*erg11A*-Δ*srbA* strain genotype was verified via PCR and Southern blot analyses (Fig. 2.4 and data not shown). Control over *erg11A* mRNA abundance in the Δ*srbA* background was confirmed via qRT-PCR. The induction of p*niiA*-*erg11A*-Δ*srbA* resulted in levels of *erg11A* mRNA comparable to those of the wild type. While under repressive conditions, *erg11A* transcript levels were similar to the Δ*srbA* background (Fig. 2.4D).

Increased FLC and VCZ Susceptibility in the Δ*srbA* Strain is the Result of *erg11A* Transcript Insufficiency

We next characterized the effect of triazoles on the p*niiA*-*erg11A*-Δ*srbA* strain in inducing and repressing conditions. By using commercially available Etest strips (AB Biodisk), we determined that the FLC susceptibility phenotype of the Δ*srbA* strain was completely rescued by restoration of *erg11A* transcript levels (Fig. 2.5A) (CBS 144.89,

>256 μg/ml; p*niiA-erg11A*-Δ*srbA*, >256 μg/ml; Δ*srbA*, 1.0 μg/ml). Importantly, FLC resistance was reversed by repression of the *niiA* promoter, restoring the MIC of the Δ*srbA* strain prior to *erg11A* induction in the p*niiA-erg11A*-Δ*srbA* strain (p*niiA-erg11A*-Δ*srbA* [induced], >256 μg/ml; p*niiA-erg11A*- Δ*srbA* [repressed], 1.0 μg/ml). As the triazole VCZ binds well to both Erg11A and Erg11B, complete amelioration of the Δ*srbA* strain susceptibility was not visualized in the p*niiA-erg11A*-Δ*srbA* strain (Fig. 2.5B) (CBS 144.89, 0.19 μg/ml; Δ*srbA*, 0.008 μg/ml; p*niiA-erg11A*-Δ*srbA*, 0.064 μg/ml). However, restoration of *erg11A* transcript levels did increase the observed MIC, further suggesting that direct regulation of the *erg11A* transcript by SrbA is involved in the observed VCZ susceptibility in the Δ*srbA* strain. These results likely suggest that additional unknown mechanisms influence the MIC of VCZ-treated *A. fumigatus*.

SrbA Directly Binds *erg11A* Upstream DNA Sequence

We next asked if *erg11A* is directly or indirectly regulated by SrbA. An electrophoretic mobility shift assay (EMSA) was employed to determine a physical interaction between an *erg11A* DNA motif and a recombinant form of SrbA that contained amino acids 1 to 275, which are predicted to encode the DNA binding domain. Recombinant SrbA protein was coincubated with a 118-bp biotin-labeled probe from the upstream region of the *erg11A* gene (Fig. 2.6A). This motif was chosen based on preliminary results from a chromatin immunoprecipitation-sequencing (ChIP-Seq) analysis of SrbA target genes that suggested an enrichment of SrbA binding in this region of the *erg11A* locus (Barker *et al.*, unpublished data). In addition, the 118-bp probe

contains a motif similar to the reported Sre1 binding motif described for *S. pombe* (5'-[A/T]TCAC[A/C]CAT-3') (323). A distinct shift in the probe band was observed when the protein and probe were coincubated, which was verified in the presence of a recombinant SrbA antibody through a supershift assay (Fig. 2.6B). This is the first documentation of SrbA binding to any ergosterol biosynthesis gene in *A. fumigatus* and strongly suggests that SrbA plays a direct role in regulating the gene expression of *erg11A*.

Hypoxia Growth Defect in the Δ*srbA* Strain is Not Directly Mediated by *erg11A*

Another clinically relevant phenotype of the Δ*srbA* strain is its inability to grow in hypoxia, which is hypothesized to contribute to the attenuated virulence of this strain. Because the ergosterol biosynthesis pathway requires significant amounts of oxygen (151), we tested this hypothesis by examining if restoration of the *erg11A* transcript could restore the hypoxia growth phenotype of the Δ*srbA* strain. The p*niiA-erg11A*-Δ*srbA* conditional strain demonstrated wild-type growth rates under atmospheric oxygen (Fig. 2.7A and B) in both inducing and repressing conditions. Restoration of *erg11A* transcript levels, however, did not restore the ability of the Δ*srbA* strain to grow in hypoxia (Fig. 2.7C). As the analysis of the Δ*srbA* phenotype reveals that several ergosterol biosynthesis pathway genes other than *erg11A* are also transcriptionally reduced in the Δ*srbA* strain (359), blockages further downstream of *erg11* likely contribute to this growth defect (36).

To verify that the lack of growth in the Δ*srbA* strain is not solely due to *erg11A*, we examined Δ*erg11A* and p*niiA-erg11A*-Δ*erg11B* conditional strains for growth under

hypoxic conditions. Both the Δ*erg11A* and p*niiA-erg11A*-Δ*erg11B* conditional strains grew in hypoxia at rates comparable to those in normoxia (Fig. 2.8), independent of nitrogen source. As expected, the p*niiA-erg11A*-Δ*erg11B* strain was lethal under repressing conditions (138). We thus conclude that the hypoxia growth defect in the Δ*srbA* strain is not directly due to *erg11A* deficiency and therefore may be due to affected ergosterol biosynthesis genes further downstream of *erg11A*.

Total Ergosterol Content is not Altered with *erg11A* Transcript Restoration in the Δ*srbA* Strain

To test the hypothesis that restoration of the *erg11A* transcript does not rescue hypoxia growth due to continued perturbations in the ergosterol pathway, we compared the ergosterol content of the Δ*srbA* strain with that of the wild type (Fig. 2.9). Total ergosterol was measured via high performance liquid chromatography (HPLC) and all concentrations were based on peak intensity comparisons with a standard curve of commercially available ergosterol (Acros Organics). Twelve hours after germination, the Δ*srbA* strain contained approximately 24.8% of the ergosterol content found in the wild type (5.71 ± 1.02 μg/mg versus 2.36 ± 0.79 μg/mg, respectively). Strikingly, induction of *erg11A* in the Δ*srbA* background had no effect on total ergosterol production (1.85 ± 0.89 μg/mg versus 2.36 ± 0.79 μg/mg, respectively). This result is consistent with our hypothesis that SrbA regulates multiple genes in the ergosterol biosynthesis pathway.

To further support this hypothesis, we analyzed the production of sterol intermediates in our strains. Previous qualitative sterol profiles of the Δ*srbA* strain demonstrated an

increase in 4-methyl sterols compared to those of wild-type *A. fumigatus* (359). Buildup of these 4-methyl sterols indicates a significant blockage at the Erg25 (C4-demethylase) step in the pathway. In particular, transcriptional analysis of the Δ*srbA* strain also indicates that the *erg25A* transcript is significantly repressed (359), suggesting that SrbA is also involved in regulation of this gene. Intriguingly, both steps at Erg11 and Erg25 require oxygen and iron as cofactors (36, 56, 187, 323). Because restoration of the *erg11A* transcript in the Δ*srbA* strain does not increase total ergosterol levels (Fig. 2.9), we hypothesized that there would be a corresponding increase in 4-methyl sterol intermediates in this strain. This hypothesis was based on the premise that SrbA regulates both *erg11A* and *erg25* and that restoration of Erg11A enzymatic activity would increase levels of intermediates in the first half of the pathway. Due to the continued blockage at Erg25, an increase in intermediates corresponding to this step would be observed, which would correlate with the lack of increase in total ergosterol. We thus examined the sterol intermediate profiles of both the Δ*srbA* and p*niiA-erg11A*-Δ*srbA* strains via GC-MS and compared their profiles to those of wild-type CBS 144.89. As previously observed, an increase in 4-methyl sterols was detected in the Δ*srbA* strain (Fig. 2.10). Consistent with an unalleviated blockage at Erg25 in the p*niiA-erg11A-Δsrb*A strain, three peaks corresponding with buildups in 4-methyl sterols were increased in the p*niiA-erg11A*-Δ*srbA* strain under inducing conditions (Fig. 2.10 [4,14,24-trimethyl cholesta-8,24(28)-dien-3β-ol, ~75% based on peak identification; 4,24- dimethyl cholesta-8(28)-dien-3β-ol and 4,4,24-trimethyl cholesta-8(28)-dien-3β-ol, ~85% identity based on peak identification]). Taken together, these results suggest that the Δ*srbA* strain also contains a

blockage downstream of Erg11, most likely in the C4-sterol oxidase enzyme, Erg25. Experiments are under way to determine if alleviation of this blockage can rescue the hypoxia growth and virulence phenotypes of the Δ*srbA* strain.

Discussion

Our study highlights the importance of SrbA in the regulation of ergosterol biosynthesis in *A. fumigatus* and provides further mechanistic insight into the inherent FLC resistance and triazole susceptibility of this important human pathogenic fungus. We began this work with the hypothesis that the reduction of the *erg11A* transcript in the Δ*srbA* strain was the likely mechanism by which the Δ*srbA* strain is susceptible to the triazole FLC and has increased susceptibility to VCZ (359). By constructing an inducible *erg11A* strain (p*niiA*-*erg11A*-Δ*srbA*), we have demonstrated that restoration of wild-type levels of the *erg11A* transcript restores the FLC resistance observed in *A. fumigatus* and also restores wild-type levels of VCZ susceptibility (Fig. 2.5). We therefore have confirmed that this regulation of *erg11A* by SrbA is likely direct, as is demonstrated by direct binding of recombinant SrbA to the promoter region of *erg11A.* Furthermore, this region contains a sequence similar to the SREBP DNA binding motif reported for *S. pombe*, though additional analyses of the actual SrbA DNA binding motif remain to be performed (Fig. 2.6).

Thus, the apparent regulation of *erg11* transcript levels by SrbA may suggest an additional target for therapeutic control over this important pathway in human pathogenic fungi. Though clinical resistance to triazoles via a defined transcriptional mechanism has

not yet been reported for *A. fumigatus*, our research may suggest a role for SrbA in the development of triazole resistance, as alterations in this transcription factor or its DNA binding affinity could transcriptionally initiate changes that alter target abundance and lead to triazole resistance. This possibility remains to be experimentally or clinically confirmed.

Precedence for the idea that alterations in a transcription factor or transcription factor DNA binding sites can affect drug resistance is found in the pathogenic yeast *C. albicans*, in tumorigenesis, and in the malarial parasite *Plasmodium vivax* (77, 85, 86, 209, 221, 279, 375). For example, mutations in *C. albicans* transcription factors that regulate drug efflux pumps are more susceptible to anti-fungal drugs (85, 221, 279, 375). Additionally, a mutation in the regulator of *erg11* in *C. albicans*, Upc2, renders the mutant more susceptible to triazole antifungals. In concordance with the role of Upc2 in regulation of *erg11*, a heterozygous gene replacement mutant of *Upc2* (*UPC2/upc2*) and a homozygous gene replacement mutant (*upc2/upc2*) in a strain with two Upc2 alleles both exhibited increased susceptibility to the triazole antifungals FLC and itraconazole (304). *upc2* gene deletion mutants have also been constructed in a strain containing four *upc2* alleles, which is thought to occur from chromosomal polysomy, and is observed occasionally in strains cultivated from patients (356). In these strains, genetic removal of two or more of the *upc2* alleles conferred a significant susceptibility to the azole ketoconazole or the triazole FLC (195). The *upc2* deletion mutant from S. MacPherson *et al.* (195) was also unable to grow in hypoxia. Overall, both of the described *C. albicans upc2* deletion mutants share strikingly similar phenotypes to that of the Δ*srbA* strain of *A.*

fumigatus and provide further support for our idea that alterations in the transcription factor SrbA may be worth monitoring in the context of triazole-resistant clinical strains of *A. fumigatus* with unknown resistance mechanisms.

Although Upc2 is not a homologue of SREBPs, these two classes of transcription factors have analogous functions, similar localization and activation patterns, and are proposed to be an example of convergent evolution in the fungal kingdom (33, 134, 198, 292, 356). The inactive protein of both classes is tethered to a cytoplasmic membrane, and both are released via proteolytic cleavage to function as transcription factors (134, 292, 356). Also, genetic deletion of either Upc2 or SREBPs results in decreased accumulation of ergosterol, increased susceptibility to antifungal drugs, and an inability to induce expression of *erg11* under antifungal drug pressure (134, 195, 292, 356). Unlike SREBPs, Upc2 seems to have no effect on the basal regulation of *erg11*, as levels of *erg11* are unchanged in the *upc2*Δ strain without the addition of antifungal drugs (134, 292).

With regard to antitumor drugs and the malarial parasite *P. vivax*, these pathosystems illustrate further examples of drug resistance mediated by transcription factor mutations (77, 85, 86, 209, 221, 279, 375). *Ex vivo* whole-genome sequencing and microarray analysis of a chloroquine-resistant *P. vivax* isolate identified six transcription factors with aberrantly high levels. These six transcription factors all contained high levels of nonsynonymous mutations and belonged to the AP class of transcription factors. These nonsynonymous mutations are thought to cause increased expression of downstream targets, resulting in drug resistance at a transcript regulation level. These results have

raised the question if modification of transcription factors may be more common in antimalarial drug resistance than previously acknowledged. The authors argue that understanding this understudied resistance mechanism will assist in the treatment of patients with malaria and extend the usefulness of the drug chloroquine (77).

Likewise, we argue that further study of the role of fungal transcription factors and antifungal drug resistance in *A. fumigatus* is likely needed in a similar context to provide additional insights into new antifungal drug design. A recent study from Albarrag *et al.* identified a transposon-mediated insertion into the promoter region of *erg11A/cyp51A* in a triazole-resistant clinical isolate of *A. fumigatus*. This strain has higher levels of the *erg11A/cyp51A* transcript, but its direct association with the transposon insertion is currently unknown and awaits further investigation (4).

The partial restoration of the VCZ MIC in the p*niiA-erg11A-*Δ*srbA* strain is another potentially interesting finding. This result suggests that additional SREBP-dependent or off-target effects exist in the p*niiA-erg11A-*Δ*srbA* strain. The persistent depletion of total ergosterol may be one mechanism whereby the p*niiA-erg11A-*Δ*srbA* strain regains only a portion of the wild-type susceptibility to VCZ. The stability of V-type ATPases, critical for maintaining vacuolar ion homeostasis, is directly related to the structural integrity of the cellular membrane and is affected by depletions of ergosterol concentration in *C. albicans* (371). Therefore, a persistent decrease of total ergosterol content in the p*niiA-erg11A-*Δ*srbA* strain, in conjunction with the dual target capabilities of VCZ, could lend itself to the partial restoration of VCZ susceptibility observed in the p*niiA-erg11A-*Δ*srbA* strain. As FLC only strongly binds *erg11B,* restoration of *erg11A* mRNA abundance

appears to be sufficient for resistance to this triazole, and off-target effects are either not observed or inefficient in the p*niiA-erg11A*-Δ*srbA* strain.

An additional important phenotype of the SrbA null mutant investigated here is its inability to grow in hypoxia and to cause lethal disease in murine models of invasive aspergillosis. Whether the attenuated virulence of fungal SREBP strains is due to their inability to adapt to *in vivo* hypoxia is still an unanswered question and is also relevant from the perspective of antifungal drug development. Thus, we explored whether restoration of the *erg11A* transcript could also restore the hypoxia growth phenotype of the Δ*srbA* strain. Although restoration of the *erg11A* transcript did not restore the ability of the Δ*srbA* strain to grow in hypoxia, both Δ*erg11A* and p*niiA-erg11A*-Δ*erg11B* strains also grow at wild-type rates in hypoxia (Fig. 2.8). Thus, blockages at Erg11 appear to not affect the ability of *A. fumigatus* to grow in hypoxia. We speculate that additional blockages downstream of Erg11 may play a more critical role. Further evidence for this hypothesis is demonstrated by the decrease in total ergosterol content (Fig. 2.9) and abundance of 4-methyl sterols (Fig. 2.10) in the p*niiA-erg11A*-Δ*srbA* strain. The intermediates identified in the sterol profile indicate that the primary blockage in both Δ*srbA* and p*niiA-erg11A*-Δ*srbA* strains occurs around the C4-demethylation step, which is initiated by the C4-methyl sterol oxidase (Erg25).

erg25 expression is induced in response to itraconazole treatment in the human pathogenic fungus *C. albicans* (73). This transcript is also increased 3-fold in *C. albicans erg11* mutants (250). In *Saccharomyces cerevisiae*, mutations in *erg25* are generally lethal but can be rescued by sterol supplementation. Thus, the *erg25* gene replacement

mutant is a sterol auxotroph in *S. cerevisiae* (103). Interestingly, the phenotypes of this mutant can be rescued by the addition of triazole antifungals, suggesting that the accumulation of 4,4,24-trimethyl cholesta-8,24(28)-dien-3β-ol is toxic to the yeast and that the corresponding reduction in *erg11* is required for membrane integrity (103). Although this sterol intermediate is observed at high levels in both Δ*srbA* and p*niiA*-*erg11A*-Δ*srbA* strains, it does not seem to have a toxic side effect on *A. fumigatus*, as these mutants grow at wild-type levels (Fig. 2.7). Potential toxic effects of this sterol intermediate may be negated, however, by the presence of adequate ergosterol, theoretically generated by ergosterol biosynthesis genes not in the regulon of SrbA, possibly such as *erg11B*. Experiments to determine the role of blockages at the C4-methyl sterol oxidase step of ergosterol biosynthesis in the Δ*srbA* strain are ongoing.

In conclusion, our study further emphasizes the important link between fungal SREBPs, ergosterol biosynthesis, and triazole drug interactions. We show that transcriptional regulation of the triazole target *erg11* in *A. fumigatus* by SrbA is an important mechanism that explains the FLC susceptibility and increased VCZ susceptibility of this strain. However, regulation of the *erg11A* transcript by SrbA does not directly appear to affect the ability of *A. fumigatus* to grow in hypoxia. Further work is needed to definitively determine what parts of the ergosterol biosynthesis pathway are directly regulated by SrbA and whether this regulation is the major mechanism behind the inability of this strain to grow in hypoxia and cause lethal disease. We also propose that examination of mutations in the DNA binding domain of SrbA or mutations in its DNA binding sequence motif may also be important future lines of inquiry in triazole-

resistant isolates that do not contain one of the canonical resistance mechanisms.

Acknowledgements

This work was supported by funding from NIH/NIAID grant RO1AI81838, NIH COBRE grant RR020185, NIH-NCRR, and the Montana State Agricultural Experiment Station. Sara Blosser is funded through an American Heart Association Predoctoral Fellowship. The mass spectrometry facility is supported by NIH NCRR COBRE P20 RR024237.

We thank Laura Alcazar-Fuoli for assistance with the sterol extraction protocols, Sven Willger for previous work and materials fundamental to this research, and Brian Bothner and Jared Bowden for GC-MS training and protocol development assistance. Special thanks to Bridget Barker, MSU, for sharing preliminary SrbA ChIP-*seq* data for use in our studies.

Figures

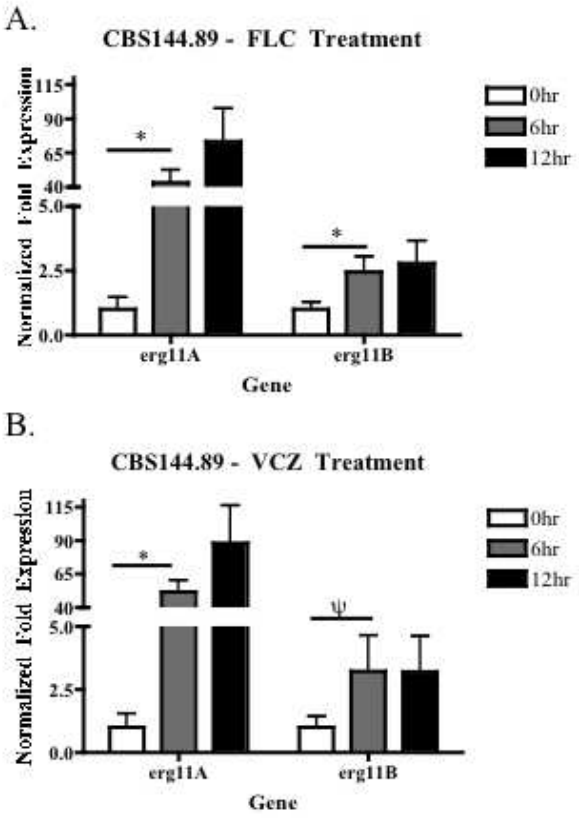


FIG 2.1 FLC and VCZ increase *erg11A* transcript levels. (A) CBS 144.89 germlings were treated with FLC (128 μg/ml) for 6 or 12 h at 37°C with agitation. (B) CBS 144.89 germlings were treated with VCZ (0.0625 μg/ml) for 6 or 12 h at 37°C with agitation. qRT-PCR was conducted to determine the effects of these triazoles on *erg11A* and *erg11B* mRNA abundance. Data represent three biological and two combined technical replicates. mRNA abundance was normalized to the housekeeping genes *tefA* and *actin* and are relative to CBS 144.89. ψ, $P < 0.05$; *, $P < 0.01$.

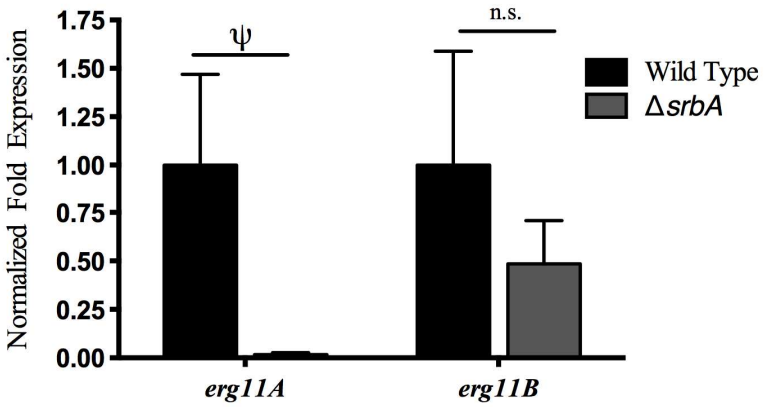


FIG 2.2 Differential *erg11A* and *erg11B* transcript abundance in the Δ*srbA* strain compared to that in CBS 144.89. RNA samples were extracted from each respective strain 12 h postgermination (in liquid GMM) at 37°C, 300 rpm. mRNA abundance was normalized to the housekeeping genes *tefA* and *actin* and are relative to CBS 144.89. Results are the means and standard deviations of two biological replicates for CBS 144.89 and three biological replicates for the Δ*srbA* strain. ψ, $P < 0.05$; n.s., not significant.

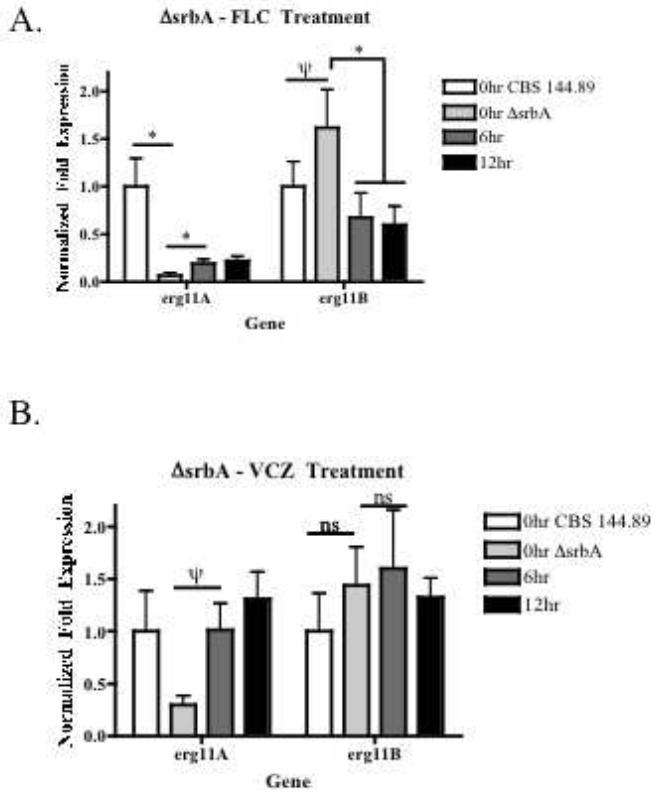


FIG 2.3 *srbA* is required for transcriptional responses to FLC and VCZ. (A) Δ*srbA* germlings were treated with FLC (0.5 μg/ml, 50% less than the observed MIC) for 6 or 12 h at 37°C with agitation. (B) Δ*srbA* germlings were treated with VCZ (0.006 μg/ml, 50% less than the observed MIC) for 6 or 12 h at 37°C with agitation. qRT-PCR was conducted to determine the effect of these triazoles on *erg11A* and *erg11B* mRNA abundance. Data represent three biological and two combined technical replicates. mRNA abundance was normalized to the housekeeping genes *tefA* and *actin* and are relative to CBS 144.89. ψ, $P < 0.05$; *, $P < 0.001$; ns, not significant.

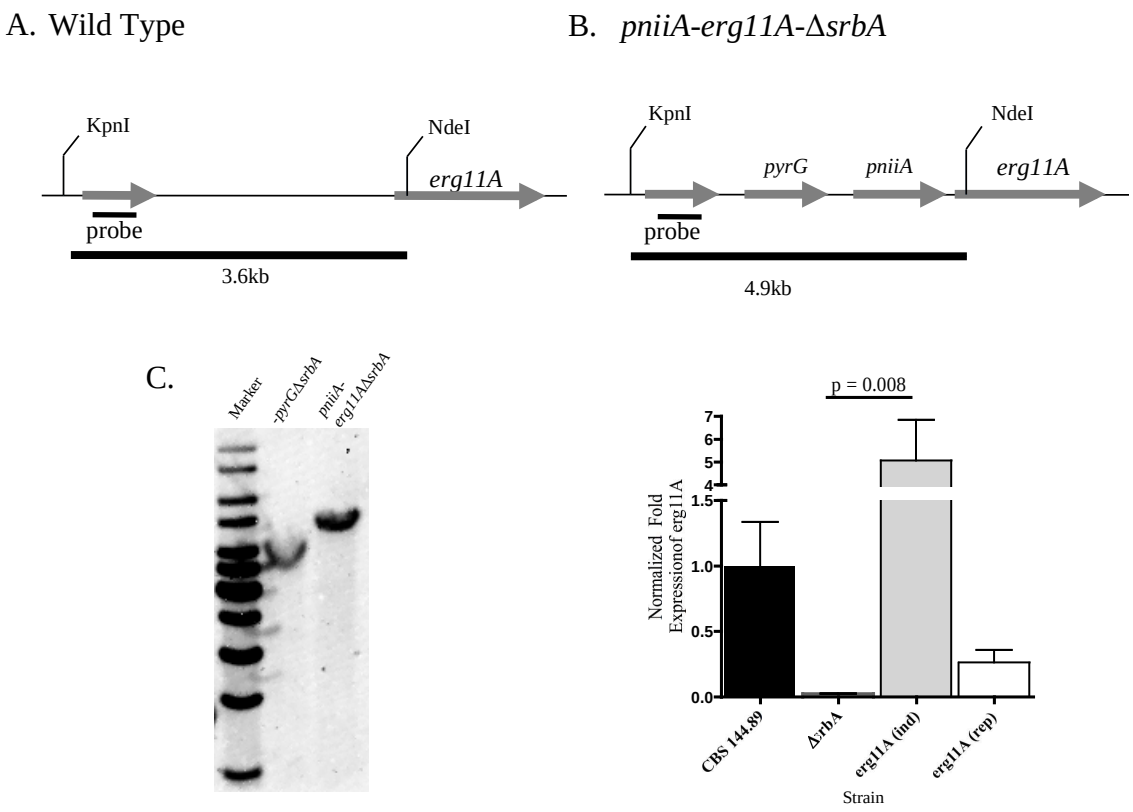


FIG 2.4 Generation of *erg11A* inducible strain in Δ*srbA* background. (A) Locus of *erg11A* locus in the CBS 144.89 (wild-type) strain. (B) Locus of the p*niiA-erg11A* construct in the Δ*srbA* background strain. (C) Southern blot analysis of the wild-type and mutant strains. XbaI/XmaI genomic DNA was digested for 18 h at 37°C. Strains were separated on a 1% agarose gel, transferred, and hybridized with a 467-bp probe. (D) Induction/repression of the p*niiA-erg11A*-Δ*srbA* strain correlates with mRNA abundance of *erg11A* ($P < 0.008$). RNA samples were extracted from each respective strain 12 h postgermination in liquid GMM plus 20 mM NO_3 (CBS 144.89, Δ*srbA*, p*niiA-erg11A*-Δ*srbA* induced [ind]) or liquid GMM plus 20 mM NH_4 (p*niiA-erg11A*-Δ*srbA* repressed [rep]) at 37°C, 300 rpm. mRNA abundance was normalized to the housekeeping genes *tefA* and *gadpH* and are relative to CBS 144.89. Results are the means and standard deviations of three biological replicates.

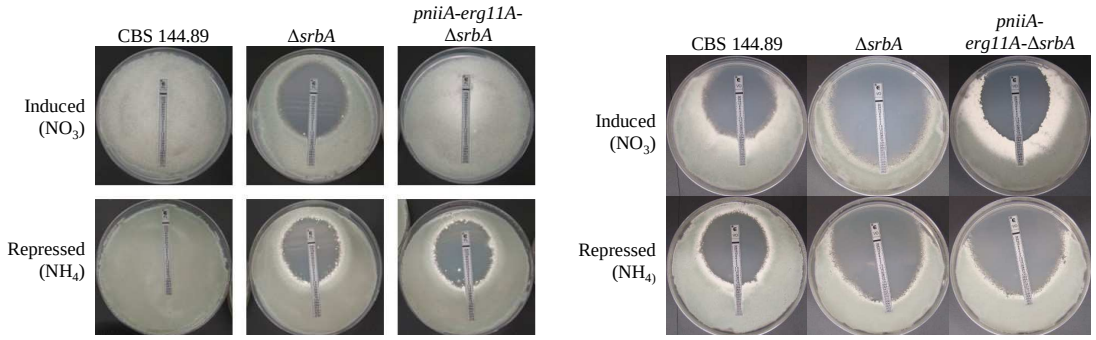


FIG 2.5 Restoration of *erg11A* transcripts restores FLC and VCZ MICs in the Δ*srbA* strain. Etest strip concentrations (in micrograms per milliliter) for *A. fumigatus* CBS 144.89, the Δ*srbA* strain, and p*niiA-erg11A*-Δ*srbA* strain. A clear ellipse indicates susceptibility to FLC or VCZ. (A) As expected, FLC has no effect on CBS 144.89, and the Δ*srbA* strain is susceptible to FLC (MIC, 1.0 μg/ml). Wild-type expression of the p*niiA-erg11A*-Δ*srbA* strain reconstitutes FLC resistance. Likewise, repression of the p*niiA-erg11A*-Δ*srbA* strain restores FLC susceptibility as observed in the Δ*srbA* strain. (B) *erg11A* transcript expression partially restores the VCZ MIC in the Δ*srbA* strain. CBS 144.89 MIC, 0.19 (induced or repressed); Δ*srbA* strain MIC, 0.008 (induced or repressed); p*niiA-erg11A*-Δ*srbA* strain MICs, 0.064 (induced) and 0.008 (repressed).

A. *Erg11A* locus

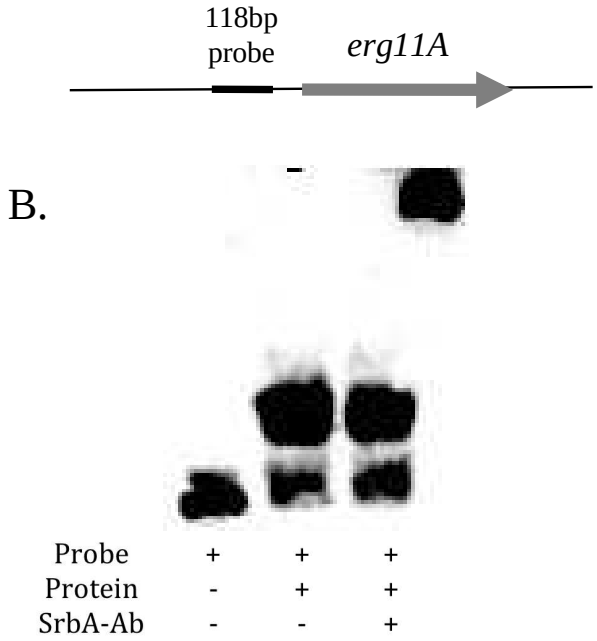


FIG 2.6 SrbA directly binds *erg11A* promoter region sequence *in vitro*. Electrophoretic mobility shift assays were conducted utilizing a LightShift chemiluminescent EMSA kit from Thermo Scientific. A total of 40 fmol of a 118-bp biotin-labeled probe from *erg11A* upstream region and 1 μg purified SrbA protein were coincubated at room temperature for 20 min. Recombinant polyclonal SrbA antibody was used for the supershift assay. Lane 1, no protein control; lane 2, EMSA reaction; lane 3, supershift assay.

A. Growth - Nitrate

	CBS 144.89		*ΔsrbA*		*pniiA-erg11A-ΔsrbA*	
	N	**H**	**N**	**H**	**N**	**H**
0 hr	0.5	0.5	0.5	0.5	0.5	0.5
24 hr	1.0	0.9 ±0.1	1.1 ± 0.07	0.5	1.0 ± 0.14	0.5
48 hr	3.4	3.3 ± 0.06	3.5 ± 0.07	0.5	3.6 ± 0.07	0.5
72 hr	5.4 ± 0.07	4.5 ± 0.10	5.4 ± 0.07	0.5	5.5	0.5
96 hr	7.1 ± 0.11	6.4	6.9	0.5	7.1 ± 0.07	0.5
120 hr	8.4	8.0	8.0 ± 0.21	0.5	8.1 ± 0.07	0.5

B. Growth - Ammonium

	CBS 144.89		*ΔsrbA*		*pniiA-erg11A-ΔsrbA*	
	N	**H**	**N**	**H**	**N**	**H**
0 hr	0.5	0.5	0.5	0.5	0.5	0.5
24 hr	0.85 ± 0.07	0.7 ± 0.06	0.9 ± 0.06	0.5	0.9 ± 0.06	0.5
72 hr	3.05 ± 0.07	3.5	3.1 ± 0.11	0.5	3.2 ± 0.17	0.5
96 hr	4.3	4.8 ± 0.10	4.3 ± 0.06	0.5	4.4 ± 0.12	0.5
120 hr	5.75 ± 0.07	6.4 ± 0.06	5.4 ± 0.1	0.5	5.7 ± 0.12	0.5

C.

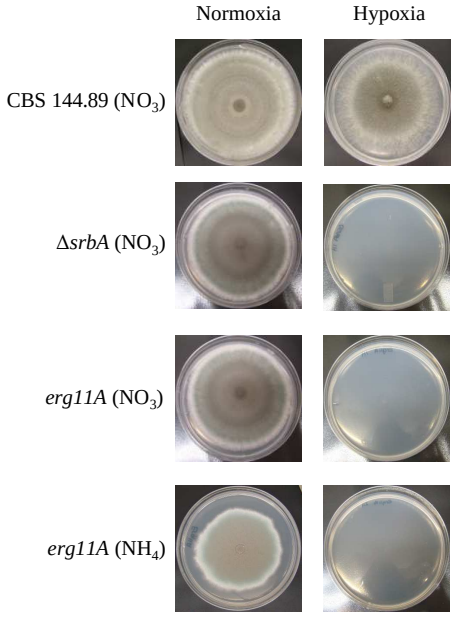
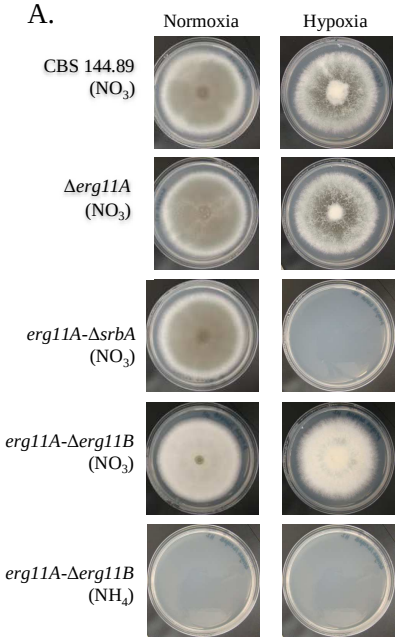


FIG 2.7 Restoration of *erg11A* does not affect hyphal growth in normoxia or restore Δ*srbA*-related hypoxic growth defect. A total of 10^5 conidia was spotted on solid media (GMM plus 20 mM NO_3 [induction] or 20 mM NH_4 [repression]) under normoxic (N) or hypoxic (H) conditions. (A) Comparison of colony growth diameter on nitrate (NO_3) in both hypoxia (H) and normoxia (N). (B) Comparison of colony growth diameter on ammonium (NH_4) in both hypoxia (H) and normoxia (N). Values represent the means and standard deviations of 3 biological replicates. (C) Radial growth at 120 h.

A.



B. Growth - Nitrate

	CBS 144.89		Δerg11A		pniiA-erg11A-Δerg11B		pniiA-erg11A-ΔsrbA	
	N	H	N	H	N	H	N	H
0 hr	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
24 hr	1.3 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.3	1.3	1.6 ± 0.2	0.5
48 hr	3.6 ± 0.1	3.6 ± 0.1	3.7	3.7 ± 0.1	3.5	3.5 ± 0.1	3.7	0.5
72 hr	5.6 ± 0.1	5.6 ± 0.1	5.7	5.7 ± 0.1	5.5	5.4 ± 0.1	5.7 ± 0.1	0.5
96 hr	7.45 ± 0.07	7.2	7.5 ± 0.07	7.3 ± 0.14	7.1	7.2 ± 0.28	7.8 ± 0.07	0.5

C. Growth - Ammonium

	CBS 144.89		Δerg11A		pniiA-erg11A-Δerg11B		pniiA-erg11A-ΔsrbA	
	N	H	N	H	N	H	N	H
0 hr	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
24 hr	1.1 ± 0.1	1.1	1.2	1.3 ± 0.1	0.5	0.5	1.3 ± 0.1	0.5
48 hr	2.8 ± 0.1	3.0	2.8 ± 0.1	2.9 ± 0.1	0.5	0.5	2.8	0.5
72 hr	4.2 ± 0.2	4.4 ± 0.1	4.0 ± 0.1	4.4 ± 0.1	0.5	0.5	4.1 ± 0.1	0.5
96 hr	5.7 ± 0.1	5.8	5.4 ± 0.2	5.7 ± 0.2	0.5	0.5	5.6	0.5

FIG 2.8 Genetic deletion of *erg11A* (Δ*erg11A*) or *erg11B* (p*niiA-erg11A*-Δ*erg11B*) does not affect hyphal growth in normoxia or hypoxia. A total of 10^5 conidia was spotted on solid media (GMM plus 20 mM NO_3 [induction] or 20 mM NH_4 [repression]) under normoxic or hypoxic conditions. (A) Radial growth at 96 h. (B) Comparison of colony growth diameter on nitrate (NO_3) in both hypoxia and normoxia. (C) Comparison of colony growth diameter on ammonium (NH_4) in both hypoxia and normoxia. Values represent the means and standard deviations of 3 biological replicates.

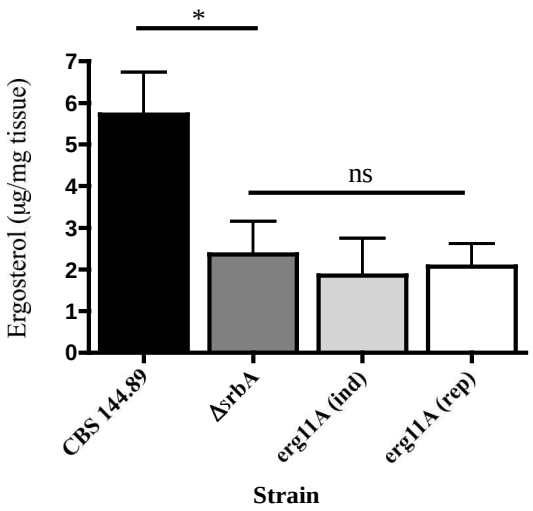


FIG 2.9 Total ergosterol is unchanged by restoration of *erg11A* transcript levels in the Δ*srbA* strain. Total ergosterol from *A. fumigatus* strains was extracted 12 h postgermination in liquid GMM with 20 mM NO_3 (p*niiA-erg11A*-Δ*srbA* ind, Δ*srbA*, and CBS 144.89) or liquid GMM with 20 mM NH4 (p*niiA-erg11A*-Δ*srbA* rep). Mycelia were harvested via vacuum filtration and then dried and weighed. Two hundred milligrams of dry fungal tissue was extracted as described previously (3). Upon preparation for injection, samples were resolved in 1 ml of methanol and syringe filtered. Total ergosterol was measured using a Shimadzu CLASS-VP HPLC and detected via a SPD-M10AVP PDA at 280 nm on a µBondapack C_{18} column (300 mm by 3.9 mm, 10 µm). *, $P < 0.001$. Values represent the means and standard deviations of 3 to 5 biological replicates and three technical replicates.

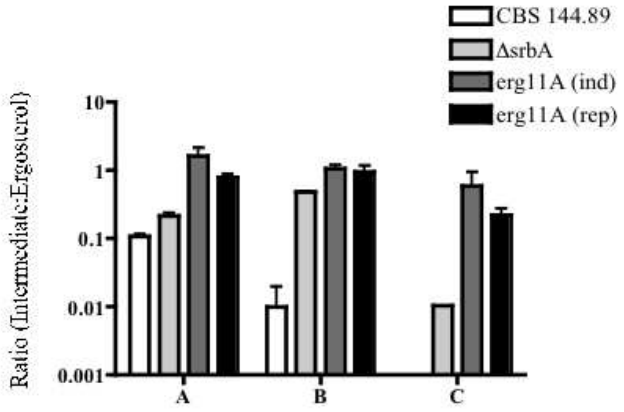


FIG 2.10 Sterol intermediates demonstrate a blockage at Erg25 in the Δ*srbA* strain. Ratio of sterol intermediates to ergosterol. Sterols were measured via GC-MS using a Shimadzu QP-2010 GC with a quadrupole mass ionizer. Peaks were identified via EI fragmentation patterns. A, 4,24-dimethyl cholesta-8,24(28)-dien-3β-ol; B, 4,4,24-trimethyl cholesta-8,24(28)-trien-3β-ol; and C, 4,14,24-trimethyl ergosta-8, 24(28)-dien-3β-ol.

CHAPTER THREE

TWO C4-STEROL METHYL OXIDASES (ERG25) CATALYZE ERGOSTEROL INTERMEDIATE DEMETHYLATION AND IMPACT ENVIRONMENTAL STRESS ADAPTATION IN *ASPERGILLUS FUMIGATUS*

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

Author: Sara J. Blosser

Contributions: Designed the study, conducted the experiments, collected and analyzed the data, wrote the manuscript.

Author: Brittney Hendrickson

Contributions: Designed and constructed strains, performed initial characterization experiments.

Author: Nora Grahl

Contributions: Designed and constructed strains, performed initial characterization experiments.

Author: Bridget M. Barker

Contributions: Assisted with murine model of Invasive Aspergillosis.

Co-Author: Robert A. Cramer

Contributions: Obtained funding for the study, assisted with study design, discussed results of the experiments and implications for this study, edited the manuscript.

Manuscript Information Page

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Journal Name: Molecular Microbiology

Status of Manuscript:

__x__ Prepared for submission to a peer-reviewed journal
_____ Officially submitted to a peer-reviewed journal
_____ Accepted by a peer-reviewed journal
_____ Published in a peer-reviewed journal

Summary

The human pathogen *Aspergillus fumigatus* adapts to stress encountered in the mammalian host as part of its ability to cause disease. The transcription factor SrbA plays a significant role in this process by regulating genes involved in hypoxia and low iron adaptation, antifungal drug responses, and virulence. SrbA is a direct transcriptional regulator of several key enzymes in the ergosterol biosynthesis pathway and Δ*srbA* accumulates C4-methyl sterols, correlating with the loss of Erg25 activity (C4-sterol methyl oxidase; SMO). As we have demonstrated that SrbA-directly regulates *erg25A* and *erg25B*, these genes of special interest as members of the SrbA-regulon (Barker, B.M. *et al*., in preparation). Characterization of the two genes encoding SMOs in *A. fumigatus* revealed that both serve as functional C4-demethylases, with Erg25A serving in a primary role over Erg25B, as Δ*erg25A* accumulated more C4-methyl sterol intermediates than Δ*erg25B*. That both *erg25* genes retain SMO function, and are not limited to a singular substrate is unique in the eukaryotic kingdom. Genetic deletion of both *erg25* genes is lethal in *A. fumigatus*. Single deletion of these SMOs revealed alterations in canonical ergosterol biosynthesis, suggesting that ergosterol is produced in an alternative fashion in the absence of SMO activity. The Δ*erg25A* strain displayed moderate sensitivity to hypoxia, reactive oxygen species, and the ER-stress inducing agent dithiothreitol, but was not required for virulence in a murine model of Invasive Aspergillosis. Overall, these findings implicate *A. fumigatus* SMOs in the maintenance of canonical ergosterol biosynthesis, and uncharacterized protein-protein interactions *in vivo* including appropriate adaptation to stress.

Introduction

Aspergillus fumigatus is a filamentous fungus that can cause significant human disease in immunocompetent and immunocompromised hosts. *A. fumigatus* infection of immunocompromised patients often results in the disease Invasive Aspergillosis (IA). Recent medical treatments, such as anti-TNF therapy for autoimmune disorders, and the unfortunate contamination of steroids used in joint therapy, have indicated that *A. fumigatus* is capable of causing lethal disease in a myriad of patients and underlying immunocompromised states (153, 240). This increase in patients susceptible to *A. fumigatus* infection has made the study of *A. fumigatus* biology and virulence mechanisms paramount to future patient health and therapeutic drug discovery.

Ergosterol is the fungal equivalent of cholesterol, and plays an important role in membrane stability and fluidity. Importantly, ergosterol and some ergosterol biosynthesis intermediates are the targets for two of the three main antifungal drug classes available today. The polyenes, including Amphotericin B, target the end product ergosterol, and are though to function simply by binding ergosterol in the membrane (115). The triazole class of antifungal drugs target the enzyme Erg11A (14α-demethylase), and may cause fungistatic activity by accumulation of toxic sterol intermediates (159). These antifungal drugs represent our best method for eradicating invasive fungal infections. Unfortunately, antifungal resistance to these compounds has been reported in the literature, especially with the emergence of intrinsicly resistant species (38, 120, 302, 303, 347). There are, however, many other biochemical steps that could be targeted in the ergosterol bisynthesis pathway for antifungal drug development. Our ability to utilize these steps is

limited, however, as little is known about other steps of the ergosterol biosynthesis pathway, or how the genes that encode for these enzymes are regulated, especially in *Aspergillus spp*.

In *A. fumigatus*, we have identified a transcription factor involved in the regulation of sterol biosynthesis, named SrbA. SrbA is a member of the helix-loop-helix family of transcription factors, and plays a significant role in fungal hypoxia and low iron adaptation, triazole drug resistance, polarized growth, and virulence (36, 347, 359). Whether the attenuated virulence of fungal SREBP-null strains is due to their inability to adapt to *in vivo* hypoxia and the mechanism behind this loss are still unanswered questions. In human pathogenic fungi, hypoxia adaptation has been hypothesized to be a critical virulence attribute, as gene-deletion mutants that are unable to grow in hypoxia are typically also unable to cause invasive disease (114, 358). Therefore, further studies involving both SrbA, and the ability of *A. fumigatus* to adapt to low oxygen, are necessary to further understand the virulence mechanisms employed by *A. fumigatus*.

As a transcription factor, SrbA directly influences the genetic response of genes in its regulon, in which the ergosterol biosynthesis genes are prominent (36). Therefore, the phenotypes observed in the Δ*srbA* strain may be attributed to direct or indirect loss of specific ergosterol biosynthesis gene function. Ergosterol biosynthesis is the pathway responsible for construction of the fungal membrane sterol ergosterol, and thus the stability and fluidity of this membrane (316, 332). Ergosterol biosynthesis is also targeted by two of the three most prominent classes of antifungal drugs currently in use today, the triazoles and the polyenes. These important attributes of ergosterol biosynthesis make the

understanding of regulatory mechanisms for this pathway of avid interest to biomedical research.

Chief among SrbA's regulon are the di-iron, di-oxygen requiring enzymes Erg11 and Erg25 (C14-demethylase and C4-sterol methyl oxidase, respectively). This regulatory relationship is evidenced by the azole susceptibility and C4-methyl sterol intermediate accumulation in the Δ*srbA* strain (37, 359). Interestingly, removal of the C14-demethylase blockage by constitutive expression of *erg11A* in Δ*srbA* aggravates the accumulation of C4-methyl sterols, suggesting that C4-demethylation is a key step in ergosterol biosynthesis regulated by SrbA in *A. fumigatus* (37). Interestingly, this predominant C4-methyl sterol accumulation is also evidenced in the null mutant of the SREBP-homologue in *C. neoformans*, Δ*sre1*(55, 180). Furthermore, the other critical phenotypes of Δ*srbA*, such as hypoxia growth and virulence are also not remediated in the *erg11A-ΔsrbA* strain, indicating that other downstream targets of SrbA, such as C4-sterol methyl oxidases (SMOs), may be responsible for these phenotypes.

Unlike *Saccharomyces cerevisiae*, *A. fumigatus'* genome encodes two SMOs, *erg25A* and *erg25B*. Although the presence of two *erg25*-encoding genes is unusual in fungi, it is more commonplace in plants, where two SMOs each remove one of two C4-methyl sterols in a subsequent but biochemically distinct fashion (31, 68). In *Arabidopsis thaliana*, removal of each of the two C4-methyl groups requires a different SMO, and mutants of the individual SMOs primarily accumulate their respective sterol intermediate. *C. albicans* is one of the only fungi characterized to-date that also encodes for two SMOs: *erg25* and *erg251*. These *C. albicans* SMOs are not redundant for one another,

however, as deletion of a single SMO is lethal, and suggests that both copies must work in tandem for efficient SMO activity (148). These examples illustrate the diverse mechanisms that organisms have evolved to remove C4-methyl sterol intermediates in the eukaryotic kingdom.

In this study we examined the individual roles of the two SMOs in *A. fumigatus*. These paralogs each serve as a functional oxidase, as they accumulate elevated levels of C4-methyl sterols, but with a probable difference in substrate affinity or timing of enzymatic reaction, as Δ*erg25A* accumulated far more C4-methylated sterol intermediates than Δ*erg25B*. These results suggest that Erg25A functions as the predominant, or primary SMO in *A. fumigatus*, with Erg25B serving in a secondary or subsequent enzymatic function. Genetic elimination of both *erg25* alleles was lethal, suggesting that *A. fumigatus* has compensated for the fragility of this critical juncture with the presence of two functioning C4-sterol methyl oxidases.

Erg25A, as the more predominant SMO, appears to also play an important role in adaptation to hypoxia, ER-stress, and reactive oxygen species, but is not essential for virulence in a murine model of Invasive Aspergillosis. These data suggest that C4-methyl sterols may function in an important signaling capacity for stress and hypoxia adaptation in *A. fumigatus* as part of the SrbA-regulon, but ultimately the dramatic phenotypes of the Δ*srbA* strain must be in concert with multiple members of its regulon. Regulation of ergosterol biosynthesis, and the key biochemical checkpoint at *erg25*, may be critical for signaling appropriate stress responses, and marshaling a subsequent adaptive response in *A. fumigatus*.

Results

Sterol Methyl Oxidase (SMO) Identification and Homology in *A. fumigatus*

Erg25, or C4-sterol methyl oxidase (SMO), is a non-heme iron-requiring enzyme, characterized by three histidine motifs that are conserved throughout Eukarya (Figure 3.1A) (20, 70, 187, 285). These three histidine motifs, HX_3H, HX_2HH, and HX_2HH, are characteristic of the sterol desaturase-like family, which include the C5-sterol desaturases (Erg3) and the C4-sterol methyl oxidases (Erg25) (285). These motifs are postulated to be iron-binding sites, and critical for the enzymatic function. Although these histidine motifs are highly conserved in *Saccharomyces cerevisiae*, *Homo sapiens*, *Candida albicans*, and *A. fumigatus* (Figure 3.1A), the divergence in remaining C4-sterol methyl oxidase inter- and intra- species amino acid homology is significant (Figure 3.1B) (20, 163, 187). This analysis clearly demonstrates that *A. fumigatus* Erg25A is evolutionarily more distant from its SMO cousins in *C. albicans* and *S. cerevisiae* than Erg25B.

Erg25A and Erg25B share 53% amino acid homology with each other, and although they are fairly similar to each other, these *Af*SMO's are not equally distant from the SMOs found in *S. cerevisiae and H. sapiens*. Interestingly, amino acid alignment with *C. albicans* indicates that Erg25B is most closely related to *Ca*Erg25. Erg25A, alternatively, is more highly diverged from *Ca*Erg25, and the lesser-studied *Ca*Erg25-1 (Figure 3.1B). This analysis demonstrates that *A. fumigatus* Erg25A is evolutionarily more distant from its SMO cousins in *C. albicans* and *S. cerevisiae* than Erg25B.

Construction of Sterol Methyl Oxidase Null Mutants: Δ*erg25A* and Δ*erg25B*

Classically, genetic deletions of C4-sterol methyl oxidases are lethal, or at a minimum, highly deleterious to the mutated organism (163, 187). Many of *A. fumigatus'* genes have another paralogous partner, owing to gene duplication. Duplication of ergosterol biosynthesis genes has been hypothesized to act as a strategy for adaptation for membrane composition and integrity (68, 231). As *A. fumigatus* has retained classical SMO protein architecture in both *erg25A* and *erg25B*, we hypothesized that single deletions of either gene would not be lethal, but instead would be able to compensate for the loss of one another.

To determine the role of these individual SMOs in *A. fumigatus*, single deletion mutants were constructed in the CEA17 background as described previously, and in the materials and methods (257, 359). These strains were viable, and were validated for single, locus-specific insertion via PCR and Southern Blot Analysis (Suppl. Fig 3.1). These mutants appeared healthy, as they demonstrated no difference in sporulation, or growth on liquid or solid minimal medium (Suppl Fig 3.1E, and data not shown). Therefore, *A. fumigatus* SMOs appear to serve in overlapping function as C4-demethylses, as single deletion of either allele was not lethal as has been observed in other organisms.

Two Viable SMOs: Sterol Intermediate Profile Verification

A. fumigatus SMOs appear to be functional oxidases, as the single deletion mutant sterol profiles accumulated the stereotypical sterol intermediates that suggest blockage or

elimination of SMO function: C4-methyl sterols. In *A. fumigatus* the C4-methyl sterol intermediates are 4-methyl fecosterol and 4,4-dimethyl fecosterol (Figure 3.2A). The C4-methyl sterol buildup was especially notable for Δ*erg25A*, which accumulated 17-fold and 32-fold wild type levels of 4-methyl fecosterol and 4,4-dimethyl fecosterol respectively (Figure 3.2A, $p<0.01$). The Δ*erg25A* mutant, conversely, only accumulated 1.5-fold and 7-fold more 4-methyl fecosterol and 4,4-dimethyl fecosterol over wild-type levels, which was not statistically significant (Figure 3.2A). These data suggest that Erg25A may serve as the predominant SMO in *A. fumigatus*, or may be the first step of two sequentially acting SMOs. More mechanistic studies would be required to elucidate the precise role of these enzymes, but this step-wise progression of enzymatic action has been demonstrated using virus-induced gene silencing of SMOs in *Nicotiana benthamiana* (71).

The Δ*srbA* strain is characterized by both a hyper-accumulation of C4-methyl sterol intermediates (Figure 3.2A, $p<0.01$ and reported previously) (37, 359), and a significant (30-70%) decrease in total ergosterol (Figure 3.2B, $p<0.01$ and reported previously) (37). Although C4-methyl sterol intermediates disproportionally accumulated in Δ*erg25A* and Δ*erg25B*, total ergosterol was not altered in either strain (Figure 3.2B). Therefore some, but not all ergosterol biosynthesis defects observed in Δ*srbA* can be attributed to decreased abundance of *erg25A* or *erg25B.*

SMO Sterol Profiles Reveal Altered
C9(11)-Intermediate Accumulation

Alterations in sterol intermediate profiles often illuminate the regulatory and compensatory mechanisms an organism utilizes to ensure adequate production of ergosterol. One feature discovered during the SMO-mutant sterol analyses was the accumulation of the sterol intermediates 9(11)-dehydroergosterol and parkeol. The intermediate parkeol was especially evident in Δ*erg25B,* whereas 9(11)-dehydroergosterol accumulated at elevated levels in both Δ*erg25A* and Δ*erg25B* (Figure 3.3A). Interestingly, 9(11)-dehydroergosterol also accumulated at higher levels in Δ*srbA,* although biological variation in replicates made statistical analysis difficult. These findings have potentially important implications for the biosynthesis of ergosterol in *A. fumigatus,* as they suggest an alternative mechanism exists to transverse from squalene to ergosterol. These findings support a non-linear model of ergosterol biosynthesis, as suggested previously by Alcazar-Fuoli, *et al* (9).

Parkeol, also known as 4,4,14-trimethyl-cholesta-9(11)-dien-3β-ol, differs from lanosterol, 4,4,14-trimethyl-cholesta-8,24-dien-3β-ol, only in the position of a single double bond. Parkeol contains a double bond between the 9 and 11 carbons of the C ring of the sterol molecule, whereas lanosterol contains a double bond between the 8 and 9 carbons on the bridge between the B and C rings. This difference may appear slight, but biochemical evidence in other fungal organisms suggests that demethylation of C14, and a subsequent accumulation of C4-methyl sterols cannot occur with the intermediate parkeol as a substrate (334). This finding is additionally significant, as the accumulation

of C14-methylated sterols is toxic when incorporated into the fungal cell membrane, as is seen with the administration of triazole antifungal drugs (159).

Strains that accumulated parkeol also accumulated the sterol-molecule 9(11)-dehydroergosterol, or 24-methyl cholesta-5,7,9(11),22-tetraen-3β-ol (Figure 3.3A), suggesting either that there are novel mechanisms for C14-demethylation of parkeol, or that C8(9) and C9(11) bonds can isomerize in *A. fumigatus* (Figure 3.3C) (334). 9(11)-dehydroergosterol has been characterized previously for its fluorescent properties, and is utilized in membrane synthesis and permeability studies. A peroxide of 9(11)-dehydroergosterol has also been identified, and is toxic to human cervical cell carcinoma cells (66). Therefore, it is plausible that aberrant accumulation of this sterol intermediate in the SMO mutants could be altering canonical sterol biosynthesis, thus highlighting possible alternative pathways heretofore unobserved in *A. fumigatus* (Figure 3.3C).

Alternative Mechanism for Episterol→ Ergosterol Transition

Although ergosterol biosynthesis is linear for much of the pathway, the steps between episterol and ergosterol are branched, allowing for three stepwise uses of the enzymes Erg5, Erg4 and Erg3. SMO mutants, however, displayed significant alterations in how they transverse between episterol and ergosterol. In Δ*erg25A*, only the Erg3→Erg4→Erg5 mechanism appears to be fully functional, as ergostatetraenol was not detectable in this strain, and 22-dihydroergosterol accumulated at diminished levels in comparison with the wild type strain (Figure 3.3B). Interestingly, 5-dihydroergosterol, the only transition maintained in Δ*erg25A* is absent in Δ*srbA* (Figure 3.3B).

Theoretically, in the absence of Erg25A, Erg25B fulfills the predominant SMO functions. We still see abundance, however diminished, of 5-dihydroergosterol in Δ*erg25B,* suggesting that either enzyme can catalyze this transition. Therefore, it is possible that either Erg25A or Erg25B function or specific substrate availability allows for the mechanism Erg5→Erg4→Erg3 to occur, whereas other mechanisms (Erg3→Erg4→Erg5 or Erg3/5→Erg4) are specific for Erg25A. Alternatively, Erg25A or Erg25B presence may dictate the stability of Erg3, Erg4 or Erg5 to interact with downstream sterol intermediates.

SMO Genes Responsive to Hypoxia

The transcription factor SrbA directly binds both *erg25A* and *erg25B* (Barker, BM. *et al*., in preparation; (36)). As SrbA is integrally involved in adaptation to hypoxia, we next examined the response of these genes in response to low oxygen environments. As expected, *srbA* mRNA was significantly induced after 30 minutes and 2 hours exposure to 1% oxygen (Figure 3.4A, *$p<0.0001$). Both SMO genes were highly induced upon exposure to hypoxia, with 13-fold and 3-fold induction of *erg25A* and *erg25B* upon thirty-minute incubation with hypoxia, and 20-fold and 7-fold induction after 2 hours incubation (Figure 3.4A, *$p<0.0001$). Overall, *erg25A* and *erg25B* are significantly induced in response to hypoxia, suggesting their possible involvement in influencing ergosterol biosynthesis during hypoxia.

Radial growth of either SMO mutant was not affected in hypoxia (1% O_2) when compared to growth in normoxia (Figure 3.4B). However, extreme hypoxia (0.2% O_2)

exposure elicited a slight growth defect and produced copious aerial hyphae, indicative of an aberrant stress response in Δ*erg25A* (Figure 3.4B) (204, 319).

Aberrant Stress Response in Δ*erg25A* and Δ*erg25B*

Alterations in *erg25* mRNA abundance in fungi have been previously characterized during stress-inducing conditions in *C. neoformans, C. albicans, S. cerevisiae,* and *Trichophyton rubrum* (19, 24, 39, 73). Intriguingly, *erg25* mRNA abundance appears to be highly elevated in stress-inducing conditions of highly diverse biological origin, including triazole antifungal drugs, hypoxia, dysregulated calcium signaling or lipid homeostasis, when encountering ER or oxidative stress, and during biofilm formation (19, 39, 78, 94, 96, 122, 140, 180, 226). As the aforementioned stress-inducing stimuli were multifaceted in their mechanisms, and thus not linked to any singular pathway or property given our current understanding, we hypothesized that Erg25-function is critical for ubiquitous stress adaptation, possibly as an integral portion of the "fungal adaptome" (125).

To test this hypothesis, we subjected Δ*erg25A* and Δ*erg25B* to various cellular stresses that *A. fumigatus* may encounter in a mammalian host, such as ER stress, low iron availability, triazole antifungal drugs, and reactive oxygen species.

ER Stress: Secretion plays an important role in the saprophytic lifestyle of *A. fumigatus*. Stress caused by the secretory system impacts the endoplasmic reticulum, the hub for the maintenance of the unfolded protein response (UPR). Disturbances in the ability of *A. fumigatus* to cope with ER-inducing stress, such as impairment of *hacA* or

ireA, render the mold more susceptible to ER-stress agents, such as paraquat, dithiothreitol, tunicamycin or brefeldin A, and render null-mutant strains less virulent in murine models of Invasive Aspergillosis (94, 264, 265). Accordingly, we examined the response of the SMO mutants to 1mM dithiothreitol (DTT), which interferes with disulfide bond formation, causing protein unfolding (94). All three mutants examined; Δ*srbA*, Δ*erg25A*, and Δ*erg25B*; displayed significant sensitivity to DTT when compared to the wild type (Figure 3.5A, $p<0.0001$). Both Δ*erg25A* and Δ*srbA* were inhibited similarly by DTT, with growth ratios 50.5% and 46.5% smaller (untreated vs. treated) than the wild type (Δ*srbA* - 0.251±0.027, Δ*erg25A* - 0.271±0.037, wild type - 0.507±0.054). The Δ*erg25B* strain was even more inhibited by DTT, with a 62.9% reduction in growth ratio compared to the wild type (Δ*erg25B* - 0.188±0.029). Overall, loss of SMO function, as exhibited in our single-SMO mutants, confers sensitivity to DTT, demonstrating the need for SMO function in either membrane stability or other potential signaling pathways in *A. fumigatus* (discussed below).

Iron Stress: Under iron-limited conditions *A. fumigatus* demonstrates a significant decrease in biomass accumulation, and is less capable of resisting oxidative stress (147, 248). The Δ*srbA* strain is even more impacted by low-iron conditions, possibly due to the overlap between iron and low-oxygen signaling mechanisms, which center around several key transcription factors (36). As Erg25 is a di-iron requiring enzyme and thus integrally impacted by the cellular iron status, we subjected Δ*erg25A* and Δ*erg25B* conidia to iron depleted conditions and evaluated subsequent biomass. Briefly, wild type and mutant strains were cultured in iron replete (30μM) or iron-free media for 24 hours.

Biomass of samples is represented as a ratio of growth under iron depleted versus iron replete conditions. Significantly, both Δ*erg25A* and Δ*erg25B* were more capable of growth in low iron conditions than the wild-type strain (Figure 3.5B, ratio 0.489 and 0.429 vs. 0.302 in Δ*erg25A*, Δ*erg25B* and wild type respectively, $p<0.0001$). This is the exact opposite of Δ*srbA*, which demonstrates a significant decrease in its ability to grow in low iron environments (Figure 3.5B, and described previously) (36).

Triazole Antifungal Drugs: Treatment of *C. albicans*, *C. neoformans*, *A. fumigatus*, or *T. rubrum* with triazole antifungal drugs elicits a robust increase in *erg25* mRNA abundance (19, 24, 68, 78). There is some suggestion that 4-methyl sterol intermediates may provide additional layers of control for ergosterol biosynthesis, as cellular stress, such as treatment with triazole antifungal drugs, ubiquitously results in an increase in *erg25* mRNA abundance. To determine the role of *erg25* in mediating resistance to triazole antifungal drugs in *A. fumigatus*, we subjected Δ*erg25A* and Δ*erg25B* to the triazole antifungal drugs fluconazole (FLC) and voriconazole (VCZ), and to the ergosterol-targeting polyene Amphotericin B (AmB). Neither Δ*erg25A* nor Δ*erg25B* exhibited altered sensitivity to the antifungal drugs tested (Table 3.1). This is in direct contrast to Δ*srbA*, which has a significant sensitivity to both FLC and VCZ. This sensitivity has been linked to a decrease in *erg11A* transcript (37). This finding is especially interesting, as mutants in Δ*erg25* in *S. cerevisiae* have been shown to have altered mitochondrial physiology and appearance, which has been linked to altered

toxicity of the triazole antifungal drugs (11, 167). The defects in the single Erg25-mull mutants is apparently insufficient to cause such a phenotype in *A. fumigatus*

Reactive Oxygen Species: Disruption of the plasma membrane by the polyene Amphotericin B generates a transcriptional increase in fungal antioxidants (125). This response is similar to the one observed when *A. fumigatus* is subjected to ROS, and suggests that plasma membrane integrity is crucial for the neutralization of ROS (125, 306). The ability to survive and neutralize ROS is highly important *in* vivo, as these environments mimic phagocytosis by host cells. To determine if *erg25* contributed to the ability of *A. fumigatus* to compensate for the presence of ROS, we subjected germlings to a transitory hydrogen peroxide shock, washed away any residual peroxide, and allowed the germlings to recover for 24 hours. Interestingly, Δ*erg25A* but not Δ*erg25B* demonstrated a significant loss of adaptability to hydrogen peroxide, suggesting this strain is impaired in its ability to counteract ROS (Figure 3.5C, *$p<0.05$).

Erg25A Not Required for Virulence of *A. fumigatus* in a Murine Model of IA

A. fumigatus mutants sensitive to ER stress-inducing agents and reactive oxygen species may have altered virulence in murine models of Invasive Aspergillosis (94, 125, 264, 265). To test whether SMO-function was critical for virulence of *A. fumigatus*, we utilized the corticosteroid model of IA, which is induced by triamcinolone. The corticosteroid model is known for its abundance of inflammation, due to the continued recruitment of host cells, which contributes to the significant incidence of hypoxia in this model (114). Survival curves demonstrate that Δ*erg25A* is not altered in its virulence in

this model of murine infection, when compared to a wild type infection (Fig 3.6). Therefore, elimination of *erg25A* alone was not sufficient to inhibit the virulence of *A. fumigatus*.

Construction of a Double-Erg25 Gene Replacement Mutant

Both Δ*erg25A* and Δ*erg25B* accumulated C4-methyl sterols, indicating they function as SMOs in *A. fumigatus.* Deletion of either SMO, however, failed to elicit any of the other predominant *erg25*-mutant phenotype characterized in the literature. Prior attempts to generate null mutants of fungal SMOs have invariably been lethal, with rescue of this lethality only by blocking the upstream C14-demethylation via triazole antifungal drugs (103). Due to the fact that Erg25A and Erg25B are likely paralogs that arose due to gene duplication, they are likely able to compensate for the loss of one another (231).

To assess the possibility that Erg25A and Erg25B could be compensating for the loss of one another we assessed the mRNA abundance of both genes in both the null mutant and wild type backgrounds. As anticipated, *erg25A* appears to be induced in Δ*erg25B* and vice versa in normoxia, and this induction is especially observable under hypoxia stress conditions (Figure 3.7A, *$p<0.05$, **$p<0.0001$). These observations suggest that Erg25A and Erg25B may serve in partially redundant functions, and therefore lethal if mutated in tandem.

To assess this hypothesis, we attempted to construct a double deletion mutant. After several transformations and attempts to generate such a strain, we determined that this double mutant was lethal. To fully demonstrate that this double mutant strain was in fact

lethal, we generated both a *-pyrGΔerg25A* and *-pyrGΔerg25B* strain, and transformed these strains with a nitrate inducible *niiA* construct for either *pniiA-erg25B* or *pniiA-erg25A* respectively (138) (Suppl. Fig. 3.2).

As hypothesized, both *pniiA-erg25A-Δerg25B* and *pniiA-erg25B-Δerg25A* were unable to grow on solid repressing media (NH_4), but grew at wild-type levels on induction media (NO_3) (Figure 3.7B, and data not shown). As reported previously, this promoter system appears to be "leaky" about twenty percent of the time, and we did see occasional growth under repression systems (138). Comparison with a *pniiA-erg11A-Δerg11B* strain verified that this was within a false-positive rate similar to this previously described strain (data not shown).

Interestingly, the repressibility of either strain was short-lived, as repression of either strain past the third passage was unsuccessful. We have observed this phenomenon several times with the *erg25* loci, utilizing both the *niiA* and *alc* promoter expression systems (138, 268). We hypothesize that these critical SMO loci are hotspots for epigenetic remodeling or suppressor mutations.

We therefore concluded that although single null mutants are in fact viable in *A. fumigatus*, that mutation of both alleles is genetically lethal. The ability for one SMO to compensate for the loss of the other suggests that these enzymes are at least partially redundant in function. However, the hierarchy in *A. fumigatus* SMO function has yet to be determined. It is plausible that substrate availability may determine enzymatic usage for C4-demethylation, or simply that Erg25B serves in an auxiliary function to Erg25A.

Importantly, maintenance of two SMOs, that appear to have the same substrate specificity, has not been previously characterized in Eukarya.

Discussion

In humans, C4-sterol demethylation represents a rate-limiting step to active cell proliferation, due to the fact that C4-sterol methyl oxidases (SMOs) are among the slowest enzymes involved in cholesterol biosynthesis (40, 260). It has been hypothesized that SMO-activity is part of the "kinetic control" of sterol biosynthesis, and a mechanism to regulate the flow of carbon in the cell.

In fungi the plausibility of limiting ergosterol biosynthesis is especially crucial as the environment encountered in the mammalian host is hostile and nutrient-poor. The ability of *A. fumigatus* to survive *in vivo* requires several key components that have recently been termed the "fungal adaptome" (140). These attributes, including the ability to grow in a low oxygen, low iron, and reactive oxygen-species rich niche provide some of the arsenal required to germinate and cause invasive disease. We therefore set out to further understand how *erg25A* and *erg25B*, two crucial members of the SrbA-regulon, not only assist *A. fumigatus* in adapting to the conditions encountered in the mammalian host, but also how the deregulation of *erg25A* and *erg25B* in Δ*srbA* contribute to the significant phenotypes observed in this mutant.

Ergosterol biosynthesis requires twelve molecules of oxygen to progress from squalene to ergosterol (180). In *A. fumigatus*, this progression requires six reactions, and involves at least sixteen different enzymes, four of which require iron cofactors (55). C4-

demethylation is required twice in these six reactions, is one of the iron-requiring steps of ergosterol biosynthesis, and requires six of the twelve molecules of oxygen to fuel SMO function. Therefore, the plausibility of Erg25 as a rate-limiting step in *A. fumigatus* ergosterol biosynthesis is credible, especially in light of the connection with oxygen and iron requirements, and its regulation by a key hypoxia-coordinated transcription factor.

In *C. albicans*, which has also maintained two paralogs of *erg25*, deletion of *Ca*Erg25 is lethal. These findings suggest that its partner, *Ca*Erg25-1 is either a non-functioning SMO, or that *Ca*Erg25-1 cannot demethylate adequately without *Ca*Erg25 (163). This finding is especially interesting given the fact that neither single SMO mutation was lethal in *A. fumigatus*. It is possible that *Ca*Erg25 and *Ca*Erg25-1 must work in tandem, unlike other examples in Eukarya, thus rendering *Ca*Erg25 lethal when deleted (163).

In *Arabidopsis thaliana*, the plant model organism, two copies of *erg25* also exist, and they both serve as C4-sterol methyl oxidases (71). Deletion of either copy of *erg25* is not lethal, and incurs an accumulation of C4-methylated sterol intermediates, much like in *A. fumigatus* (71). The entities accumulated, however, are not identical between Δ*SMO1* and Δ*SMO2*, as Δ*SMO1* accumulates mainly 4,4-dimethyl-9β,19-cyclopropylsterols, and ΔSMO2 accumulates mainly 4-methyl-Δ^7-sterols (71). These plant-specific sterol intermediates roughly correspond to an accumulation in 4,4-dimethyl fecosterol and 4-methyl fecosterol respectively. These results suggest that plants encode for distinct sets of SMOs, that are much more substrate-specific than in other eukaryotes studied to date.

This then begs the question: what is the purpose of two SMOs in *A. fumigatus*? Clearly they are both essential for the efficient demethylation of C4-sterol intermediates, as evidenced by the abundant accumulation of C4-methyl sterol intermediates in Δ*srbA* (Figure 3.2A). It appears that Erg25A may function as the predominant SMO, given Δ*erg25A*'s more pronounced accumulation of C4-methyl sterol intermediates than Δ*erg25B* (Figure 3.2A). This hypothesis is validated by the other visible phenotypes of Δ*erg25A* that are not apparent in Δ*erg25B*, including decreases in 22-dihydroergosterol and ergostatetraenol accumulation (Figure 3.3B), high mRNA induction in hypoxia (Figure 3.4A), enhanced stress response in extreme hypoxia (Figure 3.4B), and sensitivity to DTT and ROS (Figure 3.5A, 3.5C). The remaining question, then, is what is the purpose of Erg25B? Real-time PCR revealed that *erg25B* is less highly induced in response to stress, but that it partially compensates for loss of *erg25A* under stress-inducing conditions (Figures 3.7A and 3.4A). Therefore, it is possible that Erg25B functions as a backup for Erg25A in its C4-demethylation function, or may supply structural efficiency for Erg25A function. This later suggestion is based off the concept of an ergosterol "hub" or complex whose maintenance is required for full enzymatic efficiency of key proteins in the ergosterol biosynthesis pathway (218, 220). Although both *erg25A* and *erg25B* are regulated transcriptionally by SrbA, induction intensity of *srbA* mRNA does not appear to correlate equally with induction of *erg25A* and *erg25B*. Clearly there are multiple levels of transcriptional regulation at play here, placing *erg25B* in the less induced, or subordinate position to *erg25A* genetically.

Even more striking is the link between the phenotypes of Δ*erg25A* with Δ*srbA*, such as a growth defect in hypoxia (Figure 3.4B and shown before), significant mRNA induction in hypoxia (Figure 3.4A), dysregulation of the mechanism of ergosterol biosynthesis between episterol and ergosterol (Figure 3.3B), and sensitivity to ER stress-inducing agents (Figure 3.5A). These results suggest that some of the Δ*srbA* phenotypes may be directly related to a decrease in *erg25A* in this strain, as demonstrated previously (36).

Determining the biological importance in alterations of ergosterol biosynthesis from episterol to ergosterol (Figure 3.3B) is especially difficult to validate. This portion of the pathway has been described as a "fishing net;" when a disturbance pulls in one area, another section of the net takes up the load. However, if this is the case in *A. fumigatus*, then why are such distinct patterns observable in Δ*srbA* and Δ*erg25A*? Many more studies will be required to elucidate the requirement for individual enzymes or transcription factors in substrate acquisition and pathway flux. Analysis of this section of the pathway using Δ*srbA* is especially difficult, as SrbA transcriptionally regulates two of the potentially five enzymes involved in this progression (Barker, B.M. *et al.*, in preparation).

Review of the current literature pertaining to Erg25 or SMOs reveals this enzyme's importance in response to various cellular stressors. Erg25 mRNA is highly induced during stress-inducing conditions in many other fungi (19, 24, 39, 73), including triazole and polyene antifungal drug treatment, during hypoxia, with dysregulated calcium

signaling or lipid homeostasis, when encountering ER or oxidative stress, during biofilm formation, and during *in vivo* infection (19, 39, 78, 94, 96, 122, 140, 180, 226).

Growth in hypoxia results in the enrichment of proteins involved in glycolysis, ethanol fermentation, and the oxidative stress response, but a decrease in proteins involved in the TCA cycle, and the pentose phosphate shunt (23). In *S. cerevisiae*, mutants sensitive to DTT are also linked to the oxidative stress response and many display differences in regulation of glycolysis and the pentose phosphate shunt (110). Two genes in particular, *rpe1* and *tkl1*, are biochemically involved in the pentose phosphate shunt, and null-mutants display sensitivities to oxidative stress, DTT, and have defects in respiratory growth. These proteins are also essential for growth on fermentable carbon sources, linking the pentose phosphate shunt to ethanol production and usage in *S. cerevisiae* (152, 342).

DTT sensitivity has also been linked to plasma membrane and cell wall stability. This effect is observed most strikingly when the yeast *C. albicans* is treated with tetracycline. Tetracycline induces a loss of mitochondrial function, and decreases the total ergosterol, which is perhaps why tetracycline is synergistic with Amphotericin B. Interestingly, tetracycline is antagonistic to the triazole fluconazole. High concentrations of tetracycline render *C. albicans* more resistant to DTT, suggesting that alterations in the cell wall stability by DTT are countered by the effect of tetracycline on either the ergosterol content or oxidative state of the organism. This interplay between oxidative stress response, ergosterol biosynthesis, and ER-stress has been a common thread in our

studies, suggesting that these interactions are tightly controlled by central regulatory components.

In *H. sapiens*, Erg25 (SC4MOL/MSMO1) plays a critical role in the maturation of oocytes and activation of meiosis in the testis and ovary, as C4-methyl sterol intermediates serve as activating factors. One of these activating factors, follicular fluid meiosis-activating sterol (FF-MAS) is also a ligand for liver X receptors, which regulates crosstalk between inflammation and cholesterol metabolism (53, 148). Mutations in the human SC4MOL/MSMO1 contribute to altered cytokine production, including TNF-α and IL-6 (126, 315). More recently, silencing of *SC4MOL* has been linked to sensitization of refractory tumor cells to epidermal growth factor receptor (EGFR) inhibitors (315), as depletion of *SC4MOL* increased vesicular trafficking of EGFR to the lysosome.

In this same study, a yeast-two-hybrid screen identified over 140 genetic or physical interactors with Erg25 (315). These interactors belonged to many different biological processes, via GO-term analysis, including ergosterol biosynthesis, secretion, vesicle-mediated transport, localization, membrane lipid biosynthesis, sphingolipid biosynthesis, and ceramide biosynthesis (315). This evidence from *S. cerevisiae* and *H. sapiens* clearly indicates protein and/or genetic function for Erg25/*erg25* outside of ergosterol biosynthesis. It is plausible, therefore, that some of the phenotypes observed in Δ*erg25A* and Δ*erg25B* are due to uncharacterized interactions with non-ergosterol proteins, not solely due to dysregulation of membrane biosynthesis.

In conclusion, our study characterized the individual roles of C4-sterol methyl oxidase genes in *A. fumigatus*, and further emphasized the link between fungal SREBPs,

ergosterol biosynthesis, and stress adaptation. Although genetic deletion of *erg25B* demonstrated no tremendous phenotypic differences, save an enhanced sensitivity to the ER stress-inducing agent DTT, deletion of *erg25A* was more deleterious. The Δ*erg25A* strain demonstrated significant defects in the ability to compensate for low oxygen environments or ER- or ROS-induced stress, highlighting the complex interactions of fungal SMOs. In addition, SMO mutants displayed alterations in 9(11)-sterol intermediates, and a possible substrate-specific mechanism for traversing between episterol and ergosterol than previously described. Unlike other characterized two-SMO systems, *A. fumigatus* SMOs appear to retain a similar function, albeit with some substrate specificity, suggesting that Erg25A serves as the predominant SMO, and Erg25B serves subordinately to Erg25A. Both enzymes are required, however, as deletion of both alleles was lethal. Further work needs to be done to determine the contribution of *A. fumigatus* SMOs to the phenotypes of Δ*srbA*, as evidenced by the overlapping phenotypes observed in Δ*erg25A*. Also needed are more broad-scale transcriptional studies, to elucidate the covert non-ergosterol interactions occurring with these important biological mediators.

Experimental Procedures

Strains and Media

Aspergillus fumigatus strain CEA17 was used to construct the Δ*srbA* strain (359), as well as Δ*erg25A* and Δ*erg25B*. To generate the *erg25A* (Afub_084150) and *erg25B* (Afub_098170) knockout constructs, ~1kb upstream and downstream of both gene ORFs

was PCR amplified from genomic *A. fumigatus* DNA. The *pyrG* from *A. parasiticus* was amplified from plasmid pJW24 (courtesy of N. Keller), and was inserted between the upstream and downstream PCR fragments described above via fusion PCR (368). Protoplast-mediated transformation was conducted to introduce the foreign DNA into the CEA17 strain.

The *pniiA-erg25B-*Δ*erg25A* and *pniiA-erg25A-Δerg25B* inducible strains were generated in a *-pyrG*Δ*erg25A* and *-pyrG*Δ*erg25B* strain respectively, which was made by excising a 1.1kb fragment of the *pyrG* ORF from the Δ*erg25A* or Δ*erg25B* knockout plasmid and retransforming into the correct background on media containing 5-Fluoroorotic Acid (Invitrogen). The Left flank (Lflank, ~1kb approximately 200bp upstream of 5'ATG) and open reading frame (ORF) were PCR amplified from genomic DNA alongside a 3.2kb fragment containing *pyrG* and *pniiA* from the *pyrG-pniiA* plasmid, as previously described (138).

The Lflank-*pyrG-pniiA-erg25B* transformation construct was assembled via overlap-mediated fusion PCR, as described above but with the following changes: the Left flank (Lflank, ~1kb approximately 1000bp upstream of 5'ATG) was amplified, eliminating the entire putative promoter region of this construct upon transformation into *-pyrG*Δ*erg25A*.

Strains were verified via Southern Blot for single, locus-specific integration. For the Δ*erg25A* Southern Blot: genomic DNA from the Δ*erg25A*, wild type, and Δ*erg25A*+*erg25A* strains was ApaI digested for at 37˚C for 18 hours. Strains were separated on a 1% agarose gel, transferred, and hybridized with a 607-bp probe. For the Δ*erg25B* Southern Blot: genomic DNA from the wild type, Δ*erg25B*, and

Δ*erg25B+erg25B* strains was HindIII/NheI digested at 37˚C for 18 hours. Strains were separated on a 1% agarose gel, transferred, and hybridized with a 328-bp probe. The wild-type strain referred to in this article is strain CBS 144.89.

All strains are routinely grown on glucose minimal media (GMM) that contains 1% glucose, at 37°C (289). The recipe for liquid glucose minimal media is identical to that for GMM, except without agar. For induction/repression experiments, strains were grown in liquid GMM with 20mM Sodium Nitrate (NO_3) or 20mM Ammonium Tartrate (NH_4) as the sole nitrogen source (138).

Iron Stress Assay

10^8 *A. fumigatus* conidia from various strains were cultured in 150mL liquid media (glucose-minimal-media) with or without iron (ferrous sulfate heptahydrate, Fisher Scientific), for 24 hours. Following incubation, samples were harvested via vacuum filtration, frozen at -80°C, and lyophilized overnight. Samples were weighed, and the average and standard deviation of three or more biological replicates are reported. Data are represented as the ratio of growth without iron-to-iron.

ER Stress Assay

$2x10^7$ *Aspergillus fumigatus* conidia were incubated in liquid glucose minimal medium (LGMM) at 37˚C with agitation, with or without dithiothreitol (DTT, 1mM, Sigma Aldrich) for 24 hours. Cultures were harvested via vacuum filtration, frozen at -80˚C, and lyophilized until dry. Biomass of dried samples was measured, and data

expressed as the ratio of the mass of the DTT-treated samples verses control. Data are the average and standard deviation of three biological replicates per strain.

Hydrogen Peroxide Assay

Two hundred *Aspergillus fumigatus* conidia were plated onto a glucose minimal medium (GMM) plate overnight at 37˚C. Germlings were shocked with either 10mL 1.87mM hydrogen peroxide (Fisher Scientific) or sterile water for ten minutes at 37˚C, then washed with 10mL sterile water twice. Plates were incubated at 37˚C until colonies were visible, approximately 20 hours. Data are expressed as the ratio of the number of viable colonies on H_2O_2-treated versus untreated plates. Data are the average and standard deviation of three plates per strain, per condition.

Quantitative Real-Time PCR

Aspergillus fumigatus strains were cultured in liquid media (glucose-minimal-media or induction/repression minimal media) for sixteen hours, then shifted to hypoxia for the indicated times. Mycelia were harvested via vacuum filtration and lyophilized overnight prior to homogenation with 0.1mm glass beads. Total RNA was extracted using TRisure (Bioline) according to the manufacturer's instruction and purified via RNeasy column protocol (Qiagen). Genomic DNA purification was completed with Turbo DNAse I (Ambion). A secondary genomic DNA purification was done with the Qiagen QuantiTect Reverse Transcription Kit (Qiagen), as well as oligo-DT-primed cDNA synthesis. qRT-PCR was conducted in technical duplicates except where noted. The normalized fold expression graphed in each figure represents the mean and percent error of two-to-three

biological replicates as normalized to the housekeeping gene *tefA*. A no-template mRNA control was used to ensure no gDNA contamination in each analysis.

Antifungal Susceptibility Testing

Susceptibility to Fluconazole, Voriconazole, and Amphotericin B was evaluated with Etest strips (AB Biodisk). Each strip delivers a concentration gradient of drug. Strips are placed on an RPMI-MOPS (Sigma Aldrich) plate, pH 7.0, containing 10^5 conidia. Growth inhibition was evaluated at 48 hours by visualization of growth inhibition when grown at 37°C.

Total Ergosterol Assay

Total ergosterol from *A. fumigatus* strains was extracted after 24 hours shaking growth in liquid GMM. Mycelia were harvested via vacuum filtration, dried, and weighed. 200mg of dry fungal tissue were extracted as described previously (9, 37). Briefly, tissues were treated with 3mL of 25% alcoholic potassium hydroxide (3:2 methanol:ethanol) and then vortexed vigorously for 1 minute. Samples were incubated at 85°C for 1 hour. Sterols were extracted with 1mL of distilled water and 3mL of pentane and vortexed for 3 minutes. The upper aqueous layer was allowed to separate from the cellular debris and 1mL of this extract was transferred to a clean glass tube and evaporated in a fume hood at room temperature. Upon preparation for injection, samples were resolved in 1mL of methanol and syringe filtered through a 0.2μm-pore-size filter (Acrodisc, Waters). Total ergosterol was measured using a Shimadzu CLASS-VP HPLC and detected via a SPD-M10AVP PDA at 280nm on a Bondapack C18 column

(300 mm × 3.9 mm, 10 μm). The quantity of ergosterol per strain was calculated from a standard curve of ergosterol (Acros Organics 117810250).

Sterol Profile

Mycelia were grown and harvested as described for total ergosterol extraction. Extraction was done as described previously (9, 37), and identically as in the total ergosterol extraction with the exception that the neutral lipids were extracted with 1.5mL hexane. The upper aqueous layer was separated from the cellular debris and 1mL of this extract was evaporated via nitrogen. Vials were stored at -20˚C until ready for injection. Samples were resolved in 100μL toluene and derivitized with 100μL BFSTA for 1 hour at 70°C. Sterols were measured via GC-MS, using a Shimadzu QP-2010 GC with a quadrupole mass ionizer. Peaks were identified via EI (electron ionization) fragmentation pattern and retention time. All data are relative to total ergosterol content.

Virulence Assays

CD1 female mice, 6-8 weeks old were used in a chemotherapeutic model of Invasive Aspergillosis. Mice were obtained from Charles River Laboratories in Raleigh, North Carolina.

Five mice per cage were housed in a HEPA-filtered environment with autoclaved food *ad libitum*. For the corticosteroid model, a single 40mg/kg injection of Kenalog (Bristol-Myers Squibb) is administered subcutaneously (s.c.) one day prior to infection with *A. fumigatus*. For infection, $2x10^6$ conidia in 100μL 10% PBS (phospho-buffered-saline) are administered intranasally (i.n.) to 10 mice/group under isofluorine anesthesia.

Mice were observed for fourteen days after *A. fumigatus* challenge. Any animal showing severe distress; defined as ruffling of fur, hunched posture, decreased mobility, and difficulty breathing; were humanely euthanized and recorded as deceased within that 24-hour time period. A PBS-only control group was used to verify the underlying health status of these animals. No PBS-only animals perished during the course of the experiment.

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Montana State University (Federal-Wide Assurance Number: A3637-01).

Statistical Analysis

All results presented with statistical significance were analyzed with an unpaired two-tailed Student's *t*-test, with the exception of the virulence assay. Results from the murine model were done via Log Rank analysis of the Kaplan-Meir survival curve.

Acknowledgements

This work was supported by funding from NIH/NIAID grant R01AI81838, NIH/COBRE grant RR020185, and NIH-NCRR. Sara Blosser was funded through an

American Heart Association Predoctoral Fellowship for this work. The mass spectrometry facility is supported by NIH NCRR COBRE P20 RR024237.

We thank Dr. Martin Bard for his critical eye and suggestions pertaining to our sterol analysis, Dr. Ted White for review and suggestions pertaining to this project, and Drs. Brian Bothner and Jonathan Hilmer for assistance with mass-spectrometry.

Tables

Table 3.1 Antifungal minimum inhibitory concentration determination using Etests.

	FLC	AMB	VCZ
	0.016-256 µg/mL	0.002-32 µg/mL	0.002-32 µg/mL
Wild Type	>256	0.25	0.094-0.125
Δ*srbA*	1	0.25	0.004-0.006
Δ*erg25A*	>256	0.38	0.125
Δ*erg25B*	>256	0.25	0.19

FLC: fluconazole, AMB: amphotericin B, VCZ: voriconazole

Figures

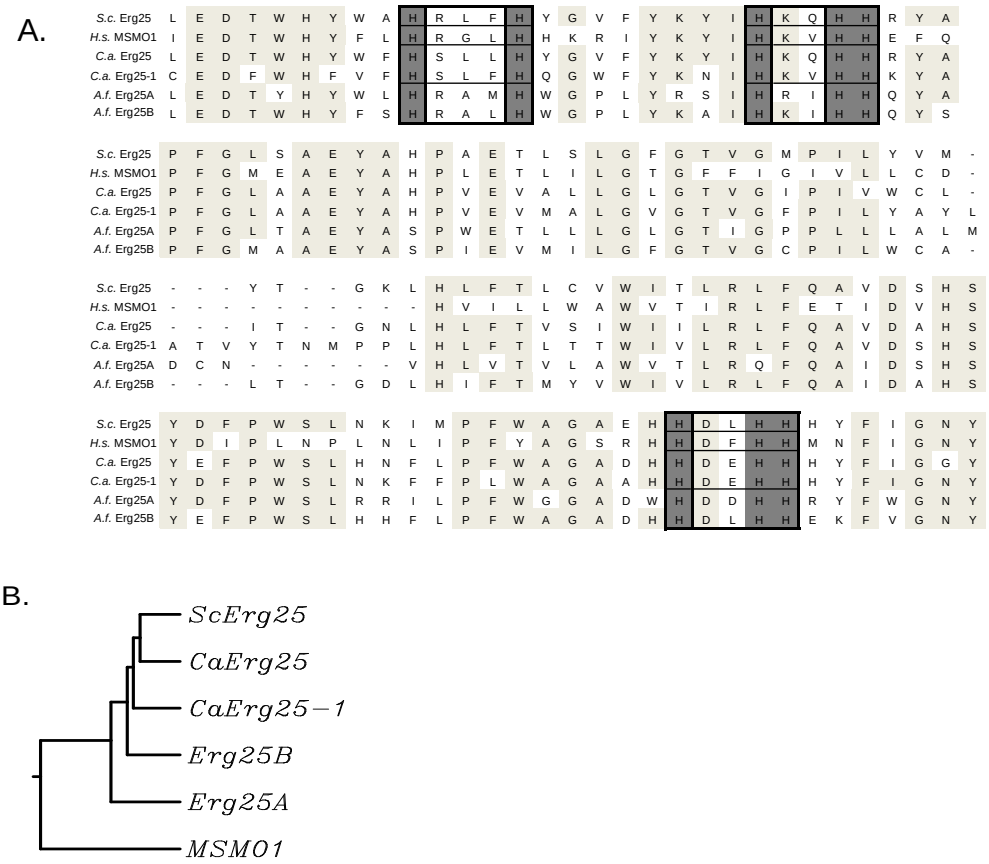


Figure 3.1 Comparative amino acid homology and motif analysis for Erg25 genes from *Aspergillus fumigatus (A.f.), Candida albicans (C.a.), Homo sapiens (H.s.),* and *Saccharomyces cerevisiae (S.c)*. A. Dark gray shaded boxes indicate conserved histidine motifs among all four examined species. Gray but unboxed residues are those conserved in at least one homolog in *S. cerevisiae*, *C. albicans*, and *A. fumigatus.* B. Rooted phylogenetic tree with branch length (UPGMA) for all examined C4-sterol methyl oxidase (SMO) genes (ClustalW, Kyoto University Bioinformatics Center). Analysis clearly demonstrates *H. sapiens* MSMO1 as the outgroup, with *A. fumigatus* Erg25B more closely related to the SMOs of *S. cerevisiae* and *C. albicans*.

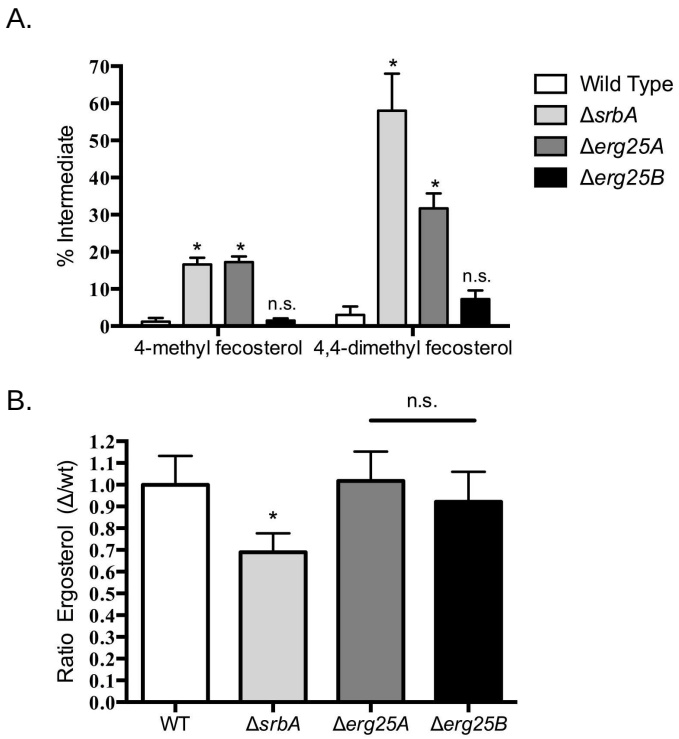


Figure 3.2 Characterization of C4-Sterol Methyl Oxidase Mutants. A. C4-methyl sterol intermediate profile demonstrates a distinct accumulation in 4-methyl fecosterol (4,24-dimethyl-cholesta-8,24(28)-dien-3β-ol) and 4,4-dimethyl fecosterol (4,4,24-trimethyl-cholesta-8,24(28)-dien-3β-ol) in Δ*srbA* and Δ*erg25A*. A trend towards accumulation of these intermediates is observed in Δ*erg25B*, but statistical significance is not obtained due to biological variation. Data are displayed as the ratio of sterol intermediates to ergosterol. Peaks were identified as described previously (37), and in the materials and methods. B. Total ergosterol is unchanged in Δ*erg25A* and Δ*erg25B* compared to the wild type strain, and does not cause the depletion in total ergosterol demonstrated in Δ*srbA*. Total ergosterol was extracted and processed as described in the materials and methods and previously (37). *$p<0.001$. Values represent the mean and standard deviation of 3-4 biological and three technical replicates for total ergosterol, and 2-3 biological replicates for sterol intermediate profiles.

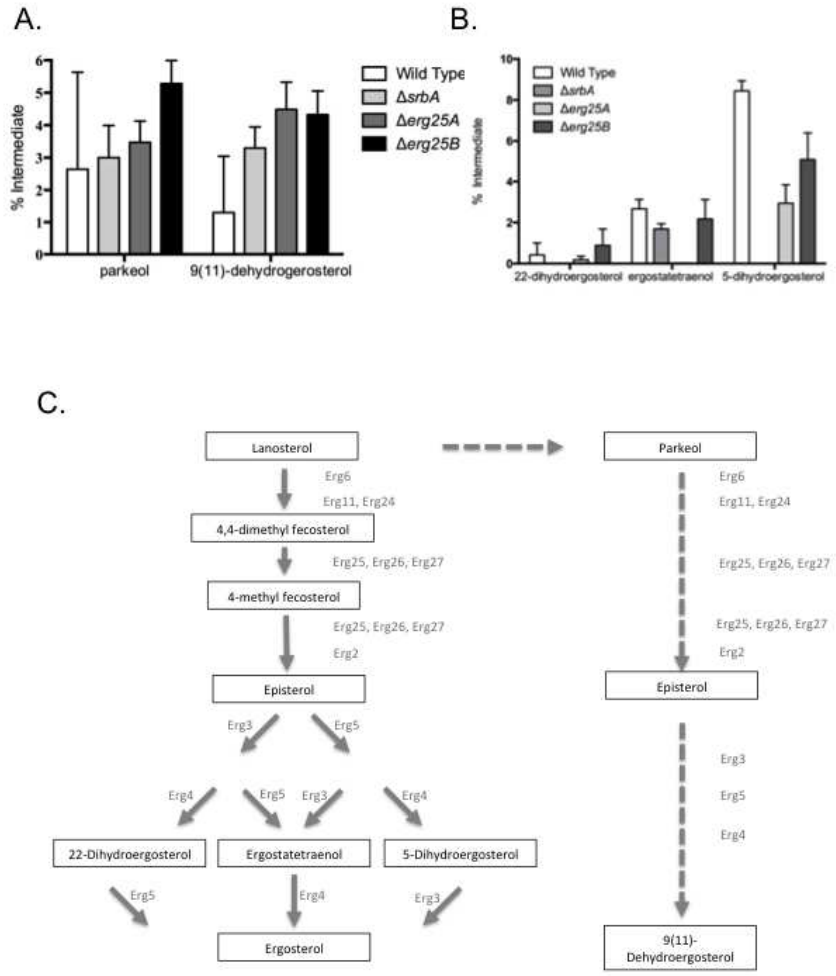


Figure 3.3 Sterol intermediate profiles for Δ*erg25A* and Δ*erg25B* and proposed alternative pathway. A. Accumulation of 9(11) intermediates in Δ*erg25A*, Δ*erg25B*, and Δ*srbA*. Much more parkeol (4,4,14-trimethyl-cholesta-9(11),24-dien-3β-ol) is observed in Δ*erg25B* than the other studied strains. Loss of *erg25A* or *erg25B* resulted in an accumulation of 9(11)-dehydroergosterol (24-methyl cholesta-5,7,9(11),22-tetraen-3β-ol). Results are displayed as the ratio of sterol intermediate to ergosterol. Peaks were identified as described in the materials and methods. B. Aberrant accumulation of sterol intermediates involving passage from episterol to ergosterol. 22-dihydroergosterol (24-methyl cholesta-7,24(28)-dien-3β-ol), ergostatetraenol (24-methyl cholesta-5,7,22,24(28)-tetraen-3β-ol), and 5-dihydroergosterol (24-methyl cholesta-7,22-dien-3β-ol). C. Schematic of ergosterol biosynthesis pathway (left) and proposed alternative mechanism of parkeol and 9(11)-dehydroergosterol intermediate accumulation (right). Dotted arrows indicate the proposed mechanism, solid arrows the mechanism proposed by Alcazar-Fuoli *et al*.

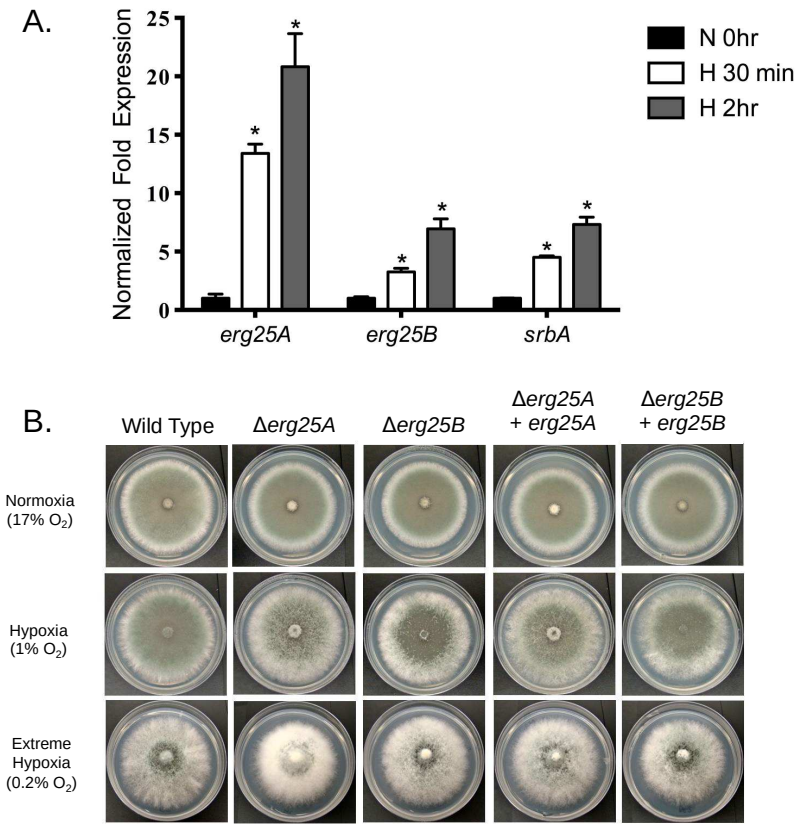


Figure 3.4 Sterol methyl oxidases (SMO) are responsive to hypoxia. A. Quantitative Real-Time PCR analysis of *erg25A*, *erg25B*, and *srbA* in response to 30 minutes and two hours exposure to hypoxia. Wild type *A. fumigatus* was grown 16 hours at 37˚C and then harvested (N 0hr) or exposed to hypoxia (1% O_2) for 30 minutes (H 30 min) or two hours (H 2hr) prior to harvesting. RNA was extracted and cDNA synthesized. mRNA abundance is normalized to the housekeeping gene *tefA* and are relative to the N 0hr sample for all genes examined. Data represent three biological and two combined technical replicates. *$p<0.0001$. B. Radial growth assay of SMO strains in hypoxia (1% O_2) and extreme hypoxia (0.2% O_2) reveals a stress-response phenotype in Δ*erg25A* but not Δ*erg25B*. 10^5 conidia were spotted on a glucose minimal media plate, and allowed to grow at 37˚C for 96 hours in the indicated condition.

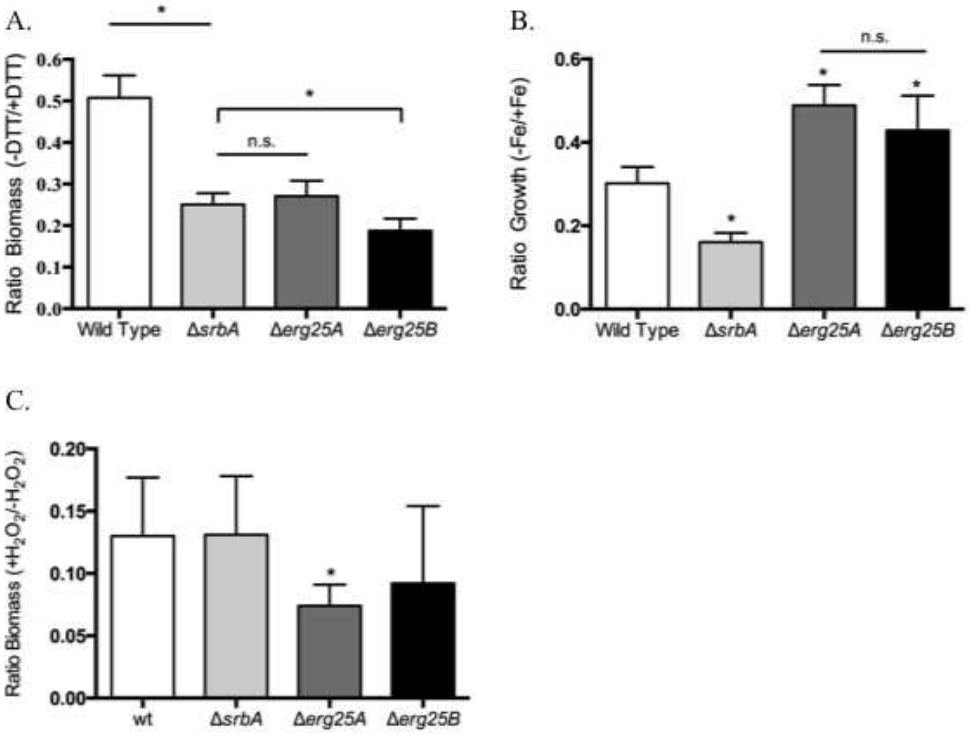


Figure 3.5 Aberrant Stress Responses in Sterol Methyl Oxidase (SMO) Mutants. A. Dithiothreitol (DTT)-ER Stress Inducing Assay. $2x10^7$ conidia were incubated in liquid glucose minimal medium (LGMM) with or without DTT (1mM) for 24 hours. Biomass was measured, and ratios express the mass of culture with DTT treatment verses control. A significant increase in sensitivity was observed for Δ*srbA*, Δ*erg25A*, and Δ*erg25B* compared to the Wild Type strain, *p<0.0001. In addition, Δ*erg25B* was significantly more sensitive than Δ*srbA* and Δ*erg25A*, *p<0.001. Clearly SMO function is crucial for adaptation to DTT. B. Iron Biomass Assay. 10^8 *A. fumigatus* conidia from various strains were cultured in 150mL liquid media (glucose-minimal-media) with or without iron (ferrous sulfate heptahydrate, Fisher Scientific), for 24 hours. Data are represented as the ratio of growth without iron versus with iron. The average and standard deviation of three or more biological replicates are reported. Both Δ*erg25A* and Δ*erg25B* are significantly more adapted to growth in low iron than Δ*srbA* and even the Wild Type strain. *p<0.0001. C. ROS susceptibility altered in Δ*erg25A* but not Δ*erg25B.* ROS susceptibility assay. Germlings were exposed to 1.87mM H_2O_2 for ten minutes at 37˚C, washed, and allowed to grow for 24 hours. Viability is evaluated compared to non-treated control. Ratios expressed as the number of colonies on H_2O_2-treated versus control-plates after 24-hour incubation. A significant increase was observed for Δ*erg25A* versus wild type and Δ*srbA*, *p<0.05. No significant change in susceptibility was observed for Δ*erg25B.*

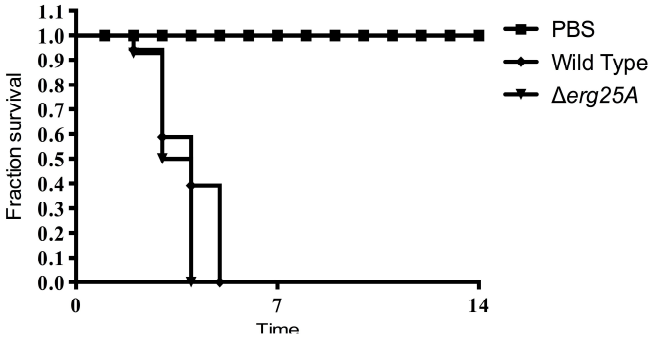


Figure 3.6 *Erg25A* is not required for virulence of *A. fumigatus*. $2x10^6$ conidia were administered intranasally to corticosteroid-treated (Kenalog, 40mg/kg) CD1 mice from Wild Type, PBS-only, and Δ*erg25A* strains. Survival was assessed for fourteen days post-inoculation. The SMO-mutant Δ*erg25A* did not have any alteration in virulence compared to the Wild Type strain.

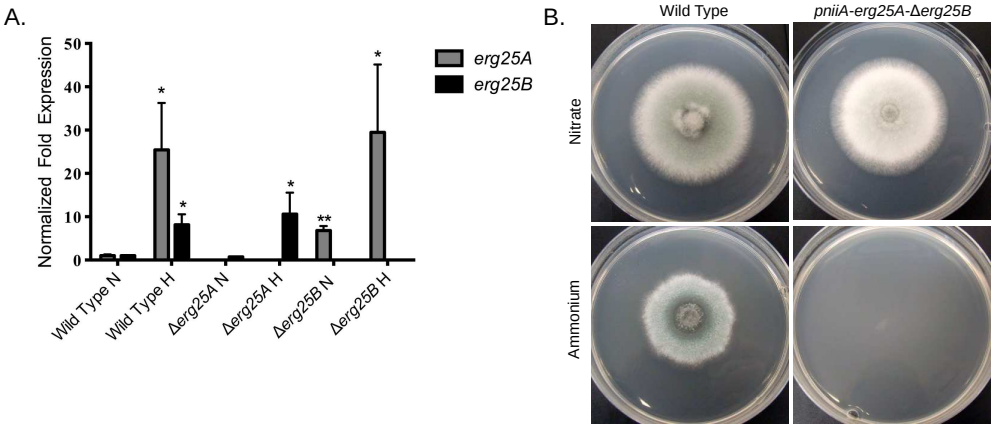
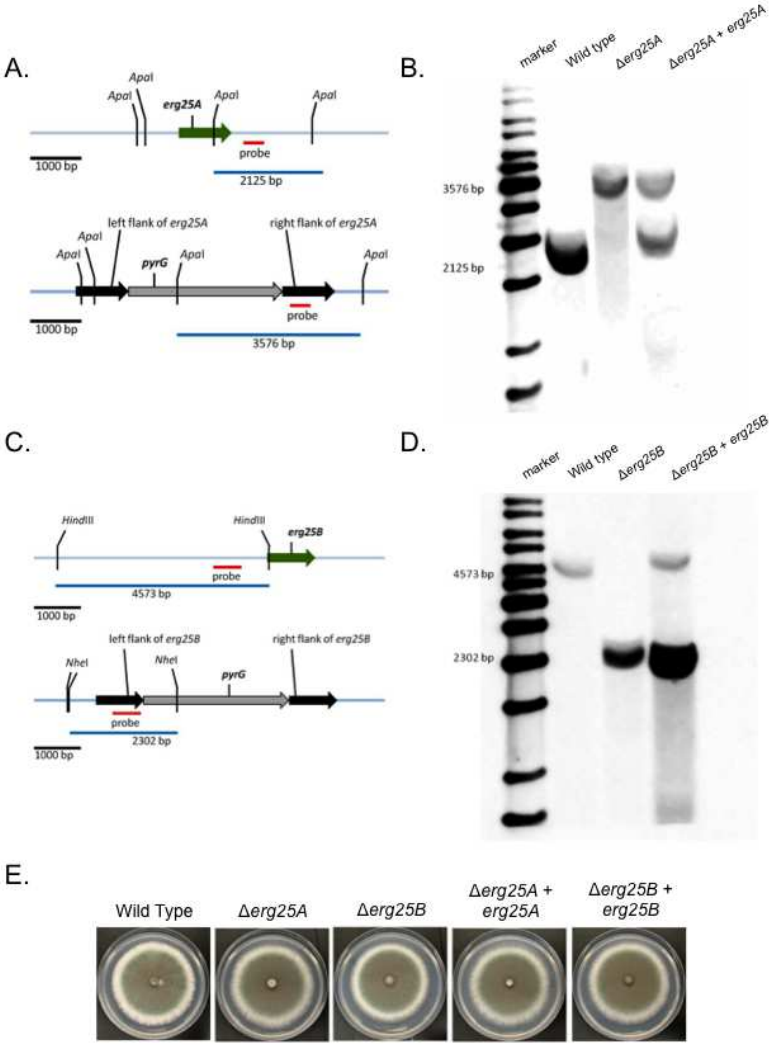
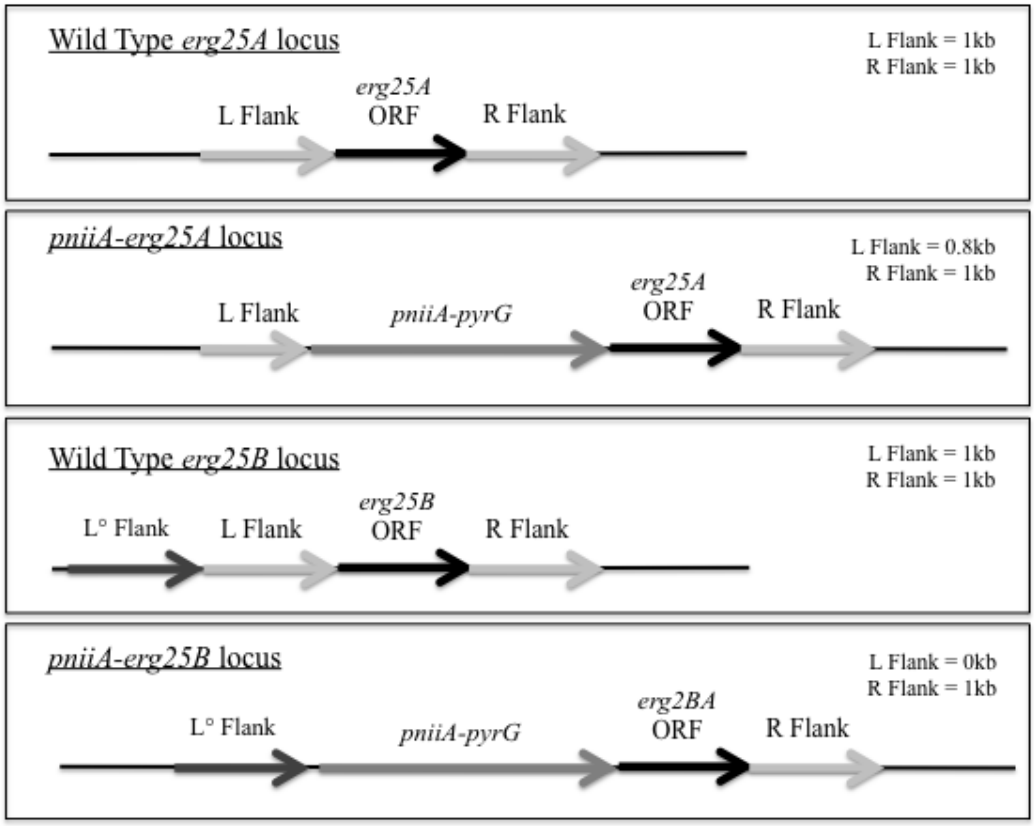


Figure 3.7 Erg25A and Erg25B can compensate for loss of one another & loss of both *erg25A* and *erg25B* lethal in *A. fumigatus*. A. Quantitative Real-Time PCR analysis of *erg25A* and *erg25B* in Δ*erg25A* and Δ*erg25B* during hypoxia stress. Wild type, Δ*erg25A*, and Δ*erg25B A. fumigatus* strains were grown 16 hours at 37˚C and then harvested (N) or exposed to hypoxia (H, 1% O_2) for four hours prior to harvesting. RNA was extracted and cDNA synthesized. mRNA abundance is normalized to the housekeeping gene *tefA* and is relative to the Wild type N sample for all genes examined. Data represent three biological and two combined technical replicates. *$p<0.05$, **$p<0.0001$. B. Lethality of a double deletion mutant was assessed via utilization of the nitrogen-inducible niiA promoter from *A. fumigatus*. Wild type or *pniiA-erg25A-*Δ*erg25B* were plated on glucose minimal media (GMM) with 20mM nitrate (induction) or ammonium (repression). A distinct lack of growth is observed for *pniiA-erg25A-*Δ*erg25B* when plated on repression media, whereas the wild type strain is fully capable of growth.



Supplementary Figure 3.1 Construction of Δ*erg25A*, Δ*erg25B* and reconstitution strains in *A. fumigatus*. A. Southern blot schematic for Δ*erg25A*. Top diagram – wild type locus of *erg25A*. Bottom diagram – locus of the Δ*erg25A* construct in the CEA17 background strain. B. Southern blot for Δ*erg25A* and Δ*erg25A* + *erg25A*. Genomic DNA was ApaI digested at 37˚C for 18 hours. Strains were probed with a 607-bp probe. All banding patterns were as expected for the various strains. C. Southern blot schematic for Δ*erg25B*. Top diagram – wild type locus of *erg25B*. Bottom diagram – locus of the Δ*erg25B* construct in the CEA17 background strain. D. Southern blot for Δ*erg25B* and Δ*erg25B* + *erg25B*. Genomic DNA was HindIII/NheI digested at 37˚C for 18 hours. Strains were probed with a 328-bp probe. All banding patterns were as expected for the various strains. E. Radial growth assay of generated strains. 10^5 conidia were spotted on a glucose minimal media plate, and allowed to grow at 37˚C for 96 hours. No difference was observed in the growth rate or colony morphology in any of the generated strains.



Supplementary Figure 3.2 Schematic for construction of *pniiA-erg25A-*Δ*erg25B* and *pniiA-erg25B-*Δ*erg25A*. Black arrows indicate the gene ORF, light gray arrows are the Left and Right flanking sequences to the ORF, sizes of these flanking sequences are indicated in each schematic. The medium gray arrows indicate the *pniiA-pyrG* cassette, including both the nitrogen inducible promoter *niiA* and the selection marker *pyrG*. The darkest gray arrows indicate the 1kb region directly upstream of the L Flank of the *erg25B* locus (L˚ Flank). Strains were constructed as detailed in the experimental procedures.

CHAPTER FOUR

REMOVAL OF C4-METHYL STEROL ACCUMULATION IN AN SREBP-NULL MUTANT OF *ASPERGILLUS FUMIGATUS* RESTORES HYPOXIA GROWTH

Contribution of Authors and Co-Authors

Manuscript in Chapter 4

Author: Sara J. Blosser

Contributions: Designed the study, conducted the experiments, collected and analyzed the data, wrote the manuscript.

Co-Author: Robert A. Cramer

Contributions: Obtained funding for the study, assisted with study design, discussed results of the experiments and implications for this study, edited the manuscript.

Manuscript Information Page

Sara J. Blosser, Robert A. Cramer

Journal Name: PLoS Pathogens

Status of Manuscript:

_x_ Prepared for submission to a peer-reviewed journal
___ Officially submitted to a peer-reviewed journal
___ Accepted by a peer-reviewed journal
___ Published in a peer-reviewed journal

Abstract

Aspergillus fumigatus is a pathogenic mold, whose ability to cause invasive diseases relies on key growth attributes, including the capacity to grow in low oxygen, or hypoxic, environments. A transcription factor in the Sterol Regulatory Binding Protein (SREBP) family, SrbA, is essential for the regulation of ergosterol biosynthesis, resistance to triazole antifungal drugs, the ability to grow in hypoxia and low iron environments, and to cause invasive disease in murine models of Invasive Aspergillosis (IA). Regulation of a key step in ergosterol biosynthesis, Erg25, assists in the ability of *A. fumigatus* to grow in hypoxia and adapt to low iron environments. Construction of a strain that constitutively expresses *erg25A* in the Δ*srbA* background restored the ability of Δ*srbA* to grow in hypoxia. This restoration, however, revealed substantial insufficiencies in *pflavA-erg25A-*Δ*srbA* when adapting to hypoxia, as this strain was hypersensitive to cell wall perturbation and reactive oxygen species (ROS). Additionally, restoration of *erg25A* impacted the triazole antifungal susceptibility of Δ*srbA*, demonstrating a complex feedback system involved in ergosterol biosynthesis. These results demonstrate SrbA's involvement in a dynamic stress adaptation program mediated in part through ergosterol biosynthesis.

Introduction

The interplay between inflammation and the generation of hypoxic microenvironments has led many researchers to evaluate the importance of hypoxia adaptation in a pathogen's virulence arsenal (60, 113, 170, 359). In human pathogenic

fungi, hypoxia adaptation has been hypothesized to be a critical virulence attribute, as gene-deletion mutants that are unable to grow in hypoxia are typically also unable to cause invasive disease (60, 113, 359). In *Aspergillus fumigatus*, a ubiquitous, saprophytic mold, a member of the Sterol Regulatory Element Binding Protein (SREBP) family, SrbA, is critical for adaptation to hypoxia and low iron conditions, resistance to triazole antifungal drugs, and virulence in murine models of Invasive Aspergillosis (IA) (36, 37, 359). As a transcription factor, SrbA directly interacts with and influences transcriptional regulation of a cohort of genes. Ergosterol biosynthesis genes appear to be enriched in the SrbA-regulon suggesting that sterol biosynthesis and regulation are mechanistically linked with fungal pathogenesis and responses to antifungal drugs. This link is also apparent in *C. neoformans*, where regulation of ergosterol biosynthesis, and sensitivity to triazole antifungals are linked to the SREBP homolog, Sre1 (55). Thus, study of the SrbA dependent regulon should lead to new insights into the role of specific sterols and their regulation in fungal pathogenesis and responses to antifungal drugs.

Ergosterol is the fungal equivalent of cholesterol, and this end product and its biosynthesis has been well studied in the context of antifungal drug design and resistance, as two of the main classes of antifungal drugs, the polyenes and the triazoles, target ergosterol or an ergosterol intermediate. However, little is known about the regulation and biological purposes of many of the other components of ergosterol biosynthesis, which have primarily been studied in fungi using the model system *Saccharomyces cerevisiae*.

In humans, Erg25, also known as SC4MOL, MSMO1, or DESP4, has been characterized for its role in cholesterol biosynthesis (53, 126, 148, 315). In addition to acting as a C4-sterol methyl oxidase (SMO), Erg25 is known to interact with many other proteins involved in vesicle transport, membrane localization, and calcium homeostasis (53, 126, 315). C4-methyl sterols are also important biological mediators, especially in the gonads, where follicular fluid meiosis-activating sterol (FF-MAS) and testis meiosis-activating sterol (T-MAS) act as maturation factors for oocytes, and mitosis in both the testis and ovary (53, 148). Interestingly, FF-MAS also act as a ligand for the liver X receptor, coordinating crosstalk between inflammation and cholesterol biosynthesis (53, 148). Thus there is a rich knowledge in other eukaryotes whereby specific sterol intermediates serve as signaling molecules to drive biological processes.

In fungi, *erg25* mRNA transcript abundance is highly induced during stress conditions, including triazole and polyene antifungal drug treatment, during hypoxia, with dysregulated calcium signaling or lipid homeostasis, when encountering ER or oxidative stress, during biofilm formation, and during *in vivo* infection (19, 39, 78, 94, 96, 122, 140, 180, 226). Either ergosterol biosynthesis is highly manipulatable during these conditions, or Erg25/C4-methylated sterols are important in determining and adapting to various stress conditions. Regardless, the underlying mechanisms of sterol biosynthesis regulation in pathogenic fungi remain largely underdeveloped.

Previously, we have demonstrated that SrbA regulates C14-demethylation via direct transcriptional activation of *erg11A* and *erg11B*, and that the loss of this regulation in Δ*srbA* contributes significantly to the azole-sensitivity phenotype of Δ*srbA* (37).

Remediation of *erg11A* in Δ*srbA* revealed a significant downstream blockage at the C4-demethylation step of ergosterol biosynthesis, as evidenced by the hyperaccumulation of C4-methyl sterol intermediates (37). Genetic deletion of either *erg25A* or *erg25B* demonstrated that *A. fumigatus* encodes for two fully functional SMOs, but that Erg25A is the predominant SMO (Blosser, S.J. *Chapter 3*). Therefore, to understand the phenotypic contributions of the loss of *erg25A* expression in Δ*srbA*, we constructed an *erg25A*-Δ*srbA* strain under the control of a new constitutive promoter based on a flavohemoglobin gene that is highly expressed in *A. fumigatus* in multiple *in vitro* conditions. Restoration of *erg25A* mRNA abundance in Δ*srbA* was functional via *pflavA*-*erg25A*-Δ*srbA* as indicated by the decrease in 4,4-dimethyl fecosterol in this strain. Importantly, *pflavA*-*erg25A*-Δ*srbA* was restored in its ability to grow in hypoxia, demonstrating the need for Erg25 in plasma membrane stability during hypoxic growth, as the accumulation of C4-methyl sterols appears to be inhibitory under these conditions. Interestingly, *pflavA*-*erg25A*-Δ*srbA* was also restored in its ability to grow in low iron environments compared to Δ*srbA*. This restoration of hypoxia and iron-replete growth was not a complete rescue for the phenotypes of Δ*srbA* however, as alterations in other SrbA-dependent ergosterol biosynthesis gene transcription occurred, as well as sensitivity to cell wall perturbation agents and reactive oxygen species in hypoxia. These finding suggest that a non-Erg25 SrbA-dependent mechanism is responsible for these phenotypes in *pflavA*-*erg25A*-Δ*srbA*, or that sterol intermediates in *pflavA*-*erg25A*-Δ*srbA* greatly alter gene expression in a deleterious manner. Overall, this study illustrates the complexity of SrbA's involvement in stress adaptation, mediated in part through transcriptional

regulation of ergosterol biosynthesis and moreover highlights the potential role of sterol intermediates in the biology of human fungal pathogens.

Results

Hypoxia Alters Response of Di-Iron, Di-Oxygen Requiring Enzymes of Ergosterol Biosynthesis

The SrbA-null mutant is incapable of growing in hypoxia, but this treatment is not lethal, as oxygenation of hypoxia-treated samples fully restores growth (358). Furthermore, ergosterol biosynthesis genes are the most predominant group of genes directly regulated by SrbA, and some of the highest expressed genes in hypoxia (23, 36). In order to dissect the relationships between hypoxia adaptation and SrbA-regulation of ergosterol biosynthesis in *Aspergillus fumigatus*, we examined the effect of hypoxia treatment on total ergosterol levels. Cultures were allowed to germinate in normoxia, then grown for the indicated times in hypoxia (1% O2) (Figure 4.1A). In *Schizosaccharomyces pombe*, a decrease in total ergosterol is a signal that allows for transcriptional activation of ergosterol biosynthesis genes through proteolytic cleavage of the SREBP Sre1 (255) (55, 140, 323). Similarly, in *A. fumigatus*, hypoxia exposure initiates a decrease in total ergosterol between time zero and approximately two hours (Figure 4.1A, *$p<0.0001$). After this time, it appears to recover or adapt to the decrease in oxygen as we see a restoration to wild-type levels at 3 and 24 hours post-switch into hypoxia. The decrease in total ergosterol at 6 hours appears to indicate that this system is in a constant state of adaptation in low oxygen environments, and that a cyclical, or

supply-and-demand mechanism may be in place to replenish the cellular ergosterol requirements.

Although ergosterol has been shown to be a crucial signaling molecule for induction of ergosterol biosynthesis genes via SREBPs in yeast, ergosterol intermediate buildup may also play a role in how *A. fumigatus* adapts to hypoxia and other stress conditions. Therefore, wild type *A. fumigatus* sterol intermediates were compared in normoxia and hypoxia twelve hours post germination to examine intermediate accumulation (Figure 4.1B). Hypoxia treatment produced the accumulation of all intermediates directly proceeding oxygen-dependent steps of ergosterol biosynthesis downstream of squalene (Figure 4.1B). Interestingly, the steps most affected were the di-iron and di-oxygen requiring enzymes Erg11, Erg25 and Erg3, which are also directly regulated by the transcription factor SrbA (Barker, B.M. *et al.*, in preparation; (36)).

C4-Methyl Sterol Intermediates in Hypoxia & Hypoxia-Induced Gene Deletion Mutants

Sterol profiles of both the wild type *A. fumigatus* in hypoxia and the SrbA-null mutant indicate altered accumulation of intermediates at the C4-demethylation or Erg25 step of ergosterol biosynthesis, and previous characterization of Δ*erg25A* illuminated an aberrant growth phenotype in hypoxia ((359), Figure 4.1B, and Blosser, S.J. *et al.*, Chapter 3). Sterol profiles of hypoxia, or hypoxia-inducible genes we have studied thus far all indicate an accumulation of C4-methyl sterol intermediates. Therefore, as this step appears to be highly important for efficient ergosterol biosynthesis in oxygen-poor

environments, we sought to understand the relationship between Erg25A and SrbA, and how the loss of *erg25A* expression in Δ*srbA* contributes to the phenotypes of the mutant.

SrbA-Regulation of *erg25A* in *A. fumigatus*

Three things link SrbA function and *erg25A* expression in *A. fumigatus*: genetically, SrbA directly binds to the promoter region of *erg25A*; phenotypically, Δ*srbA* and Δ*erg25A* both accumulate elevated levels of C4-methyl sterols; and finally, both Δ*srbA* and Δ*erg25A* demonstrate aberrant growth rates in hypoxia, though to different magnitudes ((359), Blosser *et al*, Chapters 2 & 3). Toxic sterol intermediate accumulation has been hypothesized to contribute to altered membrane function when fungi are treated with azole antifungal drugs; it is plausible, therefore, that altered accumulation of sterol intermediates could alter *A. fumigatus*' physiological response to stress.

To test this hypothesis we uncoupled *erg25A*'s dependency on SrbA for transcriptional activation by placing *erg25A* under a strong promoter in the Δ*srbA* background. Initially we intended to use the well-characterized *gpdA* promoter for these analyses, however, *pgpdA*-*erg25A*-Δ*srbA* failed to achieve >70% restoration of wild-type mRNA abundance (Suppl Fig 4.1A). Examination of RNA-*seq* analyses of *A. fumigatus* demonstrated that *erg25A* was the fifth highest abundant mRNA in normoxia with ~5500 RPKM values (Suppl Fig 4.1B). This number almost doubled after 36 hours exposure to hypoxia, validating our previous reports demonstrating that hypoxia exposure increased both *erg25A and erg25B* mRNA abundance (Blosser, S.J. *et al.*, Chapter 3). *GpdA*, however, produced ~3100 RPKM values in normoxia, and is not greatly induced in hypoxia, producing only about 33% as much mRNA as *erg25A* in our *in vitro* analyses

(Suppl Fig 4.1B). In order to express *erg25A* at wild-type levels in the Δ*srbA* background, we chose to construct a strain that would constitutively express *erg25A* with the previously uncharacterized promoter of the flavohemoglobin gene (AfuA_4g03410/Afub_099650). Flavohemoglobin is the highest expressed *A. fumigatus* mRNA according to our *in vitro* analyses with ~28,000 RPKM values reads in normoxia and ~16,600 reads after 36 hours exposure to hypoxia (Suppl Fig 4.1B).

Flavohemoglobins convert oxygen and nitric oxide to nitrate, and therefore behave as nitric oxide dehydrogenases (105). The flavohemoglobin family of proteins has been conserved among yeast, bacteria, and filamentous fungi (158, 239, 254, 323, 341), and two copies of the flavohemoglobin gene exist in the genome of *A. fumigatus*.

In *Aspergillus oryzae* and *Aspergillus nidulans*, flavohemoproteins are involved in scavenging reactive nitrogen species (273, 373). This suggests a role for flavohemoglobin proteins in protecting fungal cells from internal and external NO stress. Although flavohemoglobins have not been characterized in *A. fumigatus*, the flavohemoglobin we identified in our screens above responds transcriptionally when exposed to hypoxia, and flavohemoglobin protein accumulates in hypoxia (339).

Construction of a Constitutively Expressed *erg25A* in the SrbA-Null Mutant Background

The *pflavA-erg25A-*Δ*srbA* strain was constructed by random insertion of the *pflavA-erg25A* construct into the *-pyrG*Δ*srbA* strain. Strains were verified to have a single insertion by PCR and Southern Blot analysis (data not shown and Suppl Fig 4.2A, C, D). Insertion has no effect on normal growth parameters of the organism, as growth rates in

liquid and solid culture, as well as rates of sporulation are identical to a wild type strain. *PflavA-erg25A-*Δ*srbA* fully restores wild type *erg25A* mRNA abundance compared to Δ*srbA* (Suppl Fig 4.2B, p = 0.0002).

Restoration of *erg25A* mRNA Abundance in Δ*srbA*
Reduces 4,4-dimethyl fecosterol Accumulation

To validate that our restoration of *erg25A* mRNA abundance correlated with an increase in Erg25A enzymatic function, we subjected the Wild Type, Δ*srbA*, and *pflavA-erg25A-*Δ*srbA* to sterol profile analysis via GC-MS. The three main intermediates that contain a methylated C4 are eburicol, 4-methyl fecosterol, and 4,4-dimethyl fecosterol (4,4,14,24-tetramethyl cholesta-8,24(28)-dien-3β-ol; 4,24-dimethyl cholesta-8,24(28)-dien-3β-ol; and 4,4,24-trimethyl cholesta-8,24(28)-dien-3β-ol respectively). Restoration of *erg25A* mRNA diminished the accumulation of 4,4-dimethyl fecosterol in relation to accumulation observed in Δ*srbA*, but did not alter the accumulation of eburicol or 4-methyl fecosterol (Figure 4.2A, *$p<0.05$). This selective decrease does indicate that restoration of Erg25A is occurring in *pflavA-erg25A-*Δ*srbA*, but also highlights the importance of Erg25B in the complete removal of C4-methyl sterol intermediates. In addition, *pflavA-erg25A-*Δ*srbA* did not restore total ergosterol accumulation to wild type levels when compared with Δ*srbA* (Figure 4.2B). As SrbA also directly binds the promoter region of *erg25B*, it is plausible that restoration of both Erg25A and Erg25B in Δ*srbA* is required to remove the accumulation of eburicol and 4-methyl fecosterol, and restore wild type levels of total ergosterol.

Restoration of *erg25A* mRNA Abundance in Δ*srbA* Fully Restores Hypoxia Growth

The *pflavA-erg25A-*Δ*srbA* strain grows well at wild type levels in normoxia (Figure 4.3), however, a dramatic growth difference is observed in hypoxia for this strain. Whereas Δ*srbA* is unable to grow in hypoxia, essentially full restoration of growth in hypoxia is observed for *pflavA-erg25A-*Δ*srbA* (Figure 4.3). These data demonstrate quite clearly that reconstitution of *erg25A* mRNA abundance in the Δ*srbA* background is essential for the ability of Δ*srbA* to grow in hypoxia.

SrbA-Null Mutant Phenotypes not Associated with Toxic Diol Accumulation

Sterol intermediate buildup has been hypothesized to contribute to the sensitivity of fungi to azole antifungal drugs due to the accumulation of a 3,6-diol (159). Triazole treatment of Erg25-defective mutants in other fungi rescues lethality, possibly due to suppression of C4-methyl sterol buildup (103). Therefore we hypothesized that toxic sterol accumulation may also be playing a role in the inability of Δ*srbA* to grow and adapt to hypoxia. To test this hypothesis, we examined the sterol profiles of Wild Type, Δ*srbA*, and *pflavA-erg25A-*Δ*srbA* for the presence of 14α-methyl ergosta-8,24(28)-dien-3β,6α-diol accumulation (159, 161). This diol was not detectable in any of the examined strains, suggesting that accumulation of this 3,6-diol does not account for the lack of hypoxia growth in Δ*srbA*, or the remediation of hypoxia growth in *pflavA-erg25A-*Δ*srbA*.

Alterations in Sterol Intermediates Indicate Downstream Blockage in Δ*srbA* at *erg3* and *erg5*

Accumulation of episterol was especially pronounced in *pflavA*-*erg25A*-Δ*srbA* (Figure 4.4A), which is not unexpected, as SrbA directly binds the promoter regions of both *erg5* and *erg3B* and regulates their respective mRNA levels (Barker, B.M. *et al.*, in preparation; (36)). Although the ergosterol biosynthesis pathway between episterol and ergosterol is branched, allowing for three stepwise uses of the enzymes Erg5, Erg4 and Erg3, we have previously determined that alterations in the episterol-to-ergosterol transition occur with SMO mutants (Blosser, S.J. *et al.*, Chapter 3). Therefore, we hypothesize that the diminished levels of Erg5 and Erg3B, due to ineffective transcriptional activation, contribute to the hyperaccumulation of episterol in *pflavA*-*erg25A*-Δ*srbA*.

Interestingly, removal of a majority of C4-methyl sterol accumulation in *pflavA*-*erg25A*-Δ*srbA* enhanced accumulation of intermediates downstream of Erg25, highlighting the other enzymatic steps that are also directly regulated by SrbA. In particular the mechanisms that traverse from episterol to ergosterol via Erg5→Erg4→Erg3 and Erg3→Erg4→Erg5 but not Erg3/5→Erg4 were accumulated (5-dihydroergosterol, 22-dihydroergosterol, and ergostatetraenol respectively, Figure 4.4A), suggesting that this mechanism either is preferentially used in *A. fumigatus*, or that loss of *SrbA* transcriptional regulation alters the efficiency of this stepwise progression, perhaps due to a yet-uncharacterized stoichiometric requirement (219).

These two mechanisms that were preferentially induced versus wild type in *pflavA*-*erg25A*-Δ*srbA* are the same pathways that are absent in Δ*srbA*, suggesting that lack of

this mechanism in Δ*srbA* is directly related to loss of Erg25A function. These findings also confirm prior observations that Erg25A is involved in the episterol-to-ergosterol transition, as an Erg25A-null mutant has decreased accumulation of these intermediates (Blosser, S.J. *et al.*, Chapter 3).

We previously documented accumulation of 9(11)-dehydroergosterol, an alternative sterol intermediated postulated to be the end product when the intermediate parkeol is present in the place of lanosterol (Blosser, S.J. *et al*. Chapter 3). Elevated levels of 9(11)-dehydroergosterol were observed in *pflavA*-*erg25A*-Δ*srbA* when compared to both wild type and Δ*srbA* (Figure 4.4B, *$p<0.05$). Although parkeol accumulation was not statistically increased in *pflavA*-*erg25A*-Δ*srbA* due to biological variation, a trend towards increased accumulation of this intermediate was observed. Overall, these data suggest an important role for Erg25B in removal of this intermediate, as remediation of *erg25A* via *pflavA*-*erg25A*-Δ*srbA* exacerbated the accumulation of this atypical intermediate. Most importantly, these results reveal a substrate-specificity for downstream ergosterol biosynthesis enzymatic action, and a role for *A. fumigatus* paralogs in the multiple layers of regulatory control for this critical pathway.

Stress Adaptation of *pflavA*-*erg25A*-Δ*srbA* Altered

As we have previously described a role for SMOs in adaptation to various environmental stresses, we next examined how restoration of *erg25A* in Δ*srbA* affected other biologically relevant stresses, such as triazole antifungal drugs, reactive oxygen species, cell wall perturbation, and low iron.

Alterations in mRNA Abundance in *pflavA-erg25A-*Δ*srbA*: Little is known about the regulation of ergosterol biosynthesis in *A. fumigatus*, especially the impact of downstream intermediates on potential feedback mechanisms. In *S. pombe*, ergosterol is the primary biofeedback cue, inducing cleavage of Sre1 (32). In addition to activation of sterol-specific transcription factors, ergosterol levels may alter individual steps in ergosterol biosynthesis, as ergosterol interacts directly with several of the intermediates in *S. cerevisiae* (189). Although extensive characterization of ergosterol feedback has been documented in *S. cerevisiae* (189), there are dramatic differences in the pathways of these fungi, making direct comparison difficult. Therefore, we examined the effect that restoration of *erg25A* via *pflavA-erg25A-*Δ*srbA* has on the mRNA abundance of other SrbA-regulated ergosterol biosynthesis genes.

Previously we have characterized the transcriptional relationship between SrbA and *erg11A* (36, 37). As Erg11 functions upstream of Erg25, we hypothesized that there would be no change in expression of *erg11A* or *erg11B* mRNA in *pflavA-erg25A-*Δ*srbA* when compared with Δ*srbA*. Contrary to our hypothesis, both *erg11A* and *erg11B* mRNA abundance was induced in *pflavA-erg25A-*Δ*srbA* compared to Δ*srbA* (Figure 4.5A, $p<0.0001$). Interestingly, *erg11B* but not *erg11A* mRNA abundance was restored to wild type levels in *pflavA-erg25A-*Δ*srbA* (Figure 4.5A). These data suggest that there may be significant feedback in the ergosterol biosynthesis pathway involving Erg25A, possibly affected by differences in sterol intermediate accumulation. Overall this induction is not equal among all SrbA-regulated ergosterol biosynthetic transcripts.

As both *erg11A* and *erg11B* mRNA was substantially increased in *pflavA*-*erg25A*-Δ*srbA*, we hypothesized that the triazole sensitivity of Δ*srbA* may be partially restored. Voriconazole (VCZ) and Fluconazole (FLC) Etest analyses of *pflavA*-*erg25A*-Δ*srbA* demonstrated a significant recovery of triazole resistance, almost completely restoring minimum inhibitory concentrations (MICs) to wild type levels in both normoxia and hypoxia (Figure 4.5B, Δ*srbA* - VCZ = 0.004μg/mL, FLC = 1.0μg/mL; *pniiA*-*erg25A*-Δ*srbA* - VCZ = 0.064-0.094μg/mL, FLC = >256μg/mL). These data illustrate the need to understand the complex intricacies of ergosterol biosynthesis in *A. fumigatus*. Clearly, we have a limited understanding of the feedback mechanisms involved in sterol biosynthesis in *A. fumigatus*, including any secondary roles for the intermediates themselves as signaling molecules.

Iron Adaptation-Deficiencies of Δ*srbA* Repaired in *pflavA*-*erg25A*-Δ*srbA*: As Erg25 is a di-iron di-oxygen requiring enzyme, it is especially paramount to examine how restoration of *erg25A* impacts iron adaptation abilities of Δ*srbA*. Previously, we have demonstrated a link between iron homeostasis and hypoxia adaptation, through the interplay between the transcription factors SrbA, HapX, and SreA (36). Deletion of both SrbA and SreA renders a hyperaccumulation of intracellular iron, allowing for better adaptation to low-iron environments, and partially restores the ability of Δ*srbA* to grow in hypoxia (36). These data suggest that Δ*srbA* cells are also iron deficient due to inefficient iron uptake.

Importantly, growth in iron-depleted conditions is restored in *pflavA*-*erg25A*-Δ*srbA* equivalent to wild type levels (Figure 4.6A, *$p<0.0001$). Previous findings have

suggested that carrying two fully-functional SMOs supply an adaptive cost to *A. fumigatus* via organismal iron requirements (Blosser, S.J. *et al*., Chapter 3). When Δ*erg25A* or Δ*erg25B* were grown in depleted iron conditions their biomass was significantly increased compared with wild type, suggesting that alleviating *A. fumigatus* of a single SMO frees up some of the iron requirements of the cell. This may be particularly significant in Δ*srbA* cells that are iron deficient. Alternatively, iron transporters could be activated in *pflavA*-*erg25A*-Δ*srbA* via sterol intermediate signaling.

Reactive Oxygen Species Sensitivity in Hypoxia Reveals Internal Defect in *pflavA*-*erg25A*-Δ*srbA*: Erg25A has also been shown to be critical for the adaptation of *A. fumigatus* to reactive oxygen species (ROS), as exposure of Δ*erg25A* to hydrogen peroxide was more lethal than to a wild type control (Blosser, S.J., Chapter 3), which has also been observed for Δ*srbA*. As a link between hypoxia adaptation and the production of intracellular ROS has been described in mammals and yeast (30, 81, 256), we also were interested in examining the effect of hypoxia on ROS-adaptation.

We therefore examined the effect of hydrogen peroxide on *pflavA*-*erg25A*-Δ*srbA* under both normoxia and hypoxia conditions. As seen previously, Δ*srbA* was slightly inhibited in its ability to compensate for ROS in normoxia, and was unable to grow in hypoxia (Figure 4.6B). The wild type was more resistant to ROS in hypoxia (Figure 4.6B, *$p<0.001$), suggesting that the cell is more capable of compensating for ROS when in a low oxygen environment. This phenomenon is also observed in *S. cerevisiae* (81). Surprisingly, *pflavA*-*erg25A*-Δ*srbA* is fully resistant to ROS in normoxia, but has an

increased susceptibility to ROS in hypoxia (Figure 4.6B, p = 0.008) when compared with the wild type response. This susceptibility to ROS in hypoxia may be due to substantial intracellular ROS accumulation in the *pflavA-erg25A-*Δ*srbA* strain (75, 133, 311), as *yap1* mRNA was highly induced in this strain in hypoxia when compared with the wild type *A. fumigatus* (Figure 4.6C). These results suggest that although *pflavA-erg25A-*Δ*srbA* is restored in its ability to grow in hypoxia, and resist ROS in normoxia, that other components of its intracellular signaling or architecture are not functioning optimally.

Cell Wall Defects in β-glucan/Chitin Assembly Affect Sensitivity to Congo Red: Adaptation to ROS in fungi can be multifaceted, and genetic deletion mutants sensitive to ROS may also have sensitivities to cell wall stress (147), primarily because a structurally sound membrane is required to maintain signal transduction via membrane-bound receptors. As *pflavA-erg25A-*Δ*srbA* was more sensitive to ROS in hypoxia, we postulated that this phenotype could be due to defects in the cell wall structure. We subjected strains to stress by Congo Red (CR), a cell wall perturbation agent that affects the connection between β-glucan and chitin polymers. Wild type *A. fumigatus* is slightly inhibited by CR and is inhibited equally in both hypoxia and normoxia (Figure 4.7A). Likewise, Δ*srbA* and *pflavA-erg25A-*Δ*srbA* are slightly inhibited by CR when compared to –CR conditions.

Strikingly, *pflavA-erg25A-*Δ*srbA* has a significant growth defect on CR in hypoxia (Figure 4.7A). Comparing rates of growth on CR in normoxia and hypoxia we observe that *pflavA-erg25A-*Δ*srbA* is more inhibited in hypoxia than in normoxia (Figure 4.7B, *p<0.0001), suggesting that although hypoxia growth is restored in this strain, that the

restoration of Erg25A in *pflavA-erg25A-*Δ*srbA* reveals an SrbA-dependent underlying cell wall integrity issue.

Defect in Map Kinase Signaling in *pflavA-erg25A-*Δ*srbA*: Due to the observed defects of *pflavA-erg25A-*Δ*srbA* to adapt to cell wall perturbation in hypoxia, we hypothesized that this could be due to alterations in "stress" signal transduction. Mitogen-activated protein (MAP)-kinases are integral to stress adaptation, as they serve as the terminal component of a kinase cascade, and interact directly with transcription factors to alter cell response. Four MAP-kinases have been identified in *A. fumigatus*, *mpkA, mpkB, mpkC*, and *sakA* (202, 364), and they are integral components of the cell wall integrity (CWI) pathway, response to heat and reactive oxygen species, and nutrient sensing. Significantly, *pflavA-erg25A-*Δ*srbA* has diminished *mpkA* and *mpkC* mRNA abundance when in hypoxia compared with wild type or Δ*srbA* (Figure 4.7C, *$p<0.0001$). This finding is of interest, as these two transcripts are typically induced in hypoxia (Barker, B.M. *et al*., in preparation). On the contrary, *mpkB* mRNA abundance was partially restored in *pflavA-erg25A-*Δ*srbA* compared with Δ*srbA*, and no significant change was observed for *sakA* (Figure 4.7C, *$p<0.0001$). It is therefore plausible that *pflavA-erg25A-*Δ*srbA* is unable to accurately signal a breach of cell wall integrity in hypoxia, due to diminished activation of *mpkA* or *mpkC*.

Discussion

The purpose of this study was to examine the contribution of C4-methyl sterol accumulation on the physiology of Δ*srbA*. We previously determined that alleviation of the ergosterol biosynthesis blockage at *erg11* restores the triazole susceptibility of Δ*srbA*, but does not restore total ergosterol levels and induces a hyperaccumulation of C4-methyl sterols compared with a wild type strain. This hyper accumulation of C4-methyl sterols indicates a blockage at the Erg25-step of ergosterol biosynthesis. As SrbA directly binds upstream of *erg25A* (36), we concluded that the loss of *Erg25A* in Δ*srbA* is due to the loss of transcriptional activation of *erg25A* by SrbA. Due to the importance of ergosterol in maintenance of the membrane stability and preliminary data suggesting that *erg25A* was highly induced in hypoxia, we hypothesized that the accumulation of C4-methyl sterols in Δ*srbA* inhibited this mutant's ability to grow in hypoxia.

The inability of Δ*srbA* to grow in low oxygen environments may be explained by one of two hypotheses. First, that hyperaccumulation of C4-methyl sterol intermediates inhibits a cellular checkpoint, such as proliferation, disallowing normal growth in hypoxia. Second, that accumulation of C4-methyl sterols destabilizes the plasma membrane, and thus is unable to grow in hypoxia.

The membrane of Δ*srbA* is not unstable in normoxia, *per se*, however, we do observe a requirement for increased C4-methyl sterol oxidase activity in sterol biosynthesis in hypoxia (Figure 4.1B), as C4-methyl sterol intermediates have increased accumulation in hypoxia sterol profiles. Additionally, ergosterol biosynthesis gene mRNA levels are increased in hypoxia, and *erg25A* is one of the transcripts that is quickly increased (and

most abundant) when *A. fumigatus* is shifted from normoxia to hypoxia (23). It is plausible, therefore, that hyper accumulation of C4-methyl sterol intermediates is deleterious to adaptation to hypoxia, but has no effect on normal growth in normoxia. Another possibility from these findings is that C4-methyl sterols are modified somehow in hypoxia, not normoxia, and the modified C4-methyl sterol prohibits growth in hypoxia. Previous work in *C. neoformans* also suggested a critical role for C4-methyl sterols in the inability of the Sre1-null mutant to grow in hypoxia, suggesting that this mechanism may be conserved in fungi (180).

To test the hypothesis that hyperaccumulation of C4-methyl sterol intermediates contributes to the inability of Δ*srbA* to grow in hypoxia, we sought to restore wild-type levels of *erg25A* mRNA in Δ*srbA* using the most commonly used constitutive promoter in *A. fumigatus, gpdA.* However, repeated attempts to restore *erg25A* mRNA levels using the *gpdA* promoter were unsuccessful. While the mechanism is not clear, it may be possible that SrbA indirectly regulates *gpdA* expression. Thus, we utilized RNA-*seq* data to identify a new non-SrbA dependent promoter. We chose to utilize the promoter region of a putative flavohemoglobin gene (Suppl Fig 4.2) (372). This system was capable of restoring *erg25A* mRNA levels in Δ*srbA* in the conditions tested (Suppl Fig 4.2).

Importantly, *pflavA*-*erg25A*-Δ*srbA* completely restored the ability of Δ*srbA* to grow in hypoxia (Figure 4.3). This restoration of growth was accompanied by a decrease in 4,4-dimethyl fecosterol accumulation, indicating that Erg25A was functioning in *pflavA*-*erg25A*-Δ*srbA* (Figure 4.2A). Interestingly, no change was observed for the 4-methyl sterol intermediates 4-methyl fecosterol and eburicol, in *pflavA*-*erg25A*-Δ*srbA* versus

Δ*srbA*, suggesting a separate function for Erg25B in sterol methyl oxidation in *A. fumigatus* (Figure 4.2A).

Previously we have described single *erg25* genetic null mutants in *A. fumigatus* (Blosser, *et al*., Chapter 3). These null mutants differed substantially in the accumulation of 9(11)-dehydroergosterol, an uncharacterized sterol intermediate that may function in alternative ergosterol biosynthesis mechanisms, and possibly in production of sterol intermediate peroxides (66). Although Δ*erg25A* and Δ*srbA* accumulated significantly more 9(11)-dehydroergosterol than wild type *A. fumigatus*, Δ*erg25B* accumulated substantially more of this intermediate. Conversely, *pflavA*-*erg25A*-Δ*srbA* accumulates appreciable levels of 9(11)-dehydroergosterol to Δ*erg25B* (Figure 4.4B and Chapter 3), suggesting a potential function for Erg25B in tempering production of 9(11)-dehydroergosterol. If 9(11)-dehydroergosterol is converted into a peroxide in *A. fumigatus*, then perhaps the dual SMOs found in *A. fumigatus* serve a more complex role in regulation of ergosterol-byproducts.

The abundance of episterol accumulated in *pflavA*-*erg25A*-Δ*srbA* suggests another downstream blockage in the ergosterol biosynthesis pathway of Δ*srbA* (Figure 4.4A). As we have putatively identified SrbA-binding sites of the promoter regions of *erg5* and *erg3B*, it is highly plausible that defects in these enzymatic steps manifest as an accumulation of episterol (23). It is likely that this blockage is what does not allow for the restoration of total ergosterol in *pflavA*-*erg25A*-Δ*srbA* (Figure 4.2B). However, the full restoration of hypoxic growth of *pflavA*-*erg25A*-Δ*srbA* despite the persistent

reduction in total ergosterol strongly suggests that ergosterol levels *per se* do not influence hypoxic growth of *A. fumigatus.*

The sterol intermediate 5-dihydroergosterol is also restored in *pflavA-erg25A-*Δ*srbA*, which was absent in Δ*srbA* (Figure 4.4A). Although the specificity of these episterol-to-ergosterol intermediates has not been validated biochemically, we have previously observed substrate specificity in the accumulation of episterol-to-ergosterol intermediates with Δ*erg25A* and Δ*erg25B.* This suggests that enzymatic action of either SMO could contribute to directionality in the flow of intermediates between episterol-to-ergosterol.

The SrbA-null mutant is also sensitive to the triazole antifungal agents Fluconazole (FLC) and Voriconazole (VCZ), and this has been attributed to a direct transcriptional relationship between *erg11A* and SrbA (37). Therefore, it was unexpected to observe a substantial restoration of triazole resistance in *pflavA-erg25A-*Δ*srbA* to wild type levels (Figure 4.5B). The increased accumulation of *erg11A* mRNA abundance in *pflavA-erg25A-*Δ*srbA*, however, revealed reveals a complex interplay between downstream sterol intermediates and/or proteins was affecting the upstream transcription of key ergosterol biosynthesis genes (Figure 4.5A). These findings suggest that the accumulation of 4,4-dimethyl fecosterol has an inhibitory function on transcriptional regulation of other ergosterol biosynthesis genes.

Iron homeostasis and hypoxia adaptation are intimately linked pathways in *A. fumigatus*. Not only do the major factors regulating iron uptake and hypoxia adaptation regulate each other, but their functions overlap significantly in the regulation and control of ergosterol biosynthesis. Key enzymes in ergosterol biosynthesis require both oxygen

and iron, and intricate layers of regulation ensure that this pathway is maintained during iron- or oxygen- depleted conditions. It is perhaps not surprising, then, that restoration of hypoxia growth with *pflavA*-*erg25A*-Δ*srbA* would be accompanied by a restoration in growth under iron-depleted conditions (Figure 4.6A). It is also possible, that accumulation of 4,4-dimethyl fecosterol inhibits the iron transport machinery on the level of transcription, which would explain the iron uptake deficiency observed in Δ*srbA*. Evidence suggesting a secondary role for 4-methyl sterols in the regulation of transcription will be discussed in further detail below.

What was perhaps, atypical, or unexpected, was that restoration of hypoxia growth would be coupled with an increased sensitivity to reactive oxygen species in *pflavA*-*erg25A*-Δ*srbA* (Figure 4.6B). Intracellular accumulation of ROS activates the fungal transcription factor Yap1. Genetic deletion of *yap1* in *A. fumigatus* confers sensitivity to H_2O_2 and menadione (183), whereas a hyperactive *yap1* strain gained resistance to ROS-inducing chemicals (258). More recently, *yap1* has been identified as a critical component in defending against neutrophil-mediated attack in a corneal infection model of *A. fumigatus* (178). These studies indicate that activation of *yap1* is critical for combating ROS. Interestingly, in *pflavA*-*erg25A*-Δ*srbA* we observed a significant increase in *yap1* mRNA abundance after 4 hours in hypoxia (Figure 4.6C). These results directly opposed those of Δ*srbA*, which displayed significantly diminished levels of *yap1* when encountering hypoxia (Figure 4.6C). Therefore, *pflavA*-*erg25A*-Δ*srbA* appears to be either hypersensitive to or have increased accumulation of intracellular ROS in hypoxia.

Application of external ROS via H_2O_2 assay, therefore, overwhelmed the ROS-response, rendering *pflavA-erg25A-*Δ*srbA* hypersensitive (Figure 4.6B).

The concept of transcription-factor regulation of ROS-homeostasis is not limited to Yap1 and homologues, as the hypoxia-responsive transcription factor Rox1 also has a role in regulation of ROS status in *S. cerevisiae* (191). It is especially interesting, then, to see the disparity between *pflavA-erg25A-*Δ*srbA* and Δ*srbA yap1* expression in response to hypoxia. Alternatively, this increase in *yap1* could be due to ineffective signaling through MpkA and/or MpkC (discussed below). Future studies will be needed to determine the interplay between SrbA and C4-methyl sterols potentially involved in intracellular ROS-homeostasis.

The sensitivity of *pflavA-erg25A-ΔsrbA* to cell wall perturbation in hypoxia could be attributed to several factors including: alterations in map kinase signaling due to membrane instability, decreased membrane stability resulting in loss of cell wall structure, or potential alterations in non-ergosterol Erg25-interactions. Map kinase signaling has been substantially linked to cell wall and membrane homeostasis, as several of the MAP kinase gene deletion mutants are more sensitive to cell wall perturbation agents such as calcafluor white, congo red, caffeine, high temperature, osmo stress, and reactive oxygen species. In particular, the HOG-homologues in *A. fumigatus*, SakA and MpkC, play important roles in oxidative stress responses and nutrient change (263), and MpkC1 is induced by Congo Red in the pathogenic yeast *C. albicans* (271). However, Δ*mpkC* in *A. fumigatus* is not inhibited by levels of CR reported in the literature (164).

Conversely, the Mpk1-associated cell integrity pathway alters cell wall composition through the cell cycle and is responsive to numerous types of stress (329). MpkA is a critical component of the cell wall integrity pathway, and Δ*mpkA* demonstrates significant growth defects on the cell wall perturbation agent Congo Red (330). Additionally, MpkA-signaling interfaces with the cell wall integrity pathway and iron homeostasis, and is thus at least tangentially regulating alterations in ergosterol biosynthesis (147). As *pflavA-erg25A-*Δ*srbA* was both hypersensitive to Congo Red in hypoxia, and demonstrated decreased mRNA abundance of both *mpkA* and *mpkC*, it is plausible that a hypoxic-specific factor is not allowing adaptation to cell wall perturbation agents, and that this may be mediated through MpkA and/or MpkC defects. Conversely, it is possible that 4-methyl fecosterol levels in *pflavA-erg25A-*Δ*srbA* prohibit transcriptional activation of components of the ROS or map kinase response, as 4-methyl fecosterol levels in *pflavA-erg25A-*Δ*srbA* did not differ from Δ*srbA*. Additional experimentation would be required to definitively demonstrate the decreased activation of these MAP kinases and a possible mechanism for this loss.

Interestingly, Δ*mpkA* produces approximately 50% more siderophores than wild type *A. fumigatus* under iron-depleted conditions, and low iron is known to activate mpkA (147). This relationship with iron uptake, however, is independent of the iron-homeostasis transcriptional regulators HapX or SreA (147). These results suggest that when *A. fumigatus* encounters low iron, MpkA functions to decrease the siderophore production in an HapX- and SreA-independent manner.

This relationship between low iron levels and MpkA activation is also observed in Δ*hapX* and Δ*sreA*, where MpkA is less active in the former, and hyper-active in the later mutant (147). Not surprisingly, then, Δ*hapX* and Δ*sreA* also are more sensitive to Congo Red, connecting iron levels to the cell wall integrity pathway, and thus to MpkA (147). Unlike Δ*mpkA*, however, *pflavA-erg25A-*Δ*srbA* there is a difference in growth in iron replete and iron depleted conditions, although growth is much improved in low iron. Interestingly, the *A. nidulans* Δ*yap1* and Δ*mpkA* are sensitive to the quorum-sensing molecule farnesol, suggesting some interplay between PKA/MAPK signaling and the Yap1 response (61).

While we did not directly measure membrane integrity in the different strains, it is plausible that alterations in the sterol content of the membrane and sterol rich domains could influence the responses to drugs, iron, ROS, and cell wall stress agents. However, our data strongly suggest that C4-methyl sterols have a potential signaling role in *A. fumigatus*. This has been demonstrated in humans, where C4-methyl sterols regulate meiosis in the testis and ovaries, induce maturation, and act as ligands for nuclear receptors involved in cytokine production, and the coordination of inflammation and cholesterol biosynthesis (53, 148). In this system, C4-methyl sterols act as a ligand for the orphan receptor LXRα (liver X receptor α), and mediate cholesterol efflux, influence cellular redistribution of sterols, and impact fatty acid biosynthesis (182, 213, 262, 266, 281)**.** Although fungi lack nuclear receptor homologs, members of the zinc cluster of transcription factors are postulated to serve an analogous function (225). Therefore, it is plausible that C4-methyl sterols act as signalling molecules in *A. fumgiatus*, and that the

accumulation and dysregulation of these intermediates is directly related to the phenotypes observed in Δ*srbA*. In conclusion, more studies are required to determine the effect of these C4-methyl sterols on plasma membrane stability, synthesis, and coordination of appropriate stress responses in *A. fumigatus*

Materials and Methods

Strains and Media

Aspergillus fumigatus strain CEA17 was used to construct the *srbA* null-mutant strain as described previously (359).

The *pflavA-erg25A*-Δ*srbA* strains were constructed by PCR amplifying 1kb upstream of the flavohemoglobin ORF (AfuA_4g03410/Afub_099650), the *pyrG* gene from *A. parasiticus*, and the ORF of *erg25A*, then parsing together via fusion PCR. This *pflavA-erg25A* construct was transformed into the *–pyrG*Δ*srbA* mutant background via protoplast-mediated transformation (37).

Strains were verified via Southern Blot for single, locus-specific integration, with the exception of the *pflavA-erg25A*-Δ*srbA* strains, which contain a single, random insertion of the *pflavA-erg25A* construct into the *–pyrG*Δ*srbA* background. The wild-type strain referred to in this article is strain CBS 144.89. All strains are routinely grown on glucose minimal media (GMM) that contains 1% glucose, at 37°C (289).

Quantitative Real-Time PCR

Aspergillus fumigatus strains were cultured in liquid media (glucose-minimal-media) for sixteen hours, then shifted to hypoxia for the indicated times. Mycelia were harvested via vacuum filtration and lyophilized overnight prior to homogenation with 0.1mm glass beads. Total RNA was extracted using TRisure (Bioline) according to the manufacturer's instruction and purified via RNeasy column protocol (Qiagen). Genomic DNA purification was completed with Turbo DNAse I (Ambion). A secondary genomic DNA purification was done with the Qiagen QuantiTect Reverse Transcription Kit (Qiagen), as well as oligo-DT-primed cDNA synthesis. qRT-PCR was conducted in technical duplicates except where noted. The normalized fold expression graphed in each figure represents the mean and percent error of three biological replicates as normalized to the housekeeping gene *tefA*. A no-template mRNA control was used to ensure no gDNA contamination in each analysis.

Cell Wall Perturbation Agents

The cell wall synthesis inhibitor Congo Red (CR, Sigma Aldrich) was utilized to determine alterations in cell wall integrity in both normoxia (17% O_2) and hypoxia (1% O_2) of various strains. CR was added to GMM or GMM+UU solid media at a final concentration of 1mg/mL. 10^4 conidia of each strain were placed in the center of each plate. The radial growth at 37˚C in either normoxia or hypoxia was measured every 24 hours for 72 hours (GMM control) or 120 hours (GMM+CR). Data are represented as the average and standard deviation of three biological replicates. Photographs were taken at

72 hours for GMM or GMM+UU controls, and 120 hours form GMM+CR or GMM+UU+CR samples.

Antifungal Susceptibility Testing

Susceptibility to Fluconazole and Voriconazole was evaluated with Etest strips (AB Biodisk). Each strip delivers a concentration gradient of drug. Strips are placed on an RPMI-MOPS (Sigma Aldrich) plate, pH 7.0, containing 10^5 conidia. Growth inhibition was evaluated at 48 hours by visualization of growth inhibition when grown at 37°C in either normoxia (17% O_2) or hypoxia (1% O_2).

Hydrogen Peroxide Assay

Two hundred *Aspergillus fumigatus* conidia were plated onto a glucose minimal medium (GMM) plate overnight at 37˚C. Germlings were shocked with either 10mL 1.25mM hydrogen peroxide (Fisher Scientific) or sterile water for ten minutes at 37˚C, and then washed twice with 10mL of sterile water. Plates were incubated at 37˚C in either normoxia (17% O_2) or hypoxia (1% O_2) until colonies were visible, approximately 20 hours. Data are expressed as the ratio of the number of viable colonies on H_2O_2-treated versus untreated plates. Data are the average and standard deviation of three plates per strain, per condition.

Total Ergosterol Assay

Total ergosterol from *A. fumigatus* strains was extracted after 24 hours shaking growth in liquid GMM. Mycelia were harvested via vacuum filtration, dried, and weighed. 200mg of dry fungal tissue were extracted as described previously (9, 37).

Briefly, tissues were treated with 3mL of 25% alcoholic potassium hydroxide (3:2 methanol:ethanol) and then vortexed vigorously for 1 minute. Samples were incubated at 85°C for 1 hour. Sterols were extracted with 1mL of distilled water and 3mL of pentane and vortexed for 3 minutes. The upper aqueous layer was allowed to separate from the cellular debris and 1mL of this extract was transferred to a clean glass tube and evaporated in a fume hood at room temperature. Upon preparation for injection, samples were resolved in 1mL of methanol and syringe filtered through a 0.2μm-pore-size filter (Acrodisc, Waters). Total ergosterol was measured using a Shimadzu CLASS-VP HPLC and detected via a SPD-M10AVP PDA at 280nm on a Bondapack C18 column (300 mm × 3.9 mm, 10 μm). The quantity of ergosterol per strain was calculated from a standard curve of ergosterol (Acros Organics 117810250).

Sterol Profile

Mycelia were grown and harvested as described for total ergosterol extraction. Extraction was done as described previously (9, 37), and identically as in the total ergosterol extraction with the exception that the neutral lipids were extracted with 1.5mL hexane. The upper aqueous layer was separated from the cellular debris and 1mL of this extract was evaporated via nitrogen. Vials were stored at -20˚C until ready for injection. Samples were resolved in 100μL toluene and derivitized with 100μL BFSTA for 1 hour at 70°C. Sterols were measured via GC-MS, using a Shimadzu QP-2010 GC with a quadrupole mass ionizer. Peaks were identified via EI (electron ionization) fragmentation patterns. All data are relative to total ergosterol content.

Iron Stress Assay

10^8 *A. fumigatus* conidia from various strains were cultured in 150mL liquid media (glucose-minimal-media) with or without iron (ferrous sulfate heptahydrate, Fisher Scientific), for 24 hours. Following incubation, samples were harvested via vacuum filtration, frozen at -80°C, and lyophilized overnight. Samples were weighed, and the average and standard deviation of three or more biological replicates are reported. Data are represented as the ratio of growth without iron-to-iron.

Statistical Analysis

All results presented with statistical significance were analyzed with an unpaired two-tailed Student's *t*-test, with the exception of the virulence assay.

Acknowledgements

This work was supported by funding from NIH/NIAID grant RO1AI81838, NIH/COBRE grant RR020185, and NIH-NCRR. Sara Blosser was funded through an American Heart Association Predoctoral Fellowship for this work. The mass spectrometry facility is supported by NIH NCRR COBRE P20 RR024237.

We thank Dr. Ted White for review and suggestions pertaining to this project, and Drs. Brian Bothner and Jonathan Hilmer for assistance with mass-spectrometry.

Figures

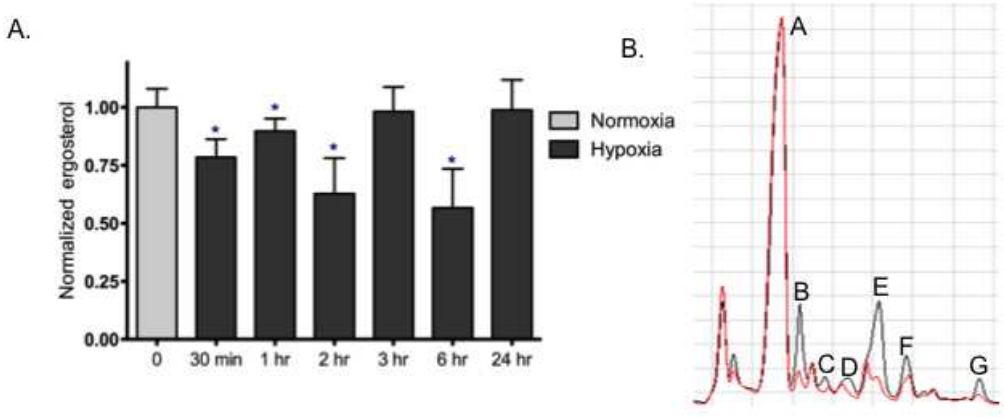


Figure 4.1 Hypoxia induces loss of total ergosterol and accumulation of intermediates at oxygen-requiring steps. A. Total ergosterol levels of wild type *A. fumigatus* at various times in hypoxia. *A. fumigatus* conidia were allowed to germinate, and then shifted to hypoxia for the given times. Following incubation in hypoxia, samples were harvested and extracted for total ergosterol. All values are normalized to total ergosterol in the normoxia control (0). *p<0.0.0001

B. Sterol profile of wild type *A. fumigatus* in normoxia (red) or hypoxia (black). A – ergosterol, B – 5-dihydroergosterol, C – 24-methyl cholesta-7,22,24(28)-trien-3β-ol, D – 22-dihydroergosterol; E – episterol; F – parkeol; G – 4,4-dimethyl fecosterol. All increased intermediates in hypoxia profile directly precede an oxygen-requiring step. Results are representative of two biological replicates per condition.

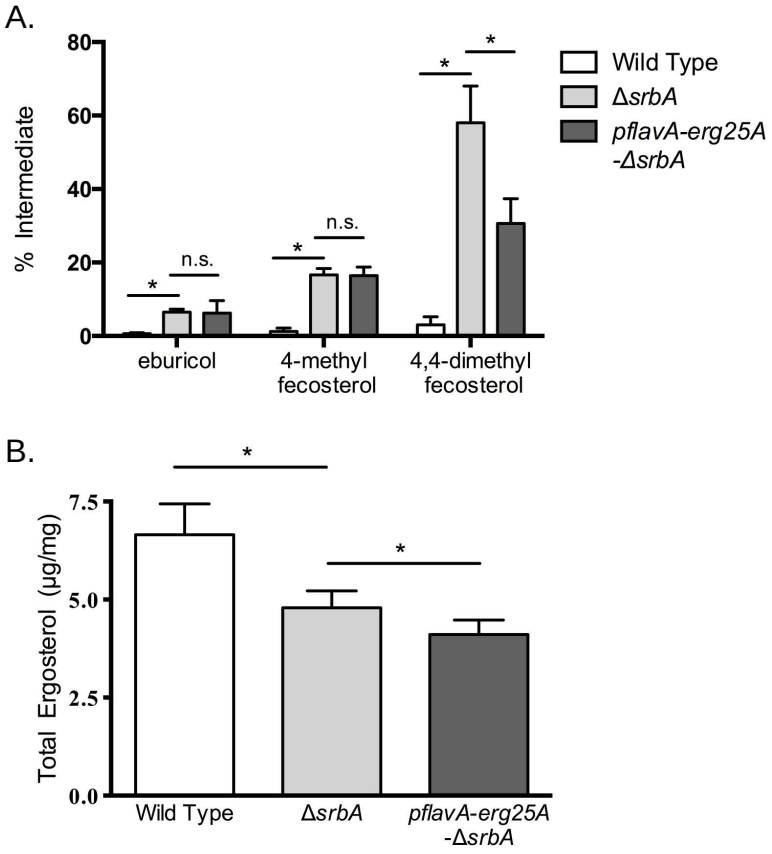


Figure 4.2 C4-methylated sterol intermediate accumulation decreases with restoration of *erg25A*. A. Sterol intermediates were identified and measured via GC-MS. Eburicol: 4,4,14,24-tetramethyl cholesta-8,24(28)-dien-3β-ol; 4-methyl fecosterol: 4,24-dimethyl cholesta-8,24(28)-dien-3β-ol; 4,4-dimethyl fecosterol: 4,4,24-trimethyl cholesta-8,24(28)-dien-3β-ol. Values are from 2-3 biological replicates per strain. *$p<0.05$, n.s. not significant. B. Total ergosterol was measured via HPLC, as described in the materials and methods. Values are from 3-4 biological replicates and 3 technical replicates per strain. *$p<0.0001$.

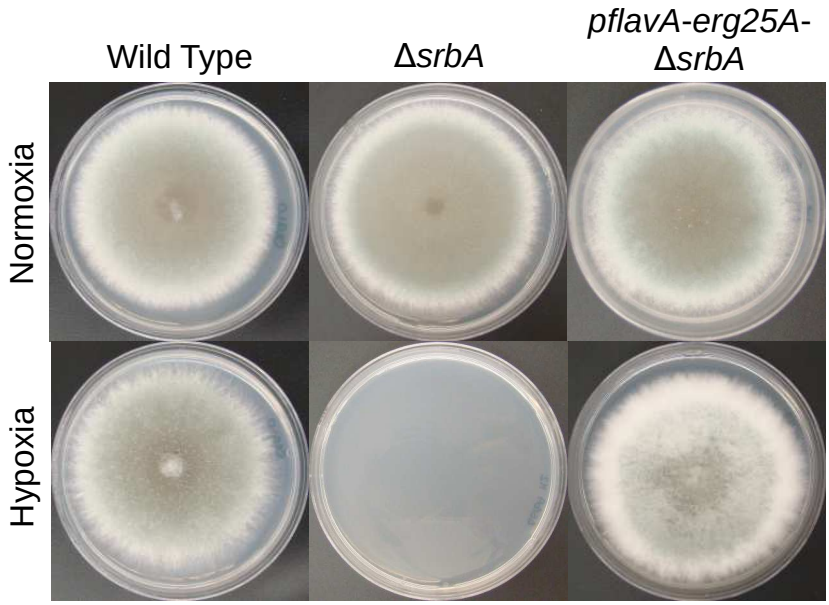


Figure 4.3 Restoration of *erg25A* recovers the ability of Δ*srbA* to grow in hypoxia. A total of 10^4 conidia was spotted on solid media (GMM) and allowed to grow at 37˚C for 96 hours in normoxia (17% O2) or hypoxia (1% O2). Plates are representative of three biological replicates per strain in each condition.

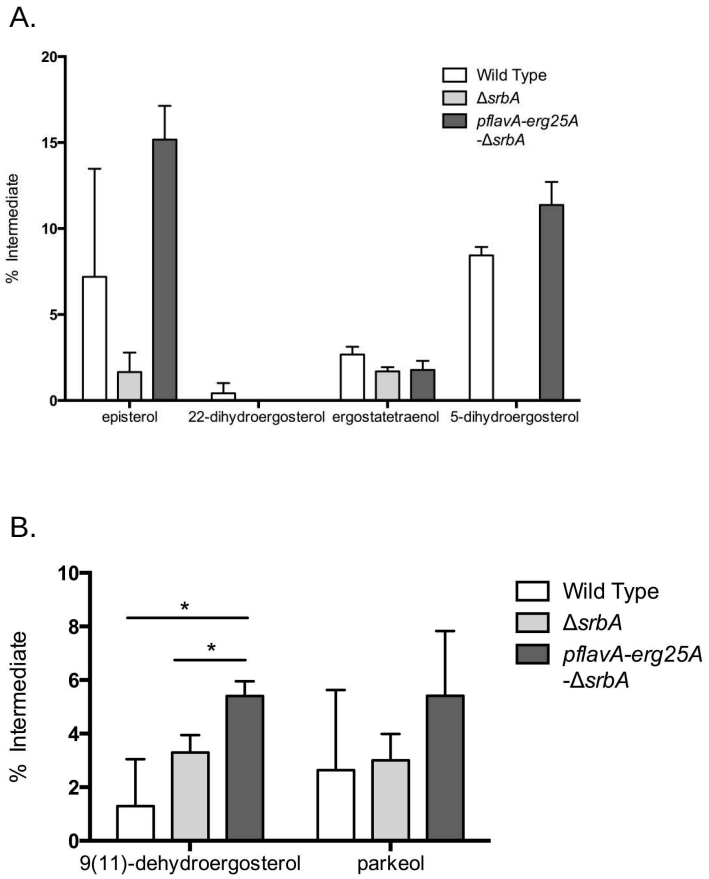


Figure 4.4 Sterol Intermediate Accumulation indicates Downstream Inhibition in Erg5/Erg3, and a role for Sterol Methyl Oxidase Substrate Specificity. A. Ergosterol sterol intermediate accumulation of episterol (24-methyl cholesta-7,24(28)-dien-3β-ol), 22-dihydroergosterol (24-methyl cholesta-7,24(28)-dien-3β-ol), ergostatetraenol (24-methyl cholesta-5,7,22,24(28)-tetraen-3β-ol), and 5-dihydroergosterol (24-methyl cholesta-7,22-dien-3β-ol). B. Ergosterol sterol intermediate accumulation of 9(11)-dehydroergosterol (24-methyl cholesta-5,7,9(11),22-tetraen-3β-ol) and parkeol (4,4,14-trimethyl-cholesta-9(11)-dien-3β-ol). Data represent the percent intermediate based on ergosterol, and are the average and standard deviation of 2-3 biological replicates. *$p<0.05$.

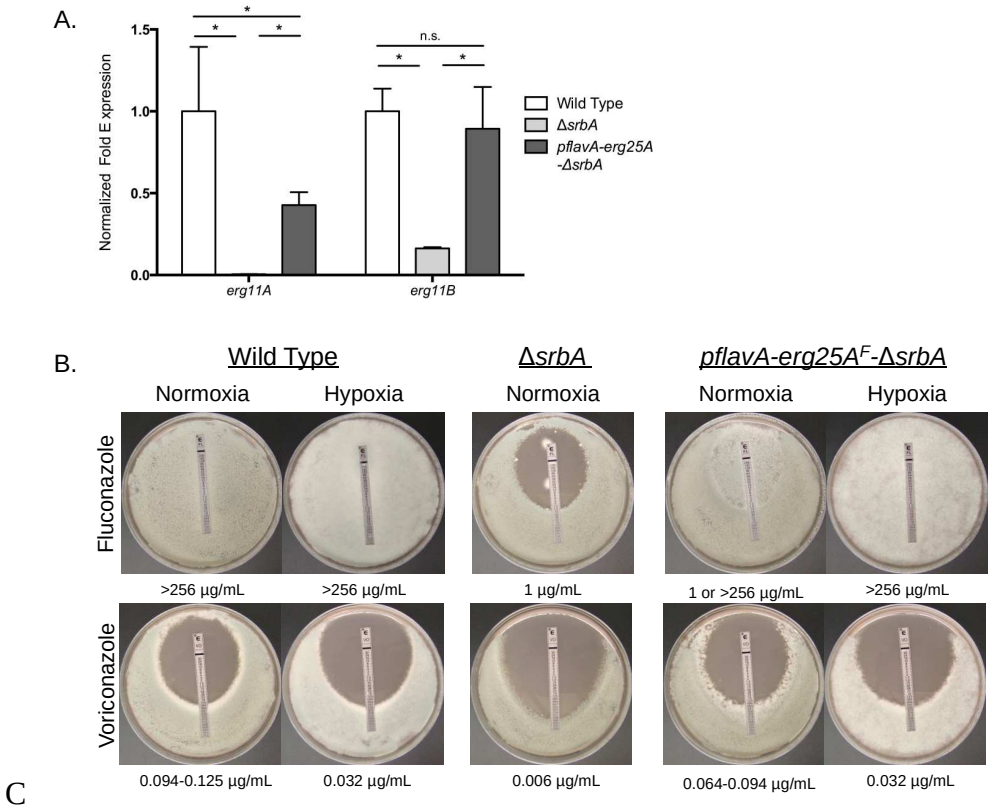


Figure 4.5 Remediation of *erg25A* in Δ*srbA* largely restores sensitivity of Δ*srbA* to triazole antifungal drugs. A. Quantitative Real-Time PCR of *pflavA-erg25A-*Δ*srbA* reveals increase in *erg11A/B* mRNA abundance. Conidia for wild type, Δ*srbA,* or *pflavA-erg25A-*Δ*srbA* were grown for 16 hours at 37˚C then transferred to hypoxia for 2 hours. *PflavA-erg25A-*Δ*srbA* levels of Ir*erg11A* and *erg11B* are higher than in Δ*srbA*. Data represent three biological and two technical replicates. mRNA abundance was normalized to the housekeeping gene *tefA* and is relative to the Wild Type strain. *p<0.001. B. Etest antifungal susceptibility testing for Wild type, Δ*srbA,* and *pflavA-erg25A-*Δ*srbA*. A clear ellipse indicates the susceptibility to fluconazole (FLC) or voriconazole (VCZ), with the exception of *pflavA-erg25A-*Δ*srbA*, which exhibits a hybrid phenotype. Top panel, *pflavA-erg25A-*Δ*srbA* appears to restore resistance of *A. fumigatus* to FLC, which is especially apparent in Hypoxia. Bottom panel, *pflavA-erg25A-*Δ*srbA* restores resistance of Δ*srbA* to voriconazole, but still may be slightly more susceptible than the Wild type strain (0.064-0.095µg/mL versus 0.095-0.125µg/mL respectively).

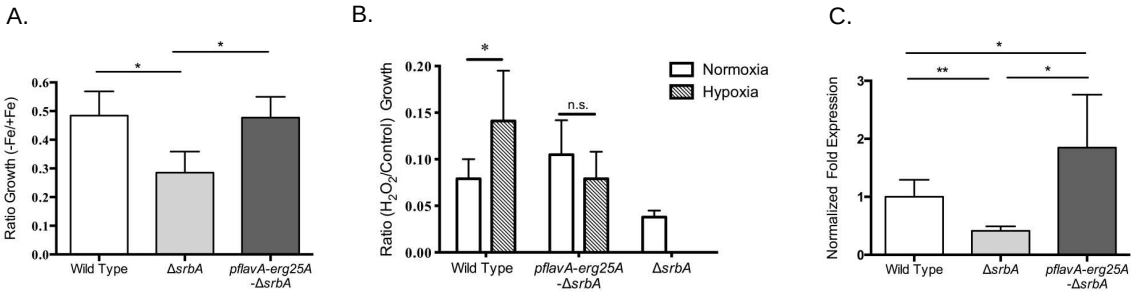


Figure 4.6 Adaptation to Reactive Oxygen Species (ROS) and Iron Altered in *pflavA-erg25A-*Δ*srbA.* A. Iron biomass assay in iron depleted and iron replete (30μM) conditions. Samples were grown for 24 hours at 37˚C. Biomass ratio is represented as the mass in iron depleted versus iron replete conditions. Data are the average and standard deviation of three biological replicates per strain. *p<0.05. B. ROS susceptibility of *pflavA-erg25A-*Δ*srbA* is altered in hypoxia (1% O_2), but not normoxia (17% O_2). To determine ROS susceptibility germlings were exposed to 1.25mM H2O2 for ten minutes at 37˚C, washed, and allowed to grow for 24 hours in either normoxia or hypoxia. Viability is evaluated compared to non-treated control. Ratios are expressed as the number of colonies on H_2O_2-treated versus control-plates after 24-hour incubation, and are the average and standard deviation of three biological replicates per strain per condition. A significant increase in resistance was observed for the Wild Type strain in hypoxia, which did not occur for *pflavA-erg25A-*Δ*srbA.* *p<0.05. No significant change in susceptibility was observed for *pflavA-erg25A-*Δ*srbA* in normoxia. C. *Yap1* mRNA is induced in *pflavA-erg25A-*Δ*srbA* during exposure to hypoxia. qRT-PCR of samples grown in normoxia for 16 hours at 37˚C, then transferred to hypoxia for 4 hours. Data are the average and percent error of three biological and two technical replicates. Data are normalized to the housekeeping gene *tefA* and are relative to wild type levels. *p<0.05, **p<0.0001.

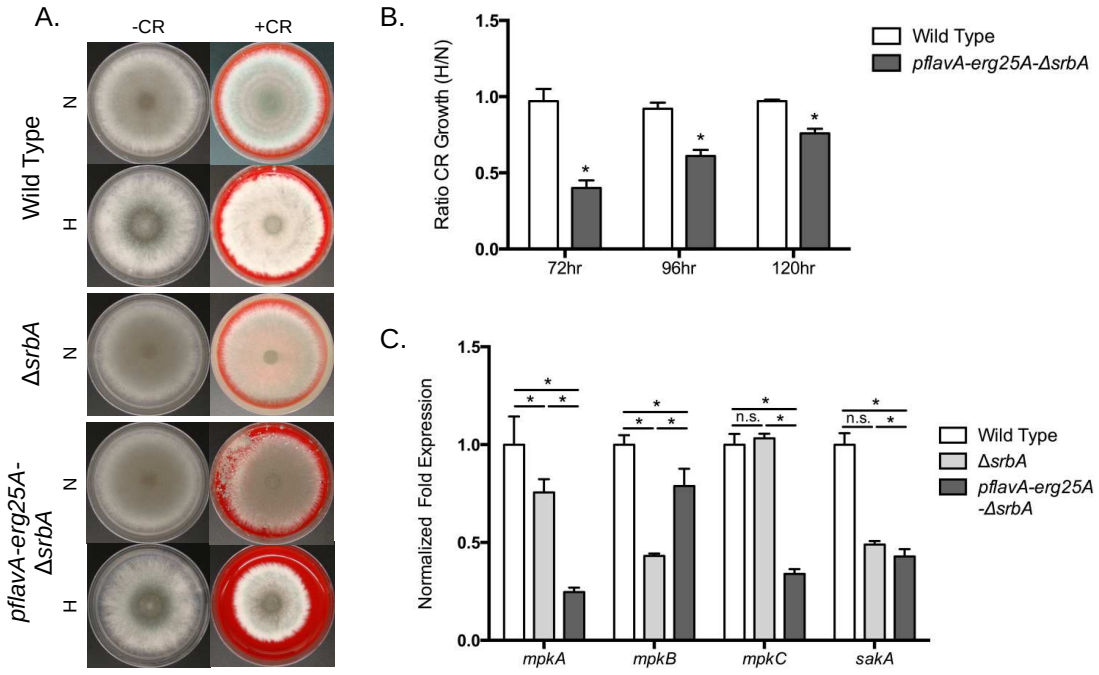
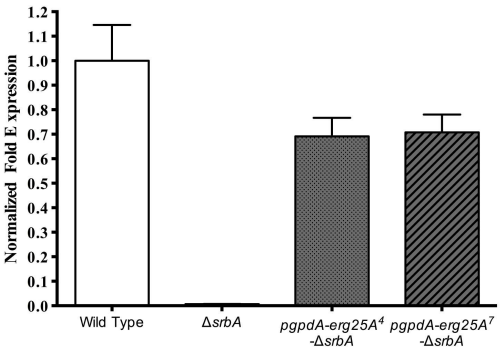


Figure 4.7 Modifying *erg25A* levels in Δ*srbA* illustrates cell wall defect in hypoxia. Cell wall defects were observed in *pflavA-erg25A-*Δ*srbA* when cultured on glucose minimum media (GMM) with Congo Red (CR) in hypoxia (H), but not normoxia (N). All strains spotted on either GMM or GMM+CR and incubated at 37˚C in either hypoxia or normoxia. A. Radial growth of all strains at 72hr for GMM or 120hr for GMM+CR conditions. 1mg/mL Congo Red utilized. Photos are representative of biological triplicates. B. Ratio of growth on CR grown in Hypoxia versus Normoxia. *PflavA-erg25A-*Δ*srbA* has a significant growth reduction in Hypoxia versus Normoxia. *P<0.0001. C. *MpkA* and *mpkC* mRNA diminished in *pflavA-erg25A-*Δ*srbA* during exposure to hypoxia compared to wild type and Δ*srbA*. qRT-PCR of samples grown in normoxia for 16 hours at 37˚C, then transferred to hypoxia for 4 hours. Data are the average and percent error of three biological and two technical replicates. Data are normalized to the housekeeping gene *tefA* and are relative to wild type levels. *p<0.05, **p<0.0001.

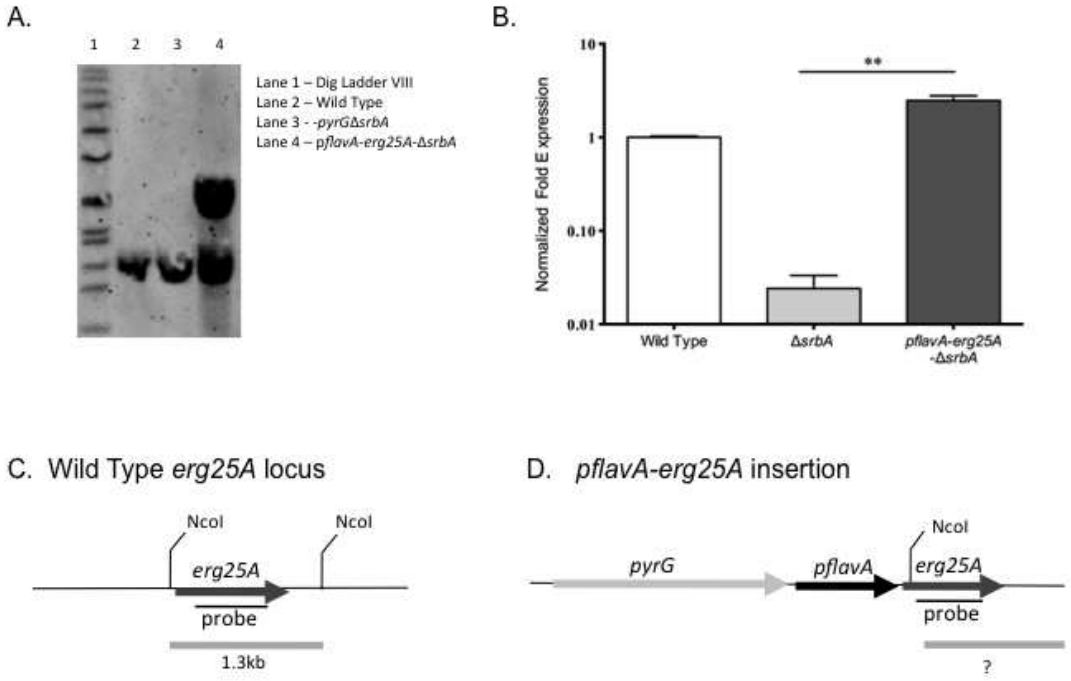
A.



B.

Afu ID	Function	T0	T12	T24	T36
AfuA_4g03410	flavohemoprotein	28452.06	29059.81	21297.62	16635.71
AfuA_8g02440	C4-sterol methyl oxidase, erg25A	5545.2	6193.64	6809.38	9137.25
AfuA_5g01970	Glyceraldehyde 3-phosphate dehyrdogenase, gpdA	3147.3	3010.89	2460.93	2960.82

Supplemental Figure 4.1 Inefficacy of *pgpdA* in restoring wild type *erg25A* mRNA abundance. A. Attempts to construct a *pgpdA-erg25A-*Δ*srbA* strain were ineffective, as transformants only expressed ~70% mRNA abundance of wild type under normoxic conditions. qRT-PCR was conducted on three biological and two technical replicates for the strains examined, and was normalized to the housekeeping gene *tefA*. All values are relative to the wild type *erg25A* mRNA abundance. B. Table of condensed RNA-*seq* results, Wild Type *A. fumigatus* subjected to hypoxia for 12, 24, or 48 hours (T12, T24, T48). Data are expressed as reads per sample, and are from three pooled biological replicates per time condition.



Supplemental Figure 4.2 Development of *pflavA* as a strong promoter system in *A. fumigatus* and Construction of *pflavA-erg25A-*Δ*srbA.* A. Southern blot to confirm number of insertions for *pflavA-erg25A-*Δ*srbA*. Genomic DNA was NcoI digested at 37˚C for 18 hours. Strains were probed with a 695-bp probe. All banding patterns were as expected for the various strains, with the exception for *pflavA-erg25A-*Δ*srbA,* which had the pattern expected for a single ectopic insertion of the *pflavA-erg25A* construct. B. qRT-PCR verification of *pflavA-erg25A-*Δ*srbA erg25A* mRNA expression. Each strain was grown in normoxia for 16 hours, and then shifted to hypoxia (1% O_2) for four hours. Three biological and two technical replicates of each strain were normalized to the housekeeping gene *tefA*. Data are normalized to *erg25A* mRNA abundance from the wild type strain. *p<0.05, **p<0.0001. C. Southern blot schematic for the native *erg25A* locus. D. Southern blot schematic for a random insertion of the *pflavA-erg25A* construct.

CHAPTER FIVE

CONCLUSIONS

Introduction

Ergosterol biosynthesis is a multi-step biochemical process for the production of the primary fungal sterol, ergosterol. Although this pathway has been characterized in the hemi-Ascomycete fungi *C. albicans*, and *S. cerevisiae*, many unanswered questions exist about this biochemical process in *A. fumigatus*. In particular, many of the genes in the ergosterol biosynthesis pathway in *A. fumigatus* have undergone a gene duplication event, which is not present in *C. albicans* or *S. cerevisiae* (231). Therefore, single genetic deletions of genes that were classically essential, such as *erg11* and *erg25*, are viable in this organism due to the presence of an alternative enzyme at these critical steps (138, 212) (Chapter 3).

Furthermore, the regulation of these ergosterol biosynthesis genes is strikingly different than in the hemi-Ascomycetes. In *S. cerevisiae* and *C. albicans* Upc2 and Ecm22, which are members of the zinc-cluster family of transcription factors, regulate sterol biosynthesis in hypoxia and normoxia respectively (292, 337). In *A. fumigatus*, SrbA, a member of the basic helix-loop-helix family of transcription factors, appears to be the primary sterol biosynthesis regulator in both normoxia and hypoxia (36, 359). Furthermore, SrbA is also a major hypoxic response regulator, as Δ*srbA* is unable to grow in low oxygen (36, 359). Interestingly, Upc2 appears to serve a similar function in *S.*

cerevisiae and *C. albicans*, but is assisted by Rox1 to regulate transcription in hypoxia (172, 292).

SrbA is involved in maintenance of polarity, triazole antifungal drug resistance, adaptation to hypoxia and low iron, and virulence in a murine model of Invasive Aspergillosis, as Δ*srbA* demonstrates significant phenotypes in these categories (36, 359). As a transcription factor, SrbA chiefly influences the transcription of many genes, including many involved in ergosterol biosynthesis (36, 37). In this thesis, I have demonstrated that two of the phenotypes observed in Δ*srbA*, triazole antifungal sensitivity and the inability to grow in hypoxia, are directly attributable to deficiencies in ergosterol biosynthesis (Chapters 2 & 4).

Summary of Findings

Initial transcriptional profiling of Δ*srbA* in hypoxia demonstrated significant decreases in *erg11A* and *erg11B* mRNA abundance, which encode the targets for the triazole antifungal drugs (Figure 2.2). Strikingly, these mRNAs, which increase in abundance in a culture treated with fluconazole (FLC) or voriconazole (VCZ), were significantly reduced in Δ*srbA* (Figures 2.1 and 2.3). This finding illustrated a potential mechanism for the triazole susceptibility of Δ*srbA*, whereby depletion of Erg11A and Erg11B, makes Δ*srbA* more susceptible to triazole antifungals, as the target is depleted. In support of this conclusion, restoration of *erg11A* mRNA levels in Δ*srbA* through use of the nitrate-inducible promoter (*niiA*) restored wild type levels of triazole susceptibility to Δ*srbA* (Figure 2.5). I also demonstrated that this relationship between SrbA and

erg11A is likely direct, as SrbA bound directly upstream of *erg11A*, in a region that contained a predicted binding motif (Figure 2.6). Interestingly, restoration of *erg11A* mRNA via *pniiA-erg11A-*Δ*srbA* did not remediate the hypoxia growth defect of this strain, or total ergosterol defect, and had hyper accumulation of 4-methyl sterols when compared to Δ*srbA* (Figures 2.8, 2.9, and 2.10).

These findings further illustrated a significant blockage at the Erg25 step of ergosterol biosynthesis in Δ*srbA*. As little is known about Erg25, or C4-Sterol Methyl Oxidase, in *A. fumigatus*, we initially characterized these genes through generation of single null mutants, Δ*erg25A* and Δ*erg25B* (Chapter 3). Although gene replacement of the *erg25* locus is lethal in *S. cerevisiae* and *C. albicans*, single mutants in *A. fumigatus* are viable, suggesting the two genes are partially redundant in their functions (20, 103, 163). Complete redundancy of these proteins, however, was not observed in my experiments as these single mutants differed in their accumulation of sterol intermediates (Figure 3.2 and 3.3), mRNA induction in hypoxia (Figure 3.4), and adaptation to reactive oxygen species and low oxygen (Figure 3.4 and 3.5). Of particular interest is the comparison of sterol intermediate accumulation in these strains, which indicates that Erg25A is the predominant C4-Sterol Methyl Oxidase in *A. fumigatus*.

Erg25A appears to not only mediate adaptation to hypoxia (Figure 3.4), but also is critical for adaptation to ER- and ROS-induced stress (Figure 3.5). Erg25B appears to have diverged slightly from Erg25A in its function, for although it accumulates more C4-methylated sterol intermediates than the wild type; this accumulation is not as robust as the C4-methyl sterol accumulation in Δ*erg25A* (Figure 3.2). Furthermore, Δ*erg25B*

accumulates more parkeol and less 5-dihydroergosterol than the wild type strain, suggesting that Erg25B contributes to production of these two intermediates (Figure 3.3). Further studies are required to evaluate the contribution of Erg25B to canonical sterol biosynthesis.

Deletion of both *erg25A* and *erg25B* was lethal, which demonstrates that although SrbA regulates both of these genes, and expression of *erg25A* and *erg25B* is significantly depleted in Δ*srbA*, that a basal level of transcription for *erg25A* and *erg25B* exists in Δ*srbA* that allows survival of this strain (Figure 3.7). 4-methyl sterol intermediates also accumulated in wild type *A. fumigatus* exposed to hypoxia, indicating that Erg25 is affected when oxygen levels are decreased. This result suggested a mechanism whereby Δ*srbA* is unable to grow in hypoxia due to accumulation of C4 methyl sterols (Figure 4.1 and Table 5.1).

Restoration of *erg25A* mRNA levels in Δ*srbA* with a highly active promoter from a flavohemoglobin gene (*pflavA)* partially restored C4-Sterol Methyl Oxidase activity to Δ*srbA*, as evidenced by the depletion of 4,4-dimethyl fecosterol (Figure 4.2). However, depletion of other 4-methyl sterols was not significant between Δ*srbA* and *pflavA*-*erg25A*-Δ*srbA*, suggesting a significant function for Erg25B in the removal of 4-methyl sterols in Δ*srbA* (Figure 4.2). Critically, restoration of Erg25A via *pflavA*-*erg25A*-Δ*srbA* fully restored the ability of Δ*srbA* to grow in hypoxia (Figure 4.3). This finding is especially interesting, given that *pflavA*-*erg25A*-Δ*srbA* and Δ*erg25A* reveal insubstantial differences in C4-methyl sterol intermediate accumulation. Considering that ratio of ergosterol-to-4,4-dimethyl fecosterol in these strains is nearly identical, we can identify a

threshold of 4-methyl sterol accumulation that inhibits growth in hypoxia. As Δ*erg25A* and *pflavA-erg25A*-Δ*srbA* but not Δ*srbA* can grow in hypoxia, the relative amount of 4,4-dimethyl fecosterol in the cell must be less than ~30% or 1/3 of the total ergosterol accumulated in the cell (Table 5.1). Although both Δ*erg25A* and *pflavA-erg25A*-Δ*srbA* can both grow in hypoxia, the later strain exhibits additional phenotypic alterations, possibly due to other sterol intermediate alterations, or the putative contributions of 4-methyl sterols as signaling molecules (discussed below).

Interestingly, *pflavA-erg25A*-Δ*srbA* hyper accumulated episterol and 9(11)-dehydroergosterol when compared to the wild type strain, and fully restored the lack of 5-dihydroergosterol apparent in Δ*srbA*, but was unable to restore wild type levels of 22-dihydroergosterol (Figure 4.4). The accumulation of episterol suggests a blockage in Δ*srbA* further downstream than Erg25, possibly due to depletion of both *erg3B* and *erg5* mRNA in Δ*srbA*, whereas the accumulation of 9(11)-dehydroergosterol suggests a predominant role for *erg25B* in balancing the accumulation of this intermediate, as it was accumulated in both Δ*srbA* and *pflavA-erg25A*-Δ*srbA* when compared to the wild type strain.

The *pflavA-erg25A*-Δ*srbA* strain provides an excellent opportunity to further investigate the complex biology surrounding not only the roles of SrbA, but also ergosterol biosynthesis in *A. fumigatus*. Several striking phenotypes are observed in *pflavA-erg25A*-Δ*srbA* that suggest a complex internal network surrounding ergosterol biosynthesis and a plausible secondary role for sterol intermediates. These phenotypes include: restoration of growth deficiency in iron depleted conditions (Figure 4.6),

sensitivity to cell wall perturbation agents and reactive oxygen species in hypoxia (Figures 4.6 and 4.7), and restoration of triazole antifungal resistance (Figure 4.5). These findings clearly demonstrate an intricate feedback system that underlies the regulation of ergosterol biosynthesis and approaches to further understand these mechanisms will be discussed in below.

Future Research Directions

Several interesting phenotypes were revealed in the *pflavA-erg25A-*Δ*srbA* analyses that warrant further investigation. I have demonstrated the impact of restoring Erg25A in Δ*srbA*, which rescued the hypoxia growth defect and sensitivity of Δ*srbA* to iron depleted conditions, but induced sensitivity to cell wall perturbation agents and reactive oxygen species in hypoxia. One of the major questions remaining from these studies is, what is mediating these phenotypic changes? There are two clear areas for research required to answer this question: first, are non-ergosterol protein-protein interactions of Erg25A mediating these phenotypic changes, or two, are they mediated through levels of 4-methyl sterol intermediates which may have direct effects or alter levels of other sterol intermediates that subsequently has phenotypic consequences on the fungal cell. I hypothesize that both mechanisms may be important in the phenotypes observed in *pflavA-erg25A-*Δ*srbA*.

Determining the Erg25A Protein-Interaction Network

To determine the contribution that the restoration of Erg25A has on hypoxia adaptation, cell wall perturbation, azole resistance, and sensitivity to reactive oxygen species, it is necessary to determine the protein-interaction network of Erg25A. These protein-protein interactions could be experimentally determined via co-immunoprecipitation with a tagged Erg25A protein, and verified via bimolecular fluorescence complementation (BiFC). Good candidates would include those whose known functions or localization would verify involvement in the cell wall integrity or in homeostasis of plasma membrane components. In addition, proteins involved with or localized to the mitochondrial membrane could potentially be of interest, as alterations in intracellular redox status may signal via similar cell response mechanisms.

Rationale for this Approach: In *S. cerevisiae,* Erg25 physically and genetically interacts with several other proteins, involved notably in vesicle transport, membrane localization, and calcium homeostasis (315). The role of Erg25 in calcium homeostasis has also been confirmed in *H. sapiens* (315). Curiously, these evolutionary relationships appear to be conserved in higher eukaryotes regardless of their ability to synthesize cholesterol (338).

Caenorhabditis elegans and *Drosophila melanogaster,* two organisms that cannot synthesize cholesterol past the farnesyl pyrophosphate stage, appear to have also retained downstream Erg genes for their non-sterol synthesizing properties (338). Expression of the *erg25* homologue in *C. elegans* and *D. melanogaster* is highly coregulated with

proteins that target to membranes, and those with oxidoreductase activity, intracellular protein transport, amino acid transport, or heat shock protein binding (338). Overall, the evidence for Erg25 functioning in a non-ergosterol biosynthetic manner is quite strong given both our phenotypic analysis of *A. fumigatus*, and the evolutionary conservation in *C. elegans*, *D. melanogaster*, *H. sapiens*, and *S. cerevisiae*.

In *A. fumigatus*, vesicle transport and membrane localization of proteins are integral components of the cell wall integrity pathway, which regulates the physiologocial structure and stress adaptation of the cell wall (79, 80, 82, 101, 147, 149, 202, 329, 330). Three main mechanisms have been identified in fungi to sense and respond to cell wall stress: the cAMP/PKA pathway (101, 102, 190, 236, 237), the calcineurin pathway (64, 98, 310), and the MAP kinase pathways (147, 329, 330). These pathways are linked together in that the signals that activate these pathways are received via G-protein coupled receptors in the fungal plasma membrane (42, 107, 132, 147). This indicates that not only are the signaling cascades for these stress responses important, but also the integrity of the membrane for the stability and function of these membrane-bound receptors.

Both the response to high osmolarity and cell wall stress are coordinated via the cAMP/PKA and MAP kinase pathways, which implement changes in β-glucan synthesis, regulation of cell wall biogenesis, and secretory vesicle targeting (147, 184, 329). As these biological pathways intersect with the putative functions of known Erg25 protein-protein interactions in other organisms, it is plausible that some phenotypes associated with *pflavA-erg25A-ΔsrbA*, such as restoration of growth in hypoxia or low iron, are due

to restoration of Erg25A protein function in this strain. Furthermore, the aberrent cell wall pertubation and reactive oxygen sensitivity in this strain in hypoxia could be explained via requrement of a coordinated interaction of Erg25A and another SrbA-regulated gene, possibly Erg25B, required for proper functioning of these stress response pathways.

Determining the Impact of Sterol Intermediates on Gene Expression

Sterol intermediates could plausibly be mediating significant changes in gene expression through unknown receptor-mediated mechanisms in *A. fumigatus*, such has been described in *H. sapiens* (182, 213, 266, 281). Therefore, the changes observed in 4-methyl sterol and other alternative sterol intermediate accumulation in Δ*srbA* and *pflavA-erg25A*-Δ*srbA*, could not only influence sterol biosynthesis, but also alter iron or triazole uptake and interfere with mechanisms required to mediate reactive oxygen species.

Alterations in iron uptake are especially interesting, as the iron regulatory machinery in *A. fumigatus* have been intimately linked to SrbA expression and function, as Δ*srbA* is deficient in iron uptake (36). Therefore, the restoration of growth of *pflavA-erg25A*-Δ*srbA* in iron-depleted conditions demonstrates that ergosterol biosynthesis is involved in iron homeostasis in both an SrbA-dependent and SrbA-independent manner. This mechanism, possibly mediated through the transcription factors HapX or SreA, illustrates how critical iron uptake and ergosterol biosynthesis are for the maintenance of fungal integrity and growth (36, 119, 135, 234, 275, 277, 278). This finding also shines a metaphorical spotlight on alternative functions for ergosterol biosynthesis enzymes or

sterol intermediates outside of canonical ergosterol biosynthesis. RNA-*seq* experiments comparing the transcriptomic changes caused by *pflavA*-*erg25A*-Δ*srbA* would allow us to further hypothesize the candidates that mediate this increased iron uptake as well as the machinery utilized to overcome the defects of Δ*srbA* in iron uptake facilitated in *pniiA*-*erg25A*-Δ*srbA*. These candidates could then be experimentally validated in order to develop a model for this phenomenon.

Transcriptional studies have indicated that Δ*srbA* may be defective in drug efflux, as several of the efflux pumps are dysregulated when Δ*srbA* is exposed to FLC or VCZ (unpublished observation). We hypothesize that one of the transporters regulated by SrbA may be responsible for drug uptake, efflux, or both (Barker B.M. *et al.*, in preparation).

This finding may be linked to the dysregulation of pumps in Δ*srbA*, both of the MFS- and ABC-class of transporters (36, 196, 359). One interpretation for the increased resistance of *pflavA*-*erg25A*-Δ*srbA* to the triazole antifungal drugs FLC and VCZ could be that restoration of Erg25 or decreased 4-methyl sterol accumulation causes the uptake of triazole from the extracellular environment to be partially restored to wild type levels. Another interpretation is that the production of sterol intermediates, in particular the 4-methyl sterols, influences transcriptional change in the triazole targets *erg11A* and *erg11B* through a yet-unidentified transcription factor. Layers of transcriptional regulation have been well documented in *S. cerevisiae* and *C. albicans*, where at least two factors induce or repress ergosterol biosynthesis under various conditions (172, 223, 292, 337).

We have recently identified another basic helix-loop-helix transcription factor in the regulon of SrbA, SrbB, which appears to function as a repressor of ergosterol biosynthesis (Barker, B.M. *et al*., in preparation). Although a conclusive repertoire of transcription factors that influence ergosterol biosynthesis has not been catalogued in *A. fumigatus*, the identification of at least one inducer, and one repressor of ergosterol biosynthesis will allow us to further characterize the regulation of this key pathway. Therefore, it is possible that 4-methyl sterols serve as a signal to activate a transcription factor, such as SrbB, influencing transcription of ergosterol biosynthesis genes in the absence of SrbA.

Also critical to these studies would be the identification of the receptor involved in sensing changes in the accumulation of specific sterol intermediates. Unfortunately, finding receptor/endogenous ligand pairs can be quite difficult, especially in the fungi, where the classification of nuclear receptors is sorely lacking (225). In other fields, researchers have attempted to construct pipelines to predict receptor-ligand interactions with varying degrees of success. One method for deducing the receptor for 4-methyl sterols is transcript profiling, whereby good candidates are identified through RNA-SEQ or ChIP-*seq* analyses and then experimentally validated *in vitro*. The main drawback with this approach is the lack of established criteria for nuclear-like receptors in fungi. As has been discussed previously, no homologs exist in fungi for the nuclear receptors identified in higher eukaryotes at least as determined by *in silico* approaches. The fungal class of zinc-finger transcription factors has been putatively identified as the best approximation of nuclear receptors in fungi, citing similarities in critical domain architecture and ligand-

binding patterns for homologs of the PXR (pregnane X receptor) and RXRs (retinoid X receptor) in *S. cerevisiae* and *C. glabrata* (225). Therefore, examination of transcriptionally altered members of the zinc family of transcription factors when wild type, Δ*srbA*, and *pflavA*-*erg25A*-Δ*srbA* are compared could provide additional insight into the identity of the receptor mediating 4-methyl sterol intermediate changes.

Also critical to these studies is empirically determining the impact of 4-methyl sterols on the various phenotypes described for *pflavA*-*erg25A*-Δ*srbA* and Δ*srbA*. Therefore, it will be necessary to synthesize the various 4-methyl sterols identified in *A. fumigatus*, and examine if *A. fumigatus* can take up exogenous sterols, and the effect of these exogenously applied sterol intermediates on the physiological responses of *A. fumigatus* to stress. Synthesis of 4-methyl sterol intermediates would also allow for the development of affinity chromatography-mass spectrometry approaches to determine receptor candidates.

Rationale for this Approach: In humans, FF-MAS, or follicular-fluid meiosis-activating sterol, and T-MAS, or testis meiosis-activating sterol, induce the resumption of meiosis (48). This action is mediated through the orphan receptor LXRα (liver X receptor α) (148). LXRs are involved in the coordination of the homeostasis between inflammation and cholesterol biosynthesis (53, 148). Among other functions, LXR agonists mediate cholesterol efflux, influence cellular redistribution of sterols, and stimulate fatty acid biosynthesis (182, 213, 262, 266, 281). LXR agonists also appear to directly suppress cholesterol synthesis by influencing transcription of squalene synthase and lanosterol demethylase (348). Importantly, LXR agonists influence fatty acid

synthesis by stimulating SREBP-1c, the SREBP isoform responsible for lipogenesis (262, 281). Although fungi lack nuclear receptor homologs, members of the zinc cluster of transcription factors are postulated to serve an analogous function (225).

In addition, these meiosis-activating sterols are also thought to have LXR-independent mechanisms of action, possibly through specific G-protein coupled receptors (127). Combined, there is significant evidence that these 4-methyl sterols serve significant functions outside of the ergosterol biosynthesis pathway. It is plausible therefore, that sterol intermediates could be involved in influencing gene expression in *A. fumigatus*.

Further Characterization of the Ergosterol Biosynthesis Pathway in *A. fumigatus*

My dissertation studies suggest a complex interchange between ergosterol biosynthesis and adaptation to various cellular stresses that has not yet been studied in *A. fumigatus*. Therefore, a more empiric study of the individual ergosterol biosynthesis genes in *A. fumigatus* is required to not only to accurately understand the implications of alterations in this crucial pathway, but also to fully capitalize upon this pathway as a target for further antifungal drug development.

A typical genetic approach to understanding the biological roles of individual genes/proteins in ergosterol biosynthesis involves the generation of single null mutants. In this vein, I have constructed single deletion mutants of all ergosterol biosynthesis genes with a demonstrated SrbA-binding site in the promoter region, except one, and are currently determining the contribution of these individual genes in the phenotypes

associated with Δ*srbA* and *pflavA*-*erg25A*-Δ*srbA*, and also the composition of their sterol intermediate profiles. The completed single deletion mutants include: Δ*erg11A*, Δ*erg25A*, Δ*erg25B*, Δ*erg3B*, and Δ*erg5*, with only the previously characterized Δ*erg11B* strain left to finish.

One of the drawbacks to this single-deletion strategy is that the ergosterol biosynthesis pathway is crucial for the growth, viability, and nutrient acquisition of *A. fumigatus*, and thus it has developed alternative mechanisms to synthesis ergosterol when a single gene has been disrupted. This lower portion of the pathway has been previously described as a "fishing net;" when a disturbance pulls in one area, another section of the net takes up the load. Therefore, researchers interested in ergosterol biosynthesis in *S. cerevisiae* and *C. albicans* found that construction of double mutants especially useful for understanding how their organism produced ergosterol, and which enzymes were critical for this mechanism.

Considering just the SrbA-dependent ergosterol biosynthesis genes, we would be required to construct thirteen double mutants to examine the relationships between *erg11A*, *erg11B*, *erg25A*, *erg25B*, *erg3B*, and *erg5*. Therefore, we propose that a next direction would be the construction of five double mutants to answer various hypothesis-driven questions about ergosterol biosynthesis in *A. fumigatus*.

These double mutants would be constructed following characterization of Δ*erg5* and Δ*erg3B*, as our hypotheses may be altered or re-prioritized based on findings in these strains. The five double mutants we propose are those that have the potential to greatly

contribute to the current knowledge about the pathway: Δ*erg11A*Δ*erg25A*, Δ*erg25A*Δ*erg3B*, Δ*erg25A*Δ*erg5*, Δ*erg11A*Δ*erg5*, and Δ*erg11B*Δ*erg5*.

The double mutant Δ*erg11A*Δ*erg25A* would be constructed to verify the contribution of *erg11A* and *erg25A* to the phenotypes of Δ*srbA*, without the contribution of other genes in the SrbA-regulon, and could illustrate any interactions between these critical enzymes of ergosterol biosynthesis. Therefore, we hypothesize that the double mutant should recapitulate all the phenotypes of the individual gene replacement mutants, but also demonstrate some synergy or loss of phenotypes, illuminating some of the complex network of interaction between these proteins. This mutant should also accumulate less 4-methyl sterol intermediates than Δ*erg25A*, as the C14-demethylation blockage at *erg11A* in Δ*erg11A*Δ*erg25A* should decrease the flow of precursor intermediates through the subsequent biosynthetic steps.

Both Δ*erg25A*Δ*erg5* and Δ*erg25A*Δ*erg3B* would determine the effect of *erg3B* and Δ*erg5* on the accumulation of 22-dehydroergosterol when Erg25A-driven substrates are unavailable. Also, these mutants should illustrate what contribution these enzymes have on the accumulation of other Erg25A-dependent ergosterol intermediate steps. We previously have hypothesized that the blockages apparent in *pflavA*-*erg25A*-Δ*srbA* are more likely due to depletion of *erg3B* than *erg5*. This hypothesis is based primarily on the known functions of *erg3* in *S. cerevisiae* and *C. albicans*, where resistance to triazole antifungal drugs results in genetic plasticity at this locus (193, 200, 217). Therefore, we extend this hypothesis to explain sterol intermediate accumulations for the episterol-to-ergosterol transition in Δ*erg25A*, whereby *erg3B* contributes more to this phenotype than

erg5. The sterol intermediate profiles of these Δ*erg25A*Δ*erg3B* and Δ*erg25A*Δ*erg5* mutants will be especially illuminating for the contribution of these downstream proteins in sterol intermediate accumulation.

As the *erg11* and *erg5* steps are the only cytochrome P450 enzymes involved in ergosterol biosynthesis, and because the triazole antifungals target *erg11*, there has been some suggestion in the literature that *erg5* could be contributing to azole resistance or sensitivity, in particular with the triazole voriconazole (199). Furthermore, a clinical *C. albicans* isolate has been characterized that contained both *erg5*-null and *erg11*-null mutations (199). This isolate was resistant to fluconazole, voriconazole, itraconazole, ketoconazole, and clotrimazole, and alternative mechanisms did not contribute to the azole resistance of this isolate (199). Therefore, we hypothesize that Δ*erg11A*Δ*erg5* may regain a partial azole sensitivity to triazole antifungal drugs when compared with the wild type MIC for Voriconazole, but very little change in MIC for Fluconazole, due to the binding preference of this pharmaceutical agent to Erg11B in *A. fumigatus* (349). In a similar vein, we hypothesize that Δ*erg11B*Δ*erg5* would demonstrate a slight susceptibility to all members of the triazole antifungal drugs. A triple mutant, Δ*erg11A*Δ*erg11B*Δ*erg5* should mimic the triazole sensitivity of Δ*srbA*, however, construction of a triple mutant is not possible in this case, due to the lethality of Δ*erg11A* in tandem with Δ*erg11B* (138).

Additionally, construction of a strain that restores both Erg25A and Erg25B in the Δ*srbA* background is essential to determine the contribution of Erg25B to restoring the phenotypes of Δ*srbA*. In particular, we are quite interested to see if restoration of Erg25B

removes the accumulation of 9(11)-dehydroergosterol present in *pniiA-erg25A-*Δ*srbA*. 9(11)-dehydroergosterol has been implicated in the formation of a 9(11)-dehydroergosterol peroxide, and some suggestions have been made regarding its anti-tumor properties in mammalian tissue culture. Whether 9(11)-dehydroergosterol can be converted into a peroxide in *A. fumigatus* has yet to be determined, as is the consequence of accumulation of such a potential bioactive molecule. Our data indicate that Erg25B is required for elimination of this intermediate, as both Δ*erg25B* and *pniiA-erg25A-*Δ*srbA* accumulated much higher levels of this intermediate than either Δ*srbA* or Δ*erg25A.* We hypothesize that this intermediate may accumulate due to unchecked expression of Erg25A in both Δ*erg25A* and *pniiA-erg25A-*Δ*srbA*.

Rationale for this Approach: In *S. cerevisiae*, Erg28 scaffolds the members of the C4-demethylation machinery, Erg25, Erg26, and Erg27, into a complex and tethers the assembled complex to the endoplasmic reticulum (220). In fact, many of the ergosterol biosynthesis genes physically interact within the Erg28-tethered complex in *S. cerevisiae*. This suggests that an intimate communication between members of the C4-demethylation machinery exists with enzymes that act both up- and down-stream of this biosynthetic step (218). Interestingly, Mo and Bard suggest that Erg11, Erg25, Erg27, and Erg28, serve as a “hub” for enzymes both earlier and later in the ergosterol biosynthesis (219), although the purpose of this hub was not biochemically evaluated. These experiments also suggested that enzymes involved in the episterol-to-ergosterol transition, Erg3, Erg4, and Erg5, are not highly dependent on the physical presence of one another, but instead their strongest interactions are with the core group of enzymes listed above. Therefore,

we propose that a loss of gene dosage, or stoichiometry, between ergosterol biosynthesis enzymes in the ergosterol "hub" of Δ*srbA* contribute to the phenotypes of this mutant.

A decrease in ergosterol "hub" stoichiometry could influence this complex in two ways: first, changes could decrease enzymatic efficiency, and second, it could change the substrate specificity of individual enzymes. Change in enzymatic efficiency could be readily discernible in the case of single gene deletion mutants. Under these conditions, loss of the mutated ergosterol biosynthesis would be accompanied by simultaneous loss of activity at other enzymatic steps, which may be evidenced by sterol intermediate accumulation. In this case, loss of the first gene, via genetic deletion, would putatively initiate secondary defects in proteins that would be adjacent to the mutant in the ergosterol complex, thus disrupting its stability. Substrate specificity could also be altered by the composition could be altered with stoichiometric disruptions in the ergosterol "hub," causing the accumulation of atypical sterol intermediates. These changes, however, may not be characterizable until double mutants are examined, as changes in single ergosterol biosynthesis genes appear to induce an alternative mechanism or plasticity to the biosynthetic process in many fungi.

Characterization of ergosterol biosynthesis has primarily focused on the fungi *S. cerevisiae* and *C. albicans,* well characterized members of the hemi-Ascomycetes. Although *S. cerevisiae, C. albicans,* and *A. fumigatus* are all fungi, the large evolutionary distance that separates the hemi-Ascomycetes from *Aspergilli* limits the correlation between the two groups. So although ergosterol biosynthesis utilizes genes similar in their architecture to proceed through this biosynthetic pathway, both the progression and

the substrate specificity differ greatly. Compounding these differences is the existence of multiple copies of most ergosterol biosynthesis genes in *A. fumigatus*, and a significant difference in the class of transcription factors that predominantly regulate sterol synthesis in the hemi-Ascomycetes or *Aspergilli*.

In general, the *Aspergillus* ergosterol biosynthesis field lags behind that of its cousins *S. cerevisiae* and *C. albicans*. Therefore, to further our understanding of the biological and structural roles that Erg25 and 4-methyl sterols play in the biological processes of *A. fumigatus*, more basic research into the functions and interactions of the major components of ergosterol biosynthesis are an absolute necessity.

Conclusions

The findings in this dissertation highlight a significant gap in understanding the molecular mechanisms of ergosterol biosynthesis in *A. fumigatus*, namely that *Aspergillus spp.* genetically encode for multiple copies of many of the genes in this pathway, but no divergence of function has been described to date. Although this lack of functional differentiation would suggest that these multiple copies remain orthologs, we propose that although these copies conserve critical domains, that they have also developed diverse functions that have yet to be determined in the *Aspergilli.* This concept of taxa-specific ergosterol biosynthesis alterations has been suggested by other investigators in the field, and ultimately yields merit for the contribution this understanding could provide the development of new antifungal drugs and understanding of emerging triazole drug resistance (6).

Through characterization of *erg11A*, *erg25A*, and *erg25B*, and the role these genes play in the phenotypes of Δ*srbA* we have begun to unveil a complex biosystem surrounding the synthesis and signaling of ergosterol intermediates (Figure 5.1). I have demonstrated a significant role between the transcription factor SrbA, and two members of its regulon, *erg11A* and *erg25A*. Through these interactions we have begun to mechanistically link SrbA to triazole antifungal resistance and hypoxia adaptation, and have revealed the need for an intricate balance between members of the SrbA-regulon and adaptation to various cellular stresses including cell wall perturbation, survival in iron depleted conditions, and resistance to reactive oxygen species.

My work raises important biological questions about how SrbA is mediating adaptation to hypoxia, triazole antifungal drugs, and the aforementioned cellular stresses, and suggests a primary role for Erg25 in this process. However, how is Erg25 mediating these dramatic changes? Is it through uncharacterized non-ergosterol protein-protein interactions with Erg25? Or perhaps this is mediated through the sterol intermediates themselves? Perhaps both of these mechanisms are required to balance this intricate dance between numerous members of the cellular protection, growth, and signaling pathways.

Tables

Table 5.1 Contribution of 4-methyl sterol intermediates to hypoxia growth

Strain	**4-methyl fecosterol**	**4,4-dimethyl fecosterol**	**Ergosterol**	**Growth in Hypoxia**
Wild Type	1.20 ± 0.99%	3.00 ± 2.26%	++	+
Δ*srbA*	16.60 ± 1.79%	58.03 ± 9.98%	+	-
Δ*erg25A*	17.23 ±1.52%	31.70 ± 4.04%	++	+
Δ*erg25B*	1.48 ± 0.59%	7.26 ± 2.32 %	++	+
*pniiA-erg25A-*ΔsrbA	16. 47 ± 2.31%	30.61 ± 6.78%	+	+

Figure

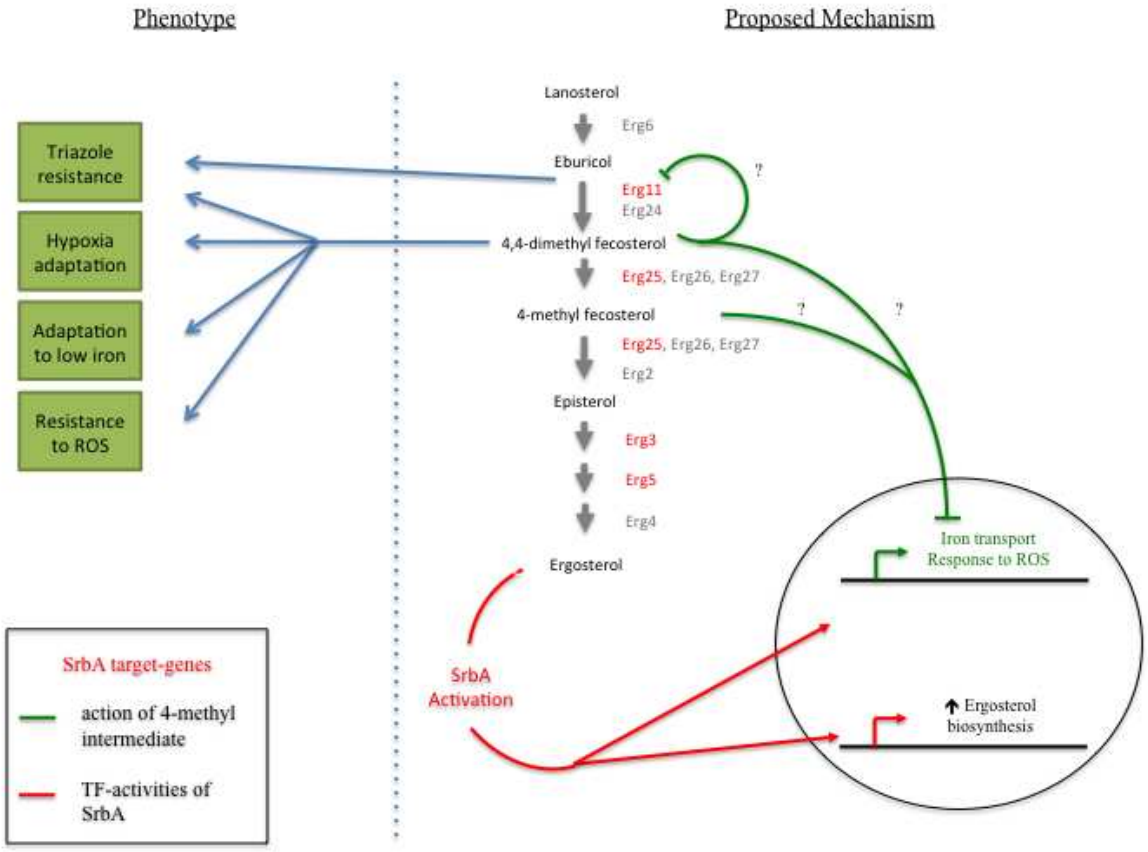


Figure 5.1 Model for SrbA-mediated Ergosterol Biosynthesis affecting Triazole Resistance, Hypoxia and Low-Iron Adaptation, and Resistance to ROS

REFERENCES CITED

1. 2007. Who Case definitions of HIV for surveillance and revised clinical staging and immunological classification of HIV-related disease in adults and children. World Health Organization.

2. Abe, F., and T. Hiraki. 2009. Mechanistic role of ergosterol in membrane rigidity and cycloheximide resistance in Saccharomyces cerevisiae. Biochim Biophys Acta 1788:743-52.

3. Aimanianda, V., J. Bayry, S. Bozza, O. Kniemeyer, K. Perruccio, S. R. Elluru, C. Clavaud, S. Paris, A. A. Brakhage, S. V. Kaveri, L. Romani, and J. P. Latge. 2009. Surface hydrophobin prevents immune recognition of airborne fungal spores. Nature 460:1117-21.

4. Al-Barrag, A., M. J. Anderson, S. J. Howard, G. D. Robson, P. A. Warn, D. Sanglard, and D. W. Denning. 2011. Interrogation of related Clinical Pan-azole Resistant Aspergillus fumigatus strains - G138C, Y431C and G434C SNPs in cyp51A, Upregulation of cyp51A and Integration and Activation of Transposon Atf1 into cyp51A Promoter. Antimicrob Agents Chemother.

5. Albarrag, A. M., M. J. Anderson, S. J. Howard, G. D. Robson, P. A. Warn, D. Sanglard, and D. W. Denning. 2011. Interrogation of related clinical pan-azole-resistant Aspergillus fumigatus strains: G138C, Y431C, and G434C single nucleotide polymorphisms in cyp51A, upregulation of cyp51A, and integration and activation of transposon Atf1 in the cyp51A promoter. Antimicrob Agents Chemother 55:5113-21.

6. Alcazar-Fuoli, L., and E. Mellado. 2012. Ergosterol biosynthesis in Aspergillus fumigatus: its relevance as an antifungal target and role in antifungal drug resistance. Front Microbiol 3:439.

7. Alcazar-Fuoli, L., E. Mellado, M. Cuenca-Estrella, and D. Sanglard. 2011. Probing the role of point mutations in the cyp51A gene from Aspergillus fumigatus in the model yeast Saccharomyces cerevisiae. Medical Mycology 49:276-284.

8. Alcazar-Fuoli, L., E. Mellado, G. Garcia-Effron, M. J. Buitrago, J. F. Lopez, J. O. Grimalt, J. M. Cuenca-Estrella, and J. L. Rodriguez-Tudela. 2006. Aspergillus fumigatus C-5 sterol desaturases Erg3A and Erg3B: role in sterol biosynthesis and antifungal drug susceptibility. Antimicrob Agents Chemother 50:453-60.

9. Alcazar-Fuoli, L., E. Mellado, G. Garcia-Effron, J. F. Lopez, J. O. Grimalt, J. M. Cuenca-Estrella, and J. L. Rodriguez-Tudela. 2008. Ergosterol biosynthesis pathway in Aspergillus fumigatus. Steroids 73:339-47.

10. Alexander, B. D., and J. R. Perfect. 1997. Antifungal resistance trends towards the year 2000. Implications for therapy and new approaches. Drugs 54:657-78.

11. Altmann, K., and B. Westermann. 2005. Role of essential genes in mitochondrial morphogenesis in Saccharomyces cerevisiae. Mol Biol Cell 16:5410-7.

12. Alvarez, F. J., L. M. Douglas, and J. B. Konopka. 2007. Sterol-rich plasma membrane domains in fungi. Eukaryot Cell 6:755-63.

13. Amin, R., A. Dupuis, S. D. Aaron, and F. Ratjen. 2010. The effect of chronic infection with Aspergillus fumigatus on lung function and hospitalization in patients with cystic fibrosis. Chest 137:171-6.

14. Anderson, J. B. 2005. Evolution of antifungal-drug resistance: mechanisms and pathogen fitness. Nat Rev Microbiol 3:547-56.

15. Ardon, O., H. Bussey, C. Philpott, D. M. Ward, S. Davis-Kaplan, S. Verroneau, B. Jiang, and J. Kaplan. 2001. Identification of a Candida albicans ferrichrome transporter and its characterization by expression in Saccharomyces cerevisiae. J Biol Chem 276:43049-55.

16. Arnold, F., D. West, and S. Kumar. 1987. Wound healing: the effect of macrophage and tumour derived angiogenesis factors on skin graft vascularization. Br J Exp Pathol 68:569-74.

17. Askew, D. S. 2008. Aspergillus fumigatus: virulence genes in a street-smart mold. Curr Opin Microbiol 11:331-7.

18. Bakare, N., V. Rickerts, J. Bargon, and G. Just-Nubling. 2003. Prevalence of Aspergillus fumigatus and other fungal species in the sputum of adult patients with cystic fibrosis. Mycoses 46:19-23.

19. Bammert, G. F., and J. M. Fostel. 2000. Genome-wide expression patterns in Saccharomyces cerevisiae: comparison of drug treatments and genetic alterations affecting biosynthesis of ergosterol. Antimicrob Agents Chemother 44:1255-65.

20. Bard, M., D. A. Bruner, C. A. Pierson, N. D. Lees, B. Biermann, L. Frye, C. Koegel, and R. Barbuch. 1996. Cloning and characterization of ERG25, the

Saccharomyces cerevisiae gene encoding C-4 sterol methyl oxidase. Proc Natl Acad Sci U S A 93:186-90.

21. Bard, M., N. D. Lees, T. Turi, D. Craft, L. Cofrin, R. Barbuch, C. Koegel, and J. C. Loper. 1993. Sterol synthesis and viability of erg11 (cytochrome P450 lanosterol demethylase) mutations in Saccharomyces cerevisiae and Candida albicans. Lipids 28:963-7.

22. Barhoom, S., and A. Sharon. 2004. cAMP regulation of "pathogenic" and "saprophytic" fungal spore germination. Fungal Genet Biol 41:317-26.

23. Barker, B. M., K. Kroll, M. Vodisch, A. Mazurie, O. Kniemeyer, and R. A. Cramer. 2012. Transcriptomic and proteomic analyses of the Aspergillus fumigatus hypoxia response using an oxygen-controlled fermenter. BMC Genomics 13:62.

24. Barker, K. S., S. Crisp, N. Wiederhold, R. E. Lewis, B. Bareither, J. Eckstein, R. Barbuch, M. Bard, and P. D. Rogers. 2004. Genome-wide expression profiling reveals genes associated with amphotericin B and fluconazole resistance in experimentally induced antifungal resistant isolates of Candida albicans. J Antimicrob Chemother 54:376-85.

25. Bashir, K., H. Inoue, S. Nagasaka, M. Takahashi, H. Nakanishi, S. Mori, and N. K. Nishizawa. 2006. Cloning and characterization of deoxymugineic acid synthase genes from graminaceous plants. J Biol Chem 281:32395-402.

26. Bassiri-Jahromi, S., and A. Doostkam. 2012. Fungal infection and increased mortality in patients with chronic granulomatous disease. J Mycol Med 22:52-7.

27. Baumann, N. A., D. P. Sullivan, H. Ohvo-Rekila, C. Simonot, A. Pottekat, Z. Klaassen, C. T. Beh, and A. K. Menon. 2005. Transport of newly synthesized sterol to the sterol-enriched plasma membrane occurs via nonvesicular equilibration. Biochemistry 44:5816-26.

28. Beffa, T., F. Staib, J. Lott Fischer, P. F. Lyon, P. Gumowski, O. E. Marfenina, S. Dunoyer-Geindre, F. Georgen, R. Roch-Susuki, L. Gallaz, and J. P. Latge. 1998. Mycological control and surveillance of biological waste and compost. Med Mycol 36 Suppl 1:137-45.

29. Beh, C. T., and J. Rine. 2004. A role for yeast oxysterol-binding protein homologs in endocytosis and in the maintenance of intracellular sterol-lipid distribution. J Cell Sci 117:2983-96.

30. Bell, E. L., B. M. Emerling, and N. S. Chandel. 2005. Mitochondrial regulation of oxygen sensing. Mitochondrion 5:322-32.

31. Benveniste, P. 2004. Biosynthesis and accumulation of sterols. Annu Rev Plant Biol 55:429-57.

32. Bhabhra, R., and D. S. Askew. 2005. Thermotolerance and virulence of Aspergillus fumigatus: role of the fungal nucleolus. Med Mycol 43 Suppl 1:S87-93.

33. Bien, C. M., and P. J. Espenshade. 2010. Sterol regulatory element binding proteins in fungi: hypoxic transcription factors linked to pathogenesis. Eukaryot Cell 9:352-9.

34. Bignell, E., S. Negrete-Urtasun, A. M. Calcagno, K. Haynes, H. N. Arst, Jr., and T. Rogers. 2005. The Aspergillus pH-responsive transcription factor PacC regulates virulence. Mol Microbiol 55:1072-84.

35. Bittner, K., A. Bittinger, and H. Lange. 1987. [Cytomegalovirus-associated superinfection of the lung following kidney transplantation]. Dtsch Med Wochenschr 112:214-8.

36. Blatzer, M., B. M. Barker, S. D. Willger, N. Beckmann, S. J. Blosser, E. J. Cornish, A. Mazurie, N. Grahl, H. Haas, and R. A. Cramer. 2011. SREBP coordinates iron and ergosterol homeostasis to mediate triazole drug and hypoxia responses in the human fungal pathogen Aspergillus fumigatus. PLoS Genet 7:e1002374.

37. Blosser, S. J., and R. A. Cramer. 2012. SREBP-dependent triazole susceptibility in Aspergillus fumigatus is mediated through direct transcriptional regulation of erg11A (cyp51A). Antimicrob Agents Chemother 56:248-57.

38. Blum, G., C. Hortnagl, E. Jukic, T. Erbeznik, T. Pumpel, H. Dietrich, M. Nagl, C. Speth, G. Rambach, and C. Lass-Florl. 2013. New Insight into Amphotericin B Resistance in Aspergillus terreus. Antimicrob Agents Chemother 57:1583-8.

39. Borecka-Melkusova, S., G. P. Moran, D. J. Sullivan, S. Kucharikova, D. Chorvat, Jr., and H. Bujdakova. 2009. The expression of genes involved in the ergosterol biosynthesis pathway in Candida albicans and Candida dubliniensis biofilms exposed to fluconazole. Mycoses 52:118-28.

40. Bouvier, F., A. Rahier, and B. Camara. 2005. Biogenesis, molecular regulation and function of plant isoprenoids. Prog Lipid Res 44:357-429.

41. Brock, M., G. Jouvion, S. Droin-Bergere, O. Dussurget, M. A. Nicola, and O. Ibrahim-Granet. 2008. Bioluminescent Aspergillus fumigatus, a new tool for drug efficiency testing and in vivo monitoring of invasive aspergillosis. Appl Environ Microbiol 74:7023-35.

42. Brodhagen, M., and N. P. Keller. 2006. Signalling pathways connecting mycotoxin production and sporulation. Mol Plant Pathol 7:285-301.

43. Brown, G. D. 2006. Dectin-1: a signalling non-TLR pattern-recognition receptor. Nat Rev Immunol 6:33-43.

44. Brown, G. D., D. W. Denning, N. A. Gow, S. M. Levitz, M. G. Netea, and T. C. White. 2012. Hidden killers: human fungal infections. Sci Transl Med 4:165rv13.

45. Bruno, C., S. Minniti, A. Vassanelli, and R. Pozzi-Mucelli. 2007. Comparison of CT features of Aspergillus and bacterial pneumonia in severely neutropenic patients. J Thorac Imaging 22:160-5.

46. Bruns, S., O. Kniemeyer, M. Hasenberg, V. Aimanianda, S. Nietzsche, A. Thywissen, A. Jeron, J. P. Latge, A. A. Brakhage, and M. Gunzer. 2010. Production of extracellular traps against Aspergillus fumigatus in vitro and in infected lung tissue is dependent on invading neutrophils and influenced by hydrophobin RodA. PLoS Pathog 6:e1000873.

47. Buied, A., C. B. Moore, D. W. Denning, and P. Bowyer. 2013. High-level expression of cyp51B in azole-resistant clinical Aspergillus fumigatus isolates. J Antimicrob Chemother 68:512-4.

48. Byskov, A. G., C. Y. Andersen, L. Nordholm, H. Thogersen, G. Xia, O. Wassmann, J. V. Andersen, E. Guddal, and T. Roed. 1995. Chemical structure of sterols that activate oocyte meiosis. Nature 374:559-62.

49. Caira, M., C. Girmenia, R. M. Fadda, M. E. Mitra, M. Picardi, M. T. Van Lint, A. Nosari, A. Candoni, A. Bonini, D. Mattei, C. de Waure, L. Fianchi, C. G. Valentini, F. Aversa, G. Leone, and L. Pagano. 2008. Invasive fungal infections in patients with acute myeloid leukemia and in those submitted to allogeneic hemopoietic stem cell transplant: who is at highest risk? Eur J Haematol 81:242-3.

50. Camps, S. M., B. E. Dutilh, M. C. Arendrup, A. J. Rijs, E. Snelders, M. A. Huynen, P. E. Verweij, and W. J. Melchers. 2012. Discovery of a HapE mutation that causes

azole resistance in Aspergillus fumigatus through whole genome sequencing and sexual crossing. PLoS One 7:e50034.

51. Carvajal, E., van den Hazel,H. B., Cybularz-Kolaczkowska, A. , Balzi, E., and A. Goffeau. 1997. Molecular and phenotypic characterization of yeast PDR1 mutants that show hyperactive transcription of various ABC multidrug transporter genes. Molecular and General Genetics MGG 256:10.

52. Casadevall, A., and L. A. Pirofski. 2003. The damage-response framework of microbial pathogenesis. Nat Rev Microbiol 1:17-24.

53. Castrillo, A., S. B. Joseph, S. A. Vaidya, M. Haberland, A. M. Fogelman, G. Cheng, and P. Tontonoz. 2003. Crosstalk between LXR and toll-like receptor signaling mediates bacterial and viral antagonism of cholesterol metabolism. Mol Cell 12:805-16.

54. Chai, L. Y., A. G. Vonk, B. J. Kullberg, P. E. Verweij, I. Verschueren, J. W. van der Meer, L. A. Joosten, J. P. Latge, and M. G. Netea. 2011. Aspergillus fumigatus cell wall components differentially modulate host TLR2 and TLR4 responses. Microbes Infect 13:151-9.

55. Chang, Y. C., C. M. Bien, H. Lee, P. J. Espenshade, and K. J. Kwon-Chung. 2007. Sre1p, a regulator of oxygen sensing and sterol homeostasis, is required for virulence in Cryptococcus neoformans. Mol Microbiol 64:614-29.

56. Chang, Y. C., H. F. Tsai, M. Karos, and K. J. Kwon-Chung. 2004. THTA, a thermotolerance gene of Aspergillus fumigatus. Fungal Genet Biol 41:888-96.

57. Chang, Y. C., L. C. Wright, R. L. Tscharke, T. C. Sorrell, C. F. Wilson, and K. J. Kwon-Chung. 2004. Regulatory roles for the homeodomain and C2H2 zinc finger regions of Cryptococcus neoformans Ste12alphap. Mol Microbiol 53:1385-96.

58. Chaudhary, N., K. Datta, F. B. Askin, J. F. Staab, and K. A. Marr. 2012. Cystic fibrosis transmembrane conductance regulator regulates epithelial cell response to Aspergillus and resultant pulmonary inflammation. Am J Respir Crit Care Med 185:301-10.

59. Chen, J., H. Li, R. Li, D. Bu, and Z. Wan. 2005. Mutations in the cyp51A gene and susceptibility to itraconazole in Aspergillus fumigatus serially isolated from a patient with lung aspergilloma. J Antimicrob Chemother 55:31-7.

60. Chun, C. D., O. W. Liu, and H. D. Madhani. 2007. A link between virulence and homeostatic responses to hypoxia during infection by the human fungal pathogen Cryptococcus neoformans. PLoS Pathog 3:e22.

61. Colabardini, A. C., P. A. De Castro, P. F. De Gouvea, M. Savoldi, I. Malavazi, M. H. Goldman, and G. H. Goldman. 2010. Involvement of the Aspergillus nidulans protein kinase C with farnesol tolerance is related to the unfolded protein response. Mol Microbiol 78:1259-79.

62. Collins, L. A., M. H. Samore, M. S. Roberts, R. Luzzati, R. L. Jenkins, W. D. Lewis, and A. W. Karchmer. 1994. Risk factors for invasive fungal infections complicating orthotopic liver transplantation. J Infect Dis 170:644-52.

63. Como, J. A., and W. E. Dismukes. 1994. Oral azole drugs as systemic antifungal therapy. N Engl J Med 330:263-72.

64. Cramer, R. A., Jr., B. Z. Perfect, N. Pinchai, S. Park, D. S. Perlin, Y. G. Asfaw, J. Heitman, J. R. Perfect, and W. J. Steinbach. 2008. Calcineurin target CrzA regulates conidial germination, hyphal growth, and pathogenesis of Aspergillus fumigatus. Eukaryot Cell 7:1085-97.

65. Crowley, J. H., F. W. Leak, Jr., K. V. Shianna, S. Tove, and L. W. Parks. 1998. A mutation in a purported regulatory gene affects control of sterol uptake in Saccharomyces cerevisiae. J Bacteriol 180:4177-83.

66. Cui, Y. J., P. Liu, and R. Y. Chen. 2002. [Studies on the chemical constituents of Spatholobus suberectus Dunn]. Yao Xue Xue Bao 37:784-7.

67. d'Enfert, C., and T. Fontaine. 1997. Molecular characterization of the Aspergillus nidulans treA gene encoding an acid trehalase required for growth on trehalose. Mol Microbiol 24:203-16.

67b. d'Enfert. 1996. Selection of multiple disruption events in *Aspergillus fumigatus* using the orotidine-5'-decarboxylase gene, *pyrG*, as a unique transformation marker. Curr Genet 30:76-82.

68. da Silva Ferreira, M. E., I. Malavazi, M. Savoldi, A. A. Brakhage, M. H. Goldman, H. S. Kim, W. C. Nierman, and G. H. Goldman. 2006. Transcriptome analysis of Aspergillus fumigatus exposed to voriconazole. Curr Genet 50:32-44.

69. Dagenais, T. R., and N. P. Keller. 2009. Pathogenesis of Aspergillus fumigatus in Invasive Aspergillosis. Clin Microbiol Rev 22:447-65.

70. Darnet, S., M. Bard, and A. Rahier. 2001. Functional identification of sterol-4alpha-methyl oxidase cDNAs from Arabidopsis thaliana by

complementation of a yeast erg25 mutant lacking sterol-4alpha-methyl oxidation. FEBS Lett 508:39-43.

71. Darnet, S., and A. Rahier. 2004. Plant sterol biosynthesis: identification of two distinct families of sterol 4alpha-methyl oxidases. Biochem J 378:889-98.

72. Davies, B. S., and J. Rine. 2006. A role for sterol levels in oxygen sensing in Saccharomyces cerevisiae. Genetics 174:191-201.

73. De Backer, M. D., T. Ilyina, X. J. Ma, S. Vandoninck, W. H. Luyten, and H. Vanden Bossche. 2001. Genomic profiling of the response of Candida albicans to itraconazole treatment using a DNA microarray. Antimicrob Agents Chemother 45:1660-70.

74. de Souza, W., and J. C. Rodrigues. 2009. Sterol Biosynthesis Pathway as Target for Anti-trypanosomatid Drugs. Interdiscip Perspect Infect Dis 2009:642502.

75. Delaunay, A., A. D. Isnard, and M. B. Toledano. 2000. H2O2 sensing through oxidation of the Yap1 transcription factor. EMBO J 19:5157-66.

76. Denning, D. W., K. Venkateswarlu, K. L. Oakley, M. J. Anderson, N. J. Manning, D. A. Stevens, D. W. Warnock, and S. L. Kelly. 1997. Itraconazole resistance in Aspergillus fumigatus. Antimicrob Agents Chemother 41:1364-8.

77. Dharia, N. V., A. T. Bright, S. J. Westenberger, S. W. Barnes, S. Batalov, K. Kuhen, R. Borboa, G. C. Federe, C. M. McClean, J. M. Vinetz, V. Neyra, A. Llanos-Cuentas, J. W. Barnwell, J. R. Walker, and E. A. Winzeler. 2010. Whole-genome sequencing and microarray analysis of ex vivo Plasmodium vivax reveal selective pressure on putative drug resistance genes. Proc Natl Acad Sci U S A 107:20045-50.

78. Diao, Y., R. Zhao, X. Deng, W. Leng, J. Peng, and Q. Jin. 2009. Transcriptional profiles of Trichophyton rubrum in response to itraconazole. Med Mycol 47:237-47.

79. Dichtl, K., F. Ebel, F. Dirr, F. H. Routier, J. Heesemann, and J. Wagener. 2010. Farnesol misplaces tip-localized Rho proteins and inhibits cell wall integrity signalling in Aspergillus fumigatus. Mol Microbiol 76:1191-204.

80. Dichtl, K., C. Helmschrott, F. Dirr, and J. Wagener. 2012. Deciphering cell wall integrity signalling in Aspergillus fumigatus: identification and functional characterization of cell wall stress sensors and relevant Rho GTPases. Mol Microbiol 83:506-19.

81. Dirmeier, R., K. M. O'Brien, M. Engle, A. Dodd, E. Spears, and R. O. Poyton. 2002. Exposure of yeast cells to anoxia induces transient oxidative stress. Implications for the induction of hypoxic genes. J Biol Chem 277:34773-84.
82. Dirr, F., B. Echtenacher, J. Heesemann, P. Hoffmann, F. Ebel, and J. Wagener. 2010. AfMkk2 is required for cell wall integrity signaling, adhesion, and full virulence of the human pathogen Aspergillus fumigatus. Int J Med Microbiol 300:496-502.

83. Dotis, J., and E. Roilides. 2004. Osteomyelitis due to Aspergillus spp. in patients with chronic granulomatous disease: comparison of Aspergillus nidulans and Aspergillus fumigatus. Int J Infect Dis 8:103-10.

84. Du, H., G. Guan, J. Xie, Y. Sun, Y. Tong, L. Zhang, and G. Huang. 2012. Roles of Candida albicans Gat2, a GATA-type zinc finger transcription factor, in biofilm formation, filamentous growth and virulence. PLoS One 7:e29707.

85. Dunkel, N., J. Blass, P. D. Rogers, and J. Morschhauser. 2008. Mutations in the multi-drug resistance regulator MRR1, followed by loss of heterozygosity, are the main cause of MDR1 overexpression in fluconazole-resistant Candida albicans strains. Mol Microbiol 69:827-40.

86. Dunkel, N., T. T. Liu, K. S. Barker, R. Homayouni, J. Morschhauser, and P. D. Rogers. 2008. A gain-of-function mutation in the transcription factor Upc2p causes upregulation of ergosterol biosynthesis genes and increased fluconazole resistance in a clinical Candida albicans isolate. Eukaryot Cell 7:1180-90.

87. Eaton, T., J. Garrett, D. Milne, A. Frankel, and A. U. Wells. 2000. Allergic bronchopulmonary aspergillosis in the asthma clinic. A prospective evaluation of CT in the diagnostic algorithm. Chest 118:66-72.

88. Emter, R., A. Heese-Peck, and A. Kralli. 2002. ERG6 and PDR5 regulate small lipophilic drug accumulation in yeast cells via distinct mechanisms. FEBS Lett 521:57-61.

89. Enoch, D. A., H. A. Ludlam, and N. M. Brown. 2006. Invasive fungal infections: a review of epidemiology and management options. J Med Microbiol 55:809-18.

90. Espenshade, P. J., and A. L. Hughes. 2007. Regulation of sterol synthesis in eukaryotes. Annu Rev Genet 41:401-27.

91. Espinel-Ingroff, A., M. Bartlett, R. Bowden, N. X. Chin, C. Cooper, Jr., A. Fothergill, M. R. McGinnis, P. Menezes, S. A. Messer, P. W. Nelson, F. C. Odds, L. Pasarell, J. Peter, M. A. Pfaller, J. H. Rex, M. G. Rinaldi, G. S. Shankland, T. J. Walsh, and I. Weitzman. 1997. Multicenter evaluation of proposed standardized procedure for antifungal susceptibility testing of filamentous fungi. J Clin Microbiol 35:139-43.

92. Fagundes, L. J. M. 1974. Estudo de esteróis e lipídeos totais em Leptomonas pessoai cultivada a 28°C e 37°C. Masters thesis. Rio de Janeiro, Brazil: Instituto de Microbiologia da Universidade Federal do Rio de Janeiro.

93. Fairs, A., J. Agbetile, B. Hargadon, M. Bourne, W. R. Monteiro, C. E. Brightling, P. Bradding, R. H. Green, K. Mutalithas, D. Desai, I. D. Pavord, A. J. Wardlaw, and C. H. Pashley. 2010. IgE sensitization to Aspergillus fumigatus is associated with reduced lung function in asthma. Am J Respir Crit Care Med 182:1362-8.

94. Feng, X., K. Krishnan, D. L. Richie, V. Aimanianda, L. Hartl, N. Grahl, M. V. Powers-Fletcher, M. Zhang, K. K. Fuller, W. C. Nierman, L. J. Lu, J. P. Latge, L. Woollett, S. L. Newman, R. A. Cramer, Jr., J. C. Rhodes, and D. S. Askew. 2011. HacA-independent functions of the ER stress sensor IreA synergize with the canonical UPR to influence virulence traits in Aspergillus fumigatus. PLoS Pathog 7:e1002330.

95. Filler, S. G., M. R. Yeaman, and D. C. Sheppard. 2005. Tumor necrosis factor inhibition and invasive fungal infections. Clin Infect Dis 41 Suppl 3:S208-12.

96. Florio, A. M., C. M. Ingram, H. A. Rakotondravony, E. E. Louis, and C. J. Raxworthy. 2012. Detecting cryptic speciation in the widespread and morphologically conservative carpet chameleon (Furcifer lateralis) of Madagascar. J Evol Biol 25:1399-414.

97. Fontaine, T., A. Beauvais, C. Loussert, B. Thevenard, C. C. Fulgsang, N. Ohno, C. Clavaud, M. C. Prevost, and J. P. Latge. 2010. Cell wall alpha1-3glucans induce the aggregation of germinating conidia of Aspergillus fumigatus. Fungal Genet Biol 47:707-12.

98. Fortwendel, J. R., P. R. Juvvadi, N. Pinchai, B. Z. Perfect, J. A. Alspaugh, J. R. Perfect, and W. J. Steinbach. 2009. Differential effects of inhibiting chitin and 1,3-{beta}-D-glucan synthesis in ras and calcineurin mutants of Aspergillus fumigatus. Antimicrob Agents Chemother 53:476-82.

99. Fraczek, M. G., M. Bromley, and P. Bowyer. 2011. An Improved Model of the Aspergillus fumigatus CYP51A Protein. Antimicrobial Agents and Chemotherapy 55:2483-2486.
100. Fryberg, M., A. C. Oehlschlager, and A. M. Unrau. 1973. Biosynthesis of ergosterol in yeast. Evidence for multiple pathways. J Am Chem Soc 95:5747-57.

101. Fuller, K. K., D. L. Richie, X. Feng, K. Krishnan, T. J. Stephens, K. A. Wikenheiser-Brokamp, D. S. Askew, and J. C. Rhodes. 2011. Divergent Protein Kinase A isoforms co-ordinately regulate conidial germination, carbohydrate metabolism and virulence in Aspergillus fumigatus. Mol Microbiol 79:1045-62.

102. Fuller, K. K., W. Zhao, D. S. Askew, and J. C. Rhodes. 2009. Deletion of the protein kinase A regulatory subunit leads to deregulation of mitochondrial activation and nuclear duplication in Aspergillus fumigatus. Eukaryot Cell 8:271-7.

103. Gachotte, D., C. A. Pierson, N. D. Lees, R. Barbuch, C. Koegel, and M. Bard. 1997. A yeast sterol auxotroph (erg25) is rescued by addition of azole antifungals and reduced levels of heme. Proc Natl Acad Sci U S A 94:11173-8.

104. Galea, A. M., and A. J. Brown. 2009. Special relationship between sterols and oxygen: were sterols an adaptation to aerobic life? Free Radic Biol Med 47:880-9.

105. Gardner, P. R., A. M. Gardner, L. A. Martin, and A. L. Salzman. 1998. Nitric oxide dioxygenase: an enzymic function for flavohemoglobin. Proc Natl Acad Sci U S A 95:10378-83.

106. Geber, A., C. A. Hitchcock, J. E. Swartz, F. S. Pullen, K. E. Marsden, K. J. Kwon-Chung, and J. E. Bennett. 1995. Deletion of the Candida glabrata ERG3 and ERG11 genes: effect on cell viability, cell growth, sterol composition, and antifungal susceptibility. Antimicrob Agents Chemother 39:2708-17.

107. Gehrke, A., T. Heinekamp, I. D. Jacobsen, and A. A. Brakhage. 2010. Heptahelical receptors GprC and GprD of Aspergillus fumigatus Are essential regulators of colony growth, hyphal morphogenesis, and virulence. Appl Environ Microbiol 76:3989-98.

108. George, M. J., D. R. Snydman, B. G. Werner, J. Griffith, M. E. Falagas, N. N. Dougherty, and R. H. Rubin. 1997. The independent role of cytomegalovirus as a risk factor for invasive fungal disease in orthotopic liver transplant recipients. Boston Center for Liver Transplantation CMVIG-Study Group. Cytogam, MedImmune, Inc. Gaithersburg, Maryland. Am J Med 103:106-13.

109. Gomez, J. E., and J. D. McKinney. 2004. M. tuberculosis persistence, latency, and drug tolerance. Tuberculosis (Edinb) 84:29-44.
110. Gorsich, S. W., B. S. Dien, N. N. Nichols, P. J. Slininger, Z. L. Liu, and C. D. Skory. 2006. Tolerance to furfural-induced stress is associated with pentose phosphate pathway genes ZWF1, GND1, RPE1, and TKL1 in Saccharomyces cerevisiae. Appl Microbiol Biotechnol 71:339-49.

111. Gottlieb, M. S. 2006. Pneumocystis pneumonia--Los Angeles. 1981. Am J Public Health 96:980-1; discussion 982-3.

112. Graham-Pole, J., B. Camitta, J. Casper, G. Elfenbein, S. Gross, R. Herzig, P. Koch, D. Mahoney, R. Marcus, L. Munoz, and et al. 1988. Intravenous immunoglobulin may lessen all forms of infection in patients receiving allogeneic bone marrow transplantation for acute lymphoblastic leukemia: a pediatric oncology group study. Bone Marrow Transplant 3:559-66.

113. Grahl, N., and R. A. Cramer, Jr. 2010. Regulation of hypoxia adaptation: an overlooked virulence attribute of pathogenic fungi? Med Mycol 48:1-15.

114. Grahl, N., S. Puttikamonkul, J. M. Macdonald, M. P. Gamcsik, L. Y. Ngo, T. M. Hohl, and R. A. Cramer. 2011. In vivo hypoxia and a fungal alcohol dehydrogenase influence the pathogenesis of invasive pulmonary aspergillosis. PLoS Pathog 7:e1002145.

115. Gray, K. C., D. S. Palacios, I. Dailey, M. M. Endo, B. E. Uno, B. C. Wilcock, and M. D. Burke. 2012. Amphotericin primarily kills yeast by simply binding ergosterol. Proc Natl Acad Sci U S A 109:2234-9.

116. Graybill, J. R., P. C. Craven, R. L. Taylor, D. M. Williams, and W. E. Magee. 1982. Treatment of murine cryptococcosis with liposome-associated amphotericin B. J Infect Dis 145:748-52.

117. Greenberger, P. A., and R. Patterson. 1988. Allergic bronchopulmonary aspergillosis and the evaluation of the patient with asthma. J Allergy Clin Immunol 81:646-50.

118. Greenberger, P. A., L. J. Smith, C. C. Hsu, M. Roberts, and J. L. Liotta. 1988. Analysis of bronchoalveolar lavage in allergic bronchopulmonary aspergillosis: divergent responses of antigen-specific antibodies and total IgE. J Allergy Clin Immunol 82:164-70.

119. Haas, H., I. Zadra, G. Stoffler, and K. Angermayr. 1999. The Aspergillus nidulans GATA factor SREA is involved in regulation of siderophore biosynthesis and control of iron uptake. J Biol Chem 274:4613-9.

120. Hadrich, I., F. Makni, S. Neji, S. Abbes, F. Cheikhrouhou, H. Trabelsi, H. Sellami, and A. Ayadi. 2012. Invasive aspergillosis: resistance to antifungal drugs. Mycopathologia 174:131-41.

121. Hall, L. A., and D. W. Denning. 1994. Oxygen requirements of Aspergillus species. J Med Microbiol 41:311-5.

122. Hameed, S., S. Dhamgaye, A. Singh, S. K. Goswami, and R. Prasad. 2011. Calcineurin signaling and membrane lipid homeostasis regulates iron mediated multidrug resistance mechanisms in Candida albicans. PLoS One 6:e18684.

123. Harold, F. M. 1999. In pursuit of the whole hypha. Fungal Genet Biol 27:128-33.

124. Harris, S. D., and M. Momany. 2004. Polarity in filamentous fungi: moving beyond the yeast paradigm. Fungal Genet Biol 41:391-400.

125. Hartmann, T., C. Sasse, A. Schedler, M. Hasenberg, M. Gunzer, and S. Krappmann. 2011. Shaping the fungal adaptome--stress responses of Aspergillus fumigatus. Int J Med Microbiol 301:408-16.

126. He, M., L. E. Kratz, J. J. Michel, A. N. Vallejo, L. Ferris, R. I. Kelley, J. J. Hoover, D. Jukic, K. M. Gibson, L. A. Wolfe, D. Ramachandran, M. E. Zwick, and J. Vockley. 2011. Mutations in the human SC4MOL gene encoding a methyl sterol oxidase cause psoriasiform dermatitis, microcephaly, and developmental delay. J Clin Invest 121:976-84.

127. Henry-Berger, J., K. Mouzat, S. Baron, C. Bernabeu, G. Marceau, J. P. Saru, V. Sapin, J. M. Lobaccaro, and F. Caira. 2008. Endoglin (CD105) expression is regulated by the liver X receptor alpha (NR1H3) in human trophoblast cell line JAR. Biol Reprod 78:968-75.

128. Herbrecht, R., D. W. Denning, T. F. Patterson, J. E. Bennett, R. E. Greene, J. W. Oestmann, W. V. Kern, K. A. Marr, P. Ribaud, O. Lortholary, R. Sylvester, R. H. Rubin, J. R. Wingard, P. Stark, C. Durand, D. Caillot, E. Thiel, P. H. Chandrasekar, M. R. Hodges, H. T. Schlamm, P. F. Troke, and B. de Pauw. 2002. Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. N Engl J Med 347:408-15.

129. Herbrecht, R., S. Natarajan-Ame, V. Letscher-Bru, and M. Canuet. 2004. Invasive pulmonary aspergillosis. Semin Respir Crit Care Med 25:191-202.

130. Herman, G. E., and L. Kratz. 2012. Disorders of sterol synthesis: beyond Smith-Lemli-Opitz syndrome. Am J Med Genet C Semin Med Genet 160C:301-21.

131. Hickman, M. J., and F. Winston. 2007. Heme levels switch the function of Hap1 of Saccharomyces cerevisiae between transcriptional activator and transcriptional repressor. Mol Cell Biol 27:7414-24.

132. Hicks, J. K., J. H. Yu, N. P. Keller, and T. H. Adams. 1997. Aspergillus sporulation and mycotoxin production both require inactivation of the FadA G alpha protein-dependent signaling pathway. EMBO J 16:4916-23.

133. Hirata, D., K. Yano, and T. Miyakawa. 1994. Stress-induced transcriptional activation mediated by YAP1 and YAP2 genes that encode the Jun family of transcriptional activators in Saccharomyces cerevisiae. Mol Gen Genet 242:250-6.

134. Hoot, S. J., A. R. Smith, R. P. Brown, and T. C. White. 2011. An A643V amino acid substitution in Upc2p contributes to azole resistance in well-characterized clinical isolates of Candida albicans. Antimicrob Agents Chemother 55:940-2.

135. Hortschansky, P., M. Eisendle, Q. Al-Abdallah, A. D. Schmidt, S. Bergmann, M. Thon, O. Kniemeyer, B. Abt, B. Seeber, E. R. Werner, M. Kato, A. A. Brakhage, and H. Haas. 2007. Interaction of HapX with the CCAAT-binding complex--a novel mechanism of gene regulation by iron. EMBO J 26:3157-68.

136. Hospenthal, D. R., K. J. Kwon-Chung, and J. E. Bennett. 1998. Concentrations of airborne Aspergillus compared to the incidence of invasive aspergillosis: lack of correlation. Med Mycol 36:165-8.

137. Howard, S. J., I. Webster, C. B. Moore, R. E. Gardiner, S. Park, D. S. Perlin, and D. W. Denning. 2006. Multi-azole resistance in Aspergillus fumigatus. Int J Antimicrob Agents 28:450-3.

138. Hu, W., S. Sillaots, S. Lemieux, J. Davison, S. Kauffman, A. Breton, A. Linteau, C. Xin, J. Bowman, J. Becker, B. Jiang, and T. Roemer. 2007. Essential gene identification and drug target prioritization in Aspergillus fumigatus. PLoS Pathog 3:e24.

139. Huang, D. B., J. J. Wu, and C. J. Lahart. 2004. Toxic epidermal necrolysis as a complication of treatment with voriconazole. South Med J 97:1116-7.

140. Hughes, A. L., C. Y. Lee, C. M. Bien, and P. J. Espenshade. 2007. 4-Methyl sterols regulate fission yeast SREBP-Scap under low oxygen and cell stress. J Biol Chem 282:24388-96.

141. Hughes, A. L., B. L. Todd, and P. J. Espenshade. 2005. SREBP pathway responds to sterols and functions as an oxygen sensor in fission yeast. Cell 120:831-42.

142. Husni, R. N., S. M. Gordon, D. L. Longworth, A. Arroliga, P. C. Stillwell, R. K. Avery, J. R. Maurer, A. Mehta, and T. Kirby. 1998. Cytomegalovirus infection is a risk factor for invasive aspergillosis in lung transplant recipients. Clin Infect Dis 26:753-5.

143. Ibrahim-Granet, O., B. Philippe, H. Boleti, E. Boisvieux-Ulrich, D. Grenet, M. Stern, and J. P. Latge. 2003. Phagocytosis and intracellular fate of Aspergillus fumigatus conidia in alveolar macrophages. Infect Immun 71:891-903.

144. Jackson, C. J., D. C. Lamb, N. J. Manning, D. E. Kelly, and S. L. Kelly. 2003. Mutations in Saccharomyces cerevisiae sterol C5-desaturase conferring resistance to the CYP51 inhibitor fluconazole. Biochem Biophys Res Commun 309:999-1004.

145. Jacobson, E. S., A. P. Goodner, and K. J. Nyhus. 1998. Ferrous iron uptake in Cryptococcus neoformans. Infect Immun 66:4169-75.

146. Jahn, B., A. Koch, A. Schmidt, G. Wanner, H. Gehringer, S. Bhakdi, and A. A. Brakhage. 1997. Isolation and characterization of a pigmentless-conidium mutant of Aspergillus fumigatus with altered conidial surface and reduced virulence. Infect Immun 65:5110-7.

147. Jain, R., V. Valiante, N. Remme, T. Docimo, T. Heinekamp, C. Hertweck, J. Gershenzon, H. Haas, and A. A. Brakhage. 2011. The MAP kinase MpkA controls cell wall integrity, oxidative stress response, gliotoxin production and iron adaptation in Aspergillus fumigatus. Mol Microbiol 82:39-53.

148. Janowski, B. A., P. J. Willy, T. R. Devi, J. R. Falck, and D. J. Mangelsdorf. 1996. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. Nature 383:728-31.

149. Ji, Y., F. Yang, D. Ma, J. Zhang, Z. Wan, W. Liu, and R. Li. 2012. HOG-MAPK signaling regulates the adaptive responses of Aspergillus fumigatus to thermal stress and other related stress. Mycopathologia 174:273-82.

150. Jia, N., B. Arthington-Skaggs, W. Lee, C. A. Pierson, N. D. Lees, J. Eckstein, R. Barbuch, and M. Bard. 2002. Candida albicans sterol C-14 reductase, encoded

by the ERG24 gene, as a potential antifungal target site. Antimicrob Agents Chemother 46:947-57.

151. Jollow, D., G. M. Kellerman, and A. W. Linnane. 1968. The biogenesis of mitochondria. 3. The lipid composition of aerobically and anaerobically grown Saccharomyces cerevisiae as related to the membrane systems of the cells. J Cell Biol 37:221-30.
152. Juhnke, H., B. Krems, P. Kotter, and K. D. Entian. 1996. Mutants that show increased sensitivity to hydrogen peroxide reveal an important role for the pentose phosphate pathway in protection of yeast against oxidative stress. Mol Gen Genet 252:456-64.

153. Kainer, M. A., D. R. Reagan, D. B. Nguyen, A. D. Wiese, M. E. Wise, J. Ward, B. J. Park, M. L. Kanago, J. Baumblatt, M. K. Schaefer, B. E. Berger, E. P. Marder, J. Y. Min, J. R. Dunn, R. M. Smith, J. Dreyzehner, and T. F. Jones. 2012. Fungal infections associated with contaminated methylprednisolone in Tennessee. N Engl J Med 367:2194-203.

154. Karthaus, M., and O. A. Cornely. 2007. Treatment options in candidaemia. Mycoses 50 Suppl 1:44-9.

155. Kawazu, M., Y. Kanda, Y. Nannya, K. Aoki, M. Kurokawa, S. Chiba, T. Motokura, H. Hirai, and S. Ogawa. 2004. Prospective comparison of the diagnostic potential of real-time PCR, double-sandwich enzyme-linked immunosorbent assay for galactomannan, and a (1-->3)-beta-D-glucan test in weekly screening for invasive aspergillosis in patients with hematological disorders. J Clin Microbiol 42:2733-41.

156. Keating, M. R., M. A. Guerrero, R. C. Daly, R. C. Walker, and S. F. Davies. 1996. Transmission of invasive aspergillosis from a subclinically infected donor to three different organ transplant recipients. Chest 109:1119-24.

157. Keber, R., D. Rozman, and S. Horvat. 2013. Sterols in spermatogenesis and sperm maturation. J Lipid Res 54:20-33.

158. Keilin, D., and A. Tissieres. 1953. Haemoglobin in moulds: Neurospora crassa and Penicillium notatum. Nature 172:393-4.

159. Kelly, S. L., D. C. Lamb, A. J. Corran, B. C. Baldwin, and D. E. Kelly. 1995. Mode of action and resistance to azole antifungals associated with the formation of 14 alpha-methylergosta-8,24(28)-dien-3 beta,6 alpha-diol. Biochem Biophys Res Commun 207:910-5.

160. Kelly, S. L., D. C. Lamb, D. E. Kelly, J. Loeffler, and H. Einsele. 1996. Resistance to fluconazole and amphotericin in Candida albicans from AIDS patients. Lancet 348:1523-4.

161. Kelly, S. L., D. C. Lamb, D. E. Kelly, N. J. Manning, J. Loeffler, H. Hebart, U. Schumacher, and H. Einsele. 1997. Resistance to fluconazole and cross-resistance to amphotericin B in Candida albicans from AIDS patients caused by defective sterol delta5,6-desaturation. FEBS Lett 400:80-2.

162. Kennedy, J. L., P. W. Heymann, and T. A. Platts-Mills. 2012. The role of allergy in severe asthma. Clin Exp Allergy 42:659-69.

163. Kennedy, M. A., T. A. Johnson, N. D. Lees, R. Barbuch, J. A. Eckstein, and M. Bard. 2000. Cloning and sequencing of the Candida albicans C-4 sterol methyl oxidase gene (ERG25) and expression of an ERG25 conditional lethal mutation in Saccharomyces cerevisiae. Lipids 35:257-62.

164. Kim, J., B. Campbell, N. Mahoney, K. Chan, R. Molyneux, and G. May. 2008. Chemosensitization prevents tolerance of Aspergillus fumigatus to antimycotic drugs. Biochem Biophys Res Commun 372:266-71.

165. Knutsen, A. P., K. R. Mueller, A. D. Levine, B. Chouhan, P. S. Hutcheson, and R. G. Slavin. 1994. Asp f I CD4+ TH2-like T-cell lines in allergic bronchopulmonary aspergillosis. J Allergy Clin Immunol 94:215-21.

166. Knutsen, A. P., and R. G. Slavin. 2011. Allergic bronchopulmonary aspergillosis in asthma and cystic fibrosis. Clin Dev Immunol 2011:843763.

167. Kontoyiannis, D. P. 2000. Modulation of fluconazole sensitivity by the interaction of mitochondria and erg3p in Saccharomyces cerevisiae. J Antimicrob Chemother 46:191-7.

168. Kontoyiannis, D. P., G. Chamilos, R. E. Lewis, S. Giralt, J. Cortes, Raad, II, J. T. Manning, and X. Han. 2007. Increased bone marrow iron stores is an independent risk factor for invasive aspergillosis in patients with high-risk hematologic malignancies and recipients of allogeneic hematopoietic stem cell transplantation. Cancer 110:1303-6.

169. Kousha, M., R. Tadi, and A. O. Soubani. 2011. Pulmonary aspergillosis: a clinical review. Eur Respir Rev 20:156-74.

170. Kronstad, J., S. Saikia, E. D. Nielson, M. Kretschmer, W. Jung, G. Hu, J. M. Geddes, E. J. Griffiths, J. Choi, B. Cadieux, M. Caza, and R. Attarian. 2012. Adaptation of Cryptococcus neoformans to mammalian hosts: integrated regulation of metabolism and virulence. Eukaryot Cell 11:109-18.

171. Kusne, S., J. S. Dummer, N. Singh, S. Iwatsuki, L. Makowka, C. Esquivel, A. G. Tzakis, T. E. Starzl, and M. Ho. 1988. Infections after liver transplantation. An analysis of 101 consecutive cases. Medicine (Baltimore) 67:132-43.

172. Kwast, K. E., L. C. Lai, N. Menda, D. T. James, 3rd, S. Aref, and P. V. Burke. 2002. Genomic analyses of anaerobically induced genes in Saccharomyces cerevisiae: functional roles of Rox1 and other factors in mediating the anoxic response. J Bacteriol 184:250-65.

173. Lamaris, G. A., R. E. Lewis, G. Chamilos, G. S. May, A. Safdar, T. J. Walsh, Raad, II, and D. P. Kontoyiannis. 2008. Caspofungin-mediated beta-glucan unmasking and enhancement of human polymorphonuclear neutrophil activity against Aspergillus and non-Aspergillus hyphae. J Infect Dis 198:186-92.

174. Lamarre, C., S. Sokol, J. P. Debeaupuis, C. Henry, C. Lacroix, P. Glaser, J. Y. Coppee, J. M. Francois, and J. P. Latge. 2008. Transcriptomic analysis of the exit from dormancy of Aspergillus fumigatus conidia. BMC Genomics 9:417.

175. Lane, S., S. Zhou, T. Pan, Q. Dai, and H. Liu. 2001. The basic helix-loop-helix transcription factor Cph2 regulates hyphal development in Candida albicans partly via TEC1. Mol Cell Biol 21:6418-28.

176. Langfelder, K., B. Jahn, H. Gehringer, A. Schmidt, G. Wanner, and A. A. Brakhage. 1998. Identification of a polyketide synthase gene (pksP) of Aspergillus fumigatus involved in conidial pigment biosynthesis and virulence. Med Microbiol Immunol 187:79-89.

177. Laufer, P., J. N. Fink, W. T. Bruns, G. F. Unger, J. H. Kalbfleisch, P. A. Greenberger, and R. Patterson. 1984. Allergic bronchopulmonary aspergillosis in cystic fibrosis. J Allergy Clin Immunol 73:44-8.

178. Leal, S. M., Jr., C. Vareechon, S. Cowden, B. A. Cobb, J. P. Latge, M. Momany, and E. Pearlman. 2012. Fungal antioxidant pathways promote survival against neutrophils during infection. J Clin Invest 122:2482-98.

179. Leclair, L. W., and D. A. Hogan. 2010. Mixed bacterial-fungal infections in the CF respiratory tract. Med Mycol 48 Suppl 1:S125-32.

180. Lee, H., C. M. Bien, A. L. Hughes, P. J. Espenshade, K. J. Kwon-Chung, and Y. C. Chang. 2007. Cobalt chloride, a hypoxia-mimicking agent, targets sterol

synthesis in the pathogenic fungus Cryptococcus neoformans. Mol Microbiol 65:1018-33.

181. Lees, N. D., B. Skaggs, D. R. Kirsch, and M. Bard. 1995. Cloning of the late genes in the ergosterol biosynthetic pathway of Saccharomyces cerevisiae--a review. Lipids 30:221-6.

182. Lehmann, J. M., S. A. Kliewer, L. B. Moore, T. A. Smith-Oliver, B. B. Oliver, J. L. Su, S. S. Sundseth, D. A. Winegar, D. E. Blanchard, T. A. Spencer, and T. M. Willson. 1997. Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. J Biol Chem 272:3137-40.

183. Lessing, F., O. Kniemeyer, I. Wozniok, J. Loeffler, O. Kurzai, A. Haertl, and A. A. Brakhage. 2007. The Aspergillus fumigatus transcriptional regulator AfYap1 represents the major regulator for defense against reactive oxygen intermediates but is dispensable for pathogenicity in an intranasal mouse infection model. Eukaryot Cell 6:2290-302.

184. Levin, D. E. 2005. Cell wall integrity signaling in Saccharomyces cerevisiae. Microbiol Mol Biol Rev 69:262-91.

185. Lewis, R. E., and D. P. Kontoyiannis. 2009. Invasive aspergillosis in glucocorticoid-treated patients. Med Mycol 47 Suppl 1:S271-81.

186. Lewis, T. L., G. A. Keesler, G. P. Fenner, and L. W. Parks. 1988. Pleiotropic mutations in Saccharomyces cerevisiae affecting sterol uptake and metabolism. Yeast 4:93-106.

187. Li, L., and J. Kaplan. 1996. Characterization of yeast methyl sterol oxidase (ERG25) and identification of a human homologue. J Biol Chem 271:16927-33.

188. Li, S., L. Du, G. Yuen, and S. D. Harris. 2006. Distinct ceramide synthases regulate polarized growth in the filamentous fungus Aspergillus nidulans. Mol Biol Cell 17:1218-27.

189. Li, X., T. A. Gianoulis, K. Y. Yip, M. Gerstein, and M. Snyder. 2010. Extensive in vivo metabolite-protein interactions revealed by large-scale systematic analyses. Cell 143:639-50.

190. Liebmann, B., S. Gattung, B. Jahn, and A. A. Brakhage. 2003. cAMP signaling in Aspergillus fumigatus is involved in the regulation of the virulence gene pksP and in defense against killing by macrophages. Mol Genet Genomics 269:420-35.

191. Liu, J., and A. Barrientos. 2012. Transcriptional Regulation of Yeast Oxidative Phosphorylation Hypoxic Genes by Oxidative Stress. Antioxid Redox Signal.

192. Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-8.

193. Lo, H. J., J. S. Wang, C. Y. Lin, C. G. Chen, T. Y. Hsiao, C. T. Hsu, C. L. Su, M. J. Fann, Y. T. Ching, and Y. L. Yang. 2005. Efg1 involved in drug resistance by regulating the expression of ERG3 in Candida albicans. Antimicrob Agents Chemother 49:1213-5.

194. Low, C., R. J. Rodriguez, and L. W. Parks. 1985. Modulation of yeast plasma membrane composition of a yeast sterol auxotroph as a function of exogenous sterol. Arch Biochem Biophys 240:530-8.

195. MacPherson, S., B. Akache, S. Weber, X. De Deken, M. Raymond, and B. Turcotte. 2005. Candida albicans zinc cluster protein Upc2p confers resistance to antifungal drugs and is an activator of ergosterol biosynthetic genes. Antimicrob Agents Chemother 49:1745-52.

196. Mansfield, B. E., H. N. Oltean, B. G. Oliver, S. J. Hoot, S. E. Leyde, L. Hedstrom, and T. C. White. 2010. Azole drugs are imported by facilitated diffusion in Candida albicans and other pathogenic fungi. PLoS Pathog 6:e1001126.

197. Marichal, P., and H. Vanden Bossche. 1995. Mechanisms of resistance to azole antifungals. Acta Biochim Pol 42:509-16.

198. Marie, C., S. Leyde, and T. C. White. 2008. Cytoplasmic localization of sterol transcription factors Upc2p and Ecm22p in S. cerevisiae. Fungal Genet Biol 45:1430-8.

199. Martel, C. M., J. E. Parker, O. Bader, M. Weig, U. Gross, A. G. Warrilow, D. E. Kelly, and S. L. Kelly. 2010. A clinical isolate of Candida albicans with mutations in ERG11 (encoding sterol 14alpha-demethylase) and ERG5 (encoding C22 desaturase) is cross resistant to azoles and amphotericin B. Antimicrob Agents Chemother 54:3578-83.

200. Martel, C. M., J. E. Parker, O. Bader, M. Weig, U. Gross, A. G. Warrilow, N. Rolley, D. E. Kelly, and S. L. Kelly. 2010. Identification and characterization of four azole-resistant erg3 mutants of Candida albicans. Antimicrob Agents Chemother 54:4527-33.

201. Martin, M. V. 1999. The use of fluconazole and itraconazole in the treatment of Candida albicans infections: a review. J Antimicrob Chemother 44:429-37.

202. May, G. S., T. Xue, D. P. Kontoyiannis, and M. C. Gustin. 2005. Mitogen activated protein kinases of Aspergillus fumigatus. Med Mycol 43 Suppl 1:S83-6.

203. McCormick, A., L. Heesemann, J. Wagener, V. Marcos, D. Hartl, J. Loeffler, J. Heesemann, and F. Ebel. 2010. NETs formed by human neutrophils inhibit growth of the pathogenic mold Aspergillus fumigatus. Microbes Infect 12:928-36.

204. McCormick, A., I. D. Jacobsen, M. Broniszewska, J. Beck, J. Heesemann, and F. Ebel. 2012. The two-component sensor kinase TcsC and its role in stress resistance of the human-pathogenic mold Aspergillus fumigatus. PLoS One 7:e38262.

205. McCormick, A., J. Loeffler, and F. Ebel. 2010. Aspergillus fumigatus: contours of an opportunistic human pathogen. Cell Microbiol 12:1535-43.

206. McDonagh, A., N. D. Fedorova, J. Crabtree, Y. Yu, S. Kim, D. Chen, O. Loss, T. Cairns, G. Goldman, D. Armstrong-James, K. Haynes, H. Haas, M. Schrettl, G. May, W. C. Nierman, and E. Bignell. 2008. Sub-telomere directed gene expression during initiation of invasive aspergillosis. PLoS Pathog 4:e1000154.

207. McKinney, J. D., and J. E. Gomez. 2003. Life on the inside for Mycobacterium tuberculosis. Nat Med 9:1356-7.

208. Meersseman, W., K. Lagrou, J. Maertens, and E. Van Wijngaerden. 2007. Invasive aspergillosis in the intensive care unit. Clin Infect Dis 45:205-16.

209. Mehta, K., and Z. H. Siddik. 2009. Drug resistance in cancer cells. Springer, New York, NY.

210. Mellado, E., T. M. Diaz-Guerra, M. Cuenca-Estrella, and J. L. Rodriguez-Tudela. 2001. Identification of two different 14-alpha sterol demethylase-related genes (cyp51A and cyp51B) in Aspergillus fumigatus and other Aspergillus species. J Clin Microbiol 39:2431-8.

211. Mellado, E., G. Garcia-Effron, L. Alcazar-Fuoli, W. J. Melchers, P. E. Verweij, M. Cuenca-Estrella, and J. L. Rodriguez-Tudela. 2007. A new Aspergillus fumigatus resistance mechanism conferring in vitro cross-resistance to azole

antifungals involves a combination of cyp51A alterations. Antimicrob Agents Chemother 51:1897-904.

212. Mellado, E., G. Garcia-Effron, M. J. Buitrago, L. Alcazar-Fuoli, M. Cuenca-Estrella, and J. L. Rodriguez-Tudela. 2005. Targeted gene disruption of the 14-alpha sterol demethylase (cyp51A) in Aspergillus fumigatus and its role in azole drug susceptibility. Antimicrob Agents Chemother 49:2536-8.

213. Menke, J. G., K. L. Macnaul, N. S. Hayes, J. Baffic, Y. S. Chao, A. Elbrecht, L. J. Kelly, M. H. Lam, A. Schmidt, S. Sahoo, J. Wang, S. D. Wright, P. Xin, G. Zhou, D. E. Moller, and C. P. Sparrow. 2002. A novel liver X receptor agonist establishes species differences in the regulation of cholesterol 7alpha-hydroxylase (CYP7a). Endocrinology 143:2548-58.

214. Meyer, R. D. 1992. Current role of therapy with amphotericin B. Clin Infect Dis 14 Suppl 1:S154-60.

215. Micheli, P. A. 1729. Nova Plantarum Genera.

216. Miller, D. D., E. W. Cowen, J. C. Nguyen, T. H. McCalmont, and L. P. Fox. 2010. Melanoma associated with long-term voriconazole therapy: a new manifestation of chronic photosensitivity. Arch Dermatol 146:300-4.

217. Miyazaki, T., Y. Miyazaki, K. Izumikawa, H. Kakeya, S. Miyakoshi, J. E. Bennett, and S. Kohno. 2006. Fluconazole treatment is effective against a Candida albicans erg3/erg3 mutant in vivo despite in vitro resistance. Antimicrob Agents Chemother 50:580-6.

218. Mo, C., and M. Bard. 2005. Erg28p is a key protein in the yeast sterol biosynthetic enzyme complex. J Lipid Res 46:1991-8.

219. Mo, C., and M. Bard. 2005. A systematic study of yeast sterol biosynthetic protein-protein interactions using the split-ubiquitin system. Biochim Biophys Acta 1737:152-60.

220. Mo, C., M. Valachovic, and M. Bard. 2004. The ERG28-encoded protein, Erg28p, interacts with both the sterol C-4 demethylation enzyme complex as well as the late biosynthetic protein, the C-24 sterol methyltransferase (Erg6p). Biochim Biophys Acta 1686:30-6.

221. Mogavero, S., A. Tavanti, S. Senesi, P. D. Rogers, and J. Morschhauser. 2011. Differential Requirement of the Transcription Factor Mcm1 for Activation of the Candida albicans Multidrug Efflux Pump MDR1 by Its Regulators Mrr1 and Cap1. Antimicrob Agents Chemother 55:2061-6.

222. Momany, M., P. J. Westfall, and G. Abramowsky. 1999. Aspergillus nidulans swo mutants show defects in polarity establishment, polarity maintenance and hyphal morphogenesis. Genetics 151:557-67.

223. Montanes, F. M., A. Pascual-Ahuir, and M. Proft. 2011. Repression of ergosterol biosynthesis is essential for stress resistance and is mediated by the Hog1 MAP kinase and the Mot3 and Rox1 transcription factors. Mol Microbiol 79:1008-23.

224. Muijsers, R. B., K. L. Goa, and L. J. Scott. 2002. Voriconazole: in the treatment of invasive aspergillosis. Drugs 62:2655-64; discussion 2665-6.

225. Naar, A. M., and J. K. Thakur. 2009. Nuclear receptor-like transcription factors in fungi. Genes Dev 23:419-32.

226. Nailis, H., D. Vandenbosch, D. Deforce, H. J. Nelis, and T. Coenye. 2010. Transcriptional response to fluconazole and amphotericin B in Candida albicans biofilms. Res Microbiol 161:284-92.

227. Nascimento, A. M., L. M. de Souza, C. H. Baggio, M. F. Werner, D. Maria-Ferreira, L. M. da Silva, G. L. Sassaki, P. A. Gorin, M. Iacomini, and T. R. Cipriani. 2013. Gastroprotective effect and structure of a rhamnogalacturonan from Acmella oleracea. Phytochemistry 85:137-42.

228. Nes, W. D., S. H. Xu, and W. F. Haddon. 1989. Evidence for similarities and differences in the biosynthesis of fungal sterols. Steroids 53:533-58.

229. Ng, A. W., K. M. Wasan, and G. Lopez-Berestein. 2003. Development of liposomal polyene antibiotics: an historical perspective. J Pharm Pharm Sci 6:67-83.

230. Nguyen, A. D., J. G. McDonald, R. K. Bruick, and R. A. DeBose-Boyd. 2007. Hypoxia stimulates degradation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase through accumulation of lanosterol and hypoxia-inducible factor-mediated induction of insigs. J Biol Chem 282:27436-46.

231. Nierman, W. C., A. Pain, M. J. Anderson, J. R. Wortman, H. S. Kim, J. Arroyo, M. Berriman, K. Abe, D. B. Archer, C. Bermejo, J. Bennett, P. Bowyer, D. Chen, M. Collins, R. Coulsen, R. Davies, P. S. Dyer, M. Farman, N. Fedorova, T. V. Feldblyum, R. Fischer, N. Fosker, A. Fraser, J. L. Garcia, M. J. Garcia, A. Goble, G. H. Goldman, K. Gomi, S. Griffith-Jones, R. Gwilliam, B. Haas, H. Haas, D. Harris, H. Horiuchi, J. Huang, S. Humphray, J. Jimenez, N. Keller, H. Khouri, K.

Kitamoto, T. Kobayashi, S. Konzack, R. Kulkarni, T. Kumagai, A. Lafon, J. P. Latge, W. Li, A. Lord, C. Lu, W. H. Majoros, G. S. May, B. L. Miller, Y. Mohamoud, M. Molina, M. Monod, I. Mouyna, S. Mulligan, L. Murphy, S. O'Neil, I. Paulsen, M. A. Penalva, M. Pertea, C. Price, B. L. Pritchard, M. A. Quail, E. Rabbinowitsch, N. Rawlins, M. A. Rajandream, U. Reichard, H. Renauld, G. D. Robson, S. Rodriguez de Cordoba, J. M. Rodriguez-Pena, C. M. Ronning, S. Rutter, S. L. Salzberg, M. Sanchez, J. C. Sanchez-Ferrero, D. Saunders, K. Seeger, R. Squares, S. Squares, M. Takeuchi, F. Tekaia, G. Turner, C. R. Vazquez de Aldana, J. Weidman, O. White, J. Woodward, J. H. Yu, C. Fraser, J. E. Galagan, K. Asai, M. Machida, N. Hall, B. Barrell, and D. W. Denning. 2005. Genomic sequence of the pathogenic and allergenic filamentous fungus Aspergillus fumigatus. Nature 438:1151-6.

232. Nobile, C. J., D. R. Andes, J. E. Nett, F. J. Smith, F. Yue, Q. T. Phan, J. E. Edwards, S. G. Filler, and A. P. Mitchell. 2006. Critical role of Bcr1-dependent adhesins in C. albicans biofilm formation in vitro and in vivo. PLoS Pathog 2:e63.

233. Novotny, C., M. Flieger, J. Panos, and F. Karst. 1992. Effect of 5,7-unsaturated sterols on ethanol tolerance in Saccharomyces cerevisiae. Biotechnol Appl Biochem 15:314-20.

234. Oberegger, H., M. Schoeser, I. Zadra, B. Abt, and H. Haas. 2001. SREA is involved in regulation of siderophore biosynthesis, utilization and uptake in Aspergillus nidulans. Mol Microbiol 41:1077-89.

235. Odds, F. C., S. L. Cheesman, and A. B. Abbott. 1986. Antifungal effects of fluconazole (UK 49858), a new triazole antifungal, in vitro. J Antimicrob Chemother 18:473-8.

236. Oliver, B. G., J. C. Panepinto, D. S. Askew, and J. C. Rhodes. 2002. cAMP alteration of growth rate of Aspergillus fumigatus and Aspergillus niger is carbon-source dependent. Microbiology 148:2627-33.

237. Oliver, B. G., J. C. Panepinto, J. R. Fortwendel, D. L. Smith, D. S. Askew, and J. C. Rhodes. 2002. Cloning and expression of pkaC and pkaR, the genes encoding the cAMP-dependent protein kinase of Aspergillus fumigatus. Mycopathologia 154:85-91.

238. Osherov, N., and G. S. May. 2001. The molecular mechanisms of conidial germination. FEMS Microbiol Lett 199:153-60.

239. Oshino, R., N. Oshino, and B. Chance. 1973. Studies on yeast hemoglobin. The properties of yeast hemoglobin and its physiological function in the cell. Eur J Biochem 35:23-33.

240. Pagano, L., M. Caira, A. Candoni, M. Offidani, L. Fianchi, B. Martino, D. Pastore, M. Picardi, A. Bonini, A. Chierichini, R. Fanci, C. Caramatti, R. Invernizzi, D. Mattei, M. E. Mitra, L. Melillo, F. Aversa, M. T. Van Lint, P. Falcucci, C. G. Valentini, C. Girmenia, and A. Nosari. 2006. The epidemiology of fungal infections in patients with hematologic malignancies: the SEIFEM-2004 study. Haematologica 91:1068-75.

241. Panicker, S. P., T. Ganguly, M. Consolo, V. Price, P. Mirmirani, K. Honda, and P. Karnik. 2012. Sterol intermediates of cholesterol biosynthesis inhibit hair growth and trigger an innate immune response in cicatricial alopecia. PLoS One 7:e38449.

242. Pappas, P. G., B. D. Alexander, D. R. Andes, S. Hadley, C. A. Kauffman, A. Freifeld, E. J. Anaissie, L. M. Brumble, L. Herwaldt, J. Ito, D. P. Kontoyiannis, G. M. Lyon, K. A. Marr, V. A. Morrison, B. J. Park, T. F. Patterson, T. M. Perl, R. A. Oster, M. G. Schuster, R. Walker, T. J. Walsh, K. A. Wannemuehler, and T. M. Chiller. 2010. Invasive fungal infections among organ transplant recipients: results of the Transplant-Associated Infection Surveillance Network (TRANSNET). Clin Infect Dis 50:1101-11.

243. Parks, L. W., C. D. Bottema, R. J. Rodriguez, and T. A. Lewis. 1985. Yeast sterols: yeast mutants as tools for the study of sterol metabolism. Methods Enzymol 111:333-46.

244. Parks, L. W., and W. M. Casey. 1995. Physiological implications of sterol biosynthesis in yeast. Annu Rev Microbiol 49:95-116.

245. Pasqualotto, A. C., G. Powell, R. Niven, and D. W. Denning. 2009. The effects of antifungal therapy on severe asthma with fungal sensitization and allergic bronchopulmonary aspergillosis. Respirology 14:1121-7.

246. Paya, C. V. 1993. Fungal infections in solid-organ transplantation. Clin Infect Dis 16:677-88.

247. Pegues, D. A., B. A. Lasker, M. M. McNeil, P. M. Hamm, J. L. Lundal, and B. M. Kubak. 2002. Cluster of cases of invasive aspergillosis in a transplant intensive care unit: evidence of person-to-person airborne transmission. Clin Infect Dis 34:412-6.

248. Philippe, B., O. Ibrahim-Granet, M. C. Prevost, M. A. Gougerot-Pocidalo, M. Sanchez Perez, A. Van der Meeren, and J. P. Latge. 2003. Killing of Aspergillus fumigatus by alveolar macrophages is mediated by reactive oxidant intermediates. Infect Immun 71:3034-42.

249. Pickering, J. W., H. W. Sant, C. A. Bowles, W. L. Roberts, and G. L. Woods. 2005. Evaluation of a (1->3)-beta-D-glucan assay for diagnosis of invasive fungal infections. J Clin Microbiol 43:5957-62.

250. Pierson, C. A., J. Eckstein, R. Barbuch, and M. Bard. 2004. Ergosterol gene expression in wild-type and ergosterol-deficient mutants of Candida albicans. Med Mycol 42:385-9.

251. Pihet, M., J. Carrere, B. Cimon, D. Chabasse, L. Delhaes, F. Symoens, and J. P. Bouchara. 2009. Occurrence and relevance of filamentous fungi in respiratory secretions of patients with cystic fibrosis--a review. Med Mycol 47:387-97.

252. Pike, L. J. 2009. The challenge of lipid rafts. J Lipid Res 50 Suppl:S323-8.

253. Pinjon, E., G. P. Moran, C. J. Jackson, S. L. Kelly, D. Sanglard, D. C. Coleman, and D. J. Sullivan. 2003. Molecular mechanisms of itraconazole resistance in Candida dubliniensis. Antimicrob Agents Chemother 47:2424-37.

254. Poole, R. K. 1994. Oxygen reactions with bacterial oxidases and globins: binding, reduction and regulation. Antonie Van Leeuwenhoek 65:289-310.

255. Porter, J. R., J. S. Burg, P. J. Espenshade, and P. A. Iglesias. 2010. Ergosterol regulates sterol regulatory element binding protein (SREBP) cleavage in fission yeast. J Biol Chem 285:41051-61.

256. Poyton, R. O., K. A. Ball, and P. R. Castello. 2009. Mitochondrial generation of free radicals and hypoxic signaling. Trends Endocrinol Metab 20:332-40.

257. Puttikamonkul, S., S. D. Willger, N. Grahl, J. R. Perfect, N. Movahed, B. Bothner, S. Park, P. Paderu, D. S. Perlin, and R. A. Cramer, Jr. 2010. Trehalose 6-phosphate phosphatase is required for cell wall integrity and fungal virulence but not trehalose biosynthesis in the human fungal pathogen Aspergillus fumigatus. Mol Microbiol.

258. Qiao, J., W. Liu, and R. Li. 2010. Truncated Afyap1 attenuates antifungal susceptibility of Aspergillus fumigatus to voriconazole and confers adaptation of the fungus to oxidative stress. Mycopathologia 170:155-60.

259. Rada, B., C. Hably, A. Meczner, C. Timar, G. Lakatos, P. Enyedi, and E. Ligeti. 2008. Role of Nox2 in elimination of microorganisms. Semin Immunopathol 30:237-53.

260. Rahier, A. 2011. Dissecting the sterol C-4 demethylation process in higher plants. From structures and genes to catalytic mechanism. Steroids 76:340-52.

261. Reichenberger, F., J. Habicht, P. Matt, R. Frei, M. Soler, C. T. Bolliger, P. Dalquen, A. Gratwohl, and M. Tamm. 1999. Diagnostic yield of bronchoscopy in histologically proven invasive pulmonary aspergillosis. Bone Marrow Transplant 24:1195-9.

262. Repa, J. J., G. Liang, J. Ou, Y. Bashmakov, J. M. Lobaccaro, I. Shimomura, B. Shan, M. S. Brown, J. L. Goldstein, and D. J. Mangelsdorf. 2000. Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. Genes Dev 14:2819-30.

263. Reyes, G., A. Romans, C. K. Nguyen, and G. S. May. 2006. Novel mitogen-activated protein kinase MpkC of Aspergillus fumigatus is required for utilization of polyalcohol sugars. Eukaryot Cell 5:1934-40.

264. Richie, D. L., X. Feng, L. Hartl, V. Aimanianda, K. Krishnan, M. V. Powers-Fletcher, D. S. Watson, A. K. Galande, S. M. White, T. Willett, J. P. Latge, J. C. Rhodes, and D. S. Askew. 2011. The virulence of the opportunistic fungal pathogen Aspergillus fumigatus requires cooperation between the endoplasmic reticulum-associated degradation pathway (ERAD) and the unfolded protein response (UPR). Virulence 2:12-21.

265. Richie, D. L., L. Hartl, V. Aimanianda, M. S. Winters, K. K. Fuller, M. D. Miley, S. White, J. W. McCarthy, J. P. Latge, M. Feldmesser, J. C. Rhodes, and D. S. Askew. 2009. A role for the unfolded protein response (UPR) in virulence and antifungal susceptibility in Aspergillus fumigatus. PLoS Pathog 5:e1000258.

266. Rigamonti, E., L. Helin, S. Lestavel, A. L. Mutka, M. Lepore, C. Fontaine, M. A. Bouhlel, S. Bultel, J. C. Fruchart, E. Ikonen, V. Clavey, B. Staels, and G. Chinetti-Gbaguidi. 2005. Liver X receptor activation controls intracellular cholesterol trafficking and esterification in human macrophages. Circ Res 97:682-9.

267. Romani, L. 2004. Immunity to fungal infections. Nat Rev Immunol 4:1-23.

268. Romero, B., G. Turner, I. Olivas, F. Laborda, and J. R. De Lucas. 2003. The Aspergillus nidulans alcA promoter drives tightly regulated conditional gene expression in Aspergillus fumigatus permitting validation of essential genes in this human pathogen. Fungal Genet Biol 40:103-14.

269. Rustad, T. R., A. M. Sherrid, K. J. Minch, and D. R. Sherman. 2009. Hypoxia: a window into Mycobacterium tuberculosis latency. Cell Microbiol 11:1151-9.

270. Ryckeboer, J., J. Mergaert, J. Coosemans, K. Deprins, and J. Swings. 2003. Microbiological aspects of biowaste during composting in a monitored compost bin. J Appl Microbiol 94:127-37.

271. Sandini, S., A. Stringaro, S. Arancia, M. Colone, F. Mondello, S. Murtas, A. Girolamo, N. Mastrangelo, and F. De Bernardis. 2011. The MP65 gene is required for cell wall integrity, adherence to epithelial cells and biofilm formation in Candida albicans. BMC Microbiol 11:106.

272. Sanglard, D., F. Ischer, T. Parkinson, D. Falconer, and J. Bille. 2003. Candida albicans mutations in the ergosterol biosynthetic pathway and resistance to several antifungal agents. Antimicrob Agents Chemother 47:2404-12.

273. Schinko, T., H. Berger, W. Lee, A. Gallmetzer, K. Pirker, R. Pachlinger, I. Buchner, T. Reichenauer, U. Guldener, and J. Strauss. 2010. Transcriptome analysis of nitrate assimilation in Aspergillus nidulans reveals connections to nitric oxide metabolism. Mol Microbiol 78:720-38.

274. Schneider, E., S. Whitmore, K. M. Glynn, K. Dominguez, A. Mitsch, and M. T. McKenna. 2008. Revised surveillance case definitions for HIV infection among adults, adolescents, and children aged <18 months and for HIV infection and AIDS among children aged 18 months to <13 years--United States, 2008. MMWR Recomm Rep 57:1-12.

275. Schrettl, M., N. Beckmann, J. Varga, T. Heinekamp, I. D. Jacobsen, C. Jochl, T. A. Moussa, S. Wang, F. Gsaller, M. Blatzer, E. R. Werner, W. C. Niermann, A. A. Brakhage, and H. Haas. 2010. HapX-mediated adaption to iron starvation is crucial for virulence of Aspergillus fumigatus. PLoS Pathog 6:e1001124.

276. Schrettl, M., E. Bignell, C. Kragl, Y. Sabiha, O. Loss, M. Eisendle, A. Wallner, H. N. Arst, Jr., K. Haynes, and H. Haas. 2007. Distinct roles for intra- and extracellular siderophores during Aspergillus fumigatus infection. PLoS Pathog 3:1195-207.

277. Schrettl, M., and H. Haas. 2011. Iron homeostasis--Achilles' heel of Aspergillus fumigatus? Curr Opin Microbiol 14:400-5.

278. Schrettl, M., H. S. Kim, M. Eisendle, C. Kragl, W. C. Nierman, T. Heinekamp, E. R. Werner, I. Jacobsen, P. Illmer, H. Yi, A. A. Brakhage, and H. Haas. 2008. SreA-mediated iron regulation in Aspergillus fumigatus. Mol Microbiol 70:27-43.

279. Schubert, S., K. S. Barker, S. Znaidi, S. Schneider, F. Dierolf, N. Dunkel, M. Aid, G. Boucher, P. D. Rogers, M. Raymond, and J. Morschhauser. 2011. Regulation of Efflux Pump Expression and Drug Resistance by the Transcription Factors Mrr1, Upc2, and Cap1 in Candida albicans. Antimicrobial Agents and Chemotherapy 55:2212-2223.

280. Schuck, S., and K. Simons. 2004. Polarized sorting in epithelial cells: raft clustering and the biogenesis of the apical membrane. J Cell Sci 117:5955-64.

281. Schultz, J. R., H. Tu, A. Luk, J. J. Repa, J. C. Medina, L. Li, S. Schwendner, S. Wang, M. Thoolen, D. J. Mangelsdorf, K. D. Lustig, and B. Shan. 2000. Role of LXRs in control of lipogenesis. Genes Dev 14:2831-8.

282. Segal, B. H., and L. R. Romani. 2009. Invasive aspergillosis in chronic granulomatous disease. Med Mycol 47 Suppl 1:S282-90.

283. Sepkowitz, K. A. 2002. Opportunistic infections in patients with and patients without Acquired Immunodeficiency Syndrome. Clin Infect Dis 34:1098-107.

284. Servouse, M., and F. Karst. 1986. Regulation of early enzymes of ergosterol biosynthesis in Saccharomyces cerevisiae. Biochem J 240:541-7.

285. Shanklin, J., E. Whittle, and B. G. Fox. 1994. Eight histidine residues are catalytically essential in a membrane-associated iron enzyme, stearoyl-CoA desaturase, and are conserved in alkane hydroxylase and xylene monooxygenase. Biochemistry 33:12787-94.

286. Shao, W., and P. J. Espenshade. 2012. Expanding roles for SREBP in metabolism. Cell Metab 16:414-9.

287. Sharpless, K. E., and S. D. Harris. 2002. Functional characterization and localization of the Aspergillus nidulans formin SEPA. Mol Biol Cell 13:469-79.

288. Shepardson, K. M., L. Y. Ngo, V. Aimanianda, J. P. Latge, B. M. Barker, S. J. Blosser, Y. Iwakura, T. M. Hohl, and R. A. Cramer. 2012. Hypoxia enhances innate immune activation to Aspergillus fumigatus through cell wall modulation. Microbes Infect.

289. Shimizu, K., and N. P. Keller. 2001. Genetic involvement of a cAMP-dependent protein kinase in a G protein signaling pathway regulating morphological and chemical transitions in Aspergillus nidulans. Genetics 157:591-600.

290. Shobayashi, M., S. Mitsueda, M. Ago, T. Fujii, K. Iwashita, and H. Iefuji. 2005. Effects of culture conditions on ergosterol biosynthesis by Saccharomyces cerevisiae. Biosci Biotechnol Biochem 69:2381-8.

291. Silveira, F. P., and S. Husain. 2007. Fungal infections in solid organ transplantation. Med Mycol 45:305-20.

292. Silver, P. M., B. G. Oliver, and T. C. White. 2004. Role of Candida albicans transcription factor Upc2p in drug resistance and sterol metabolism. Eukaryot Cell 3:1391-7.

293. Simmen, H. P., H. Battaglia, P. Giovanoli, and J. Blaser. 1994. Analysis of pH, pO2 and pCO2 in drainage fluid allows for rapid detection of infectious complications during the follow-up period after abdominal surgery. Infection 22:386-9.

294. Simons, K., and D. Toomre. 2000. Lipid rafts and signal transduction. Nat Rev Mol Cell Biol 1:31-9.

295. Singh, N. 2003. Fungal infections in the recipients of solid organ transplantation. Infect Dis Clin North Am 17:113-34, viii.

296. Skov, M., K. McKay, C. Koch, and P. J. Cooper. 2005. Prevalence of allergic bronchopulmonary aspergillosis in cystic fibrosis in an area with a high frequency of atopy. Respir Med 99:887-93.

297. Slaven, J. W., M. J. Anderson, D. Sanglard, G. K. Dixon, J. Bille, I. S. Roberts, and D. W. Denning. 2002. Increased expression of a novel Aspergillus fumigatus ABC transporter gene, atrF, in the presence of itraconazole in an itraconazole resistant clinical isolate. Fungal Genet Biol 36:199-206.

298. Snelders, E., A. Karawajczyk, R.J. Verhoeven, H. Venselaar, G. Schaftenaar, P.E. Verweij, W.J. Melchers. 2011. The structure-function relationship of the Aspergillus fumigatus cyp51A L98H conversion by site-directed mutagenesis: The mechanism of L98H azole resistance. Fungal Genet Biol 48:9.

299. Snelders, E., S. M. Camps, A. Karawajczyk, G. Schaftenaar, G. H. Kema, H. A. van der Lee, C. H. Klaassen, W. J. Melchers, and P. E. Verweij. 2012. Triazole fungicides can induce cross-resistance to medical triazoles in Aspergillus fumigatus. PLoS One 7:e31801.

300. Snelders, E., R. A. Huis In 't Veld, A. J. Rijs, G. H. Kema, W. J. Melchers, and P. E. Verweij. 2009. Possible environmental origin of resistance of Aspergillus fumigatus to medical triazoles. Appl Environ Microbiol 75:4053-7.

301. Snelders, E., A. Karawajczyk, G. Schaftenaar, P. E. Verweij, and W. J. Melchers. 2010. Azole resistance profile of amino acid changes in Aspergillus fumigatus CYP51A based on protein homology modeling. Antimicrob Agents Chemother 54:2425-30.

302. Snelders, E., A. Karawajczyk, R. J. Verhoeven, H. Venselaar, G. Schaftenaar, P. E. Verweij, and W. J. Melchers. 2011. The structure-function relationship of the Aspergillus fumigatuscyp51A L98H conversion by site-directed mutagenesis: the mechanism of L98H azole resistance. Fungal Genet Biol 48:1062-70.

303. Snelders, E., W. J. Melchers, and P. E. Verweij. 2011. Azole resistance in Aspergillus fumigatus: a new challenge in the management of invasive aspergillosis? Future Microbiol 6:335-47.

304. Snelders, E., H. A. van der Lee, J. Kuijpers, A. J. Rijs, J. Varga, R. A. Samson, E. Mellado, A. R. Donders, W. J. Melchers, and P. E. Verweij. 2008. Emergence of azole resistance in Aspergillus fumigatus and spread of a single resistance mechanism. PLoS Med 5:e219.

305. Snydman, D. R., B. G. Werner, B. Heinze-Lacey, V. P. Berardi, N. L. Tilney, R. L. Kirkman, E. L. Milford, S. I. Cho, H. L. Bush, Jr., A. S. Levey, and et al. 1987. Use of cytomegalovirus immune globulin to prevent cytomegalovirus disease in renal-transplant recipients. N Engl J Med 317:1049-54.

306. Sokol-Anderson, M. L., J. Brajtburg, and G. Medoff. 1986. Amphotericin B-induced oxidative damage and killing of Candida albicans. J Infect Dis 154:76-83.

307. Speirs, J. J., C. K. van der Ent, and J. M. Beekman. 2012. Effects of Aspergillus fumigatus colonization on lung function in cystic fibrosis. Curr Opin Pulm Med 18:632-8.

308. Starzl, T. E., S. Iwatsuki, G. Klintmalm, G. P. Schroter, R. Weil, 3rd, L. J. Koep, and K. A. Porter. 1981. Liver transplantation, 1980, with particular reference to cyclosporin-A. Transplant Proc 13:281-5.

309. Stein, D. K., and A. M. Sugar. 1989. Fungal infections in the immunocompromised host. Diagn Microbiol Infect Dis 12:221S-228S.

310. Steinbach, W. J., R. A. Cramer, Jr., B. Z. Perfect, C. Henn, K. Nielsen, J. Heitman, and J. R. Perfect. 2007. Calcineurin inhibition or mutation enhances cell wall inhibitors against Aspergillus fumigatus. Antimicrob Agents Chemother 51:2979-81.

311. Stephen, D. W., S. L. Rivers, and D. J. Jamieson. 1995. The role of the YAP1 and YAP2 genes in the regulation of the adaptive oxidative stress responses of Saccharomyces cerevisiae. Mol Microbiol 16:415-23.

312. Stevens, D. A., H. J. Schwartz, J. Y. Lee, B. L. Moskovitz, D. C. Jerome, A. Catanzaro, D. M. Bamberger, A. J. Weinmann, C. U. Tuazon, M. A. Judson, T. A. Platts-Mills, and A. C. DeGraff, Jr. 2000. A randomized trial of itraconazole in allergic bronchopulmonary aspergillosis. N Engl J Med 342:756-62.

313. Stewart, E. V., S. J. Lloyd, J. S. Burg, C. C. Nwosu, R. E. Lintner, R. Daza, C. Russ, K. Ponchner, C. Nusbaum, and P. J. Espenshade. 2012. Yeast sterol regulatory element-binding protein (SREBP) cleavage requires Cdc48 and Dsc5, a ubiquitin regulatory X domain-containing subunit of the Golgi Dsc E3 ligase. J Biol Chem 287:672-81.

314. Stewart, E. V., C. C. Nwosu, Z. Tong, A. Roguev, T. D. Cummins, D. U. Kim, J. Hayles, H. O. Park, K. L. Hoe, D. W. Powell, N. J. Krogan, and P. J. Espenshade. 2011. Yeast SREBP cleavage activation requires the Golgi Dsc E3 ligase complex. Mol Cell 42:160-71.

315. Sukhanova, A., A. Gorin, I. G. Serebriiskii, L. Gabitova, H. Zheng, D. Restifo, B. L. Egleston, D. Cunningham, T. Bagnyukova, H. Liu, A. Nikonova, G. P. Adams, Y. Zhou, D. H. Yang, R. Mehra, B. Burtness, K. Q. Cai, A. Klein-Szanto, L. E. Kratz, R. I. Kelley, L. M. Weiner, G. E. Herman, E. A. Golemis, and I. Astsaturov. 2013. Targeting C4-Demethylating Genes in the Cholesterol Pathway Sensitizes Cancer Cells to EGF Receptor Inhibitors via Increased EGF Receptor Degradation. Cancer Discov 3:96-111.

316. T.C. White, J. H., B.G. Oliver. 2003. Antifungal Drug Resistance: Pumps and Permutations, p. 319-338. *In* G. S. K. J.E. Domer (ed.), Human Fungal Pathogens, The Mycota, vol. 12. Springer.

317. Taheri-Talesh, N., T. Horio, L. Araujo-Bazan, X. Dou, E. A. Espeso, M. A. Penalva, S. A. Osmani, and B. R. Oakley. 2008. The tip growth apparatus of Aspergillus nidulans. Mol Biol Cell 19:1439-49.

318. Takeshita, N., G. Diallinas, and R. Fischer. 2012. The role of flotillin FloA and stomatin StoA in the maintenance of apical sterol-rich membrane domains

and polarity in the filamentous fungus Aspergillus nidulans. Mol Microbiol 83:1136-52.

319. Tamame, M., F. Antequera, J. R. Villanueva, and T. Santos. 1983. High-frequency conversion to a "fluffy" developmental phenotype in Aspergillus spp. by 5-azacytidine treatment: evidence for involvement of a single nuclear gene. Mol Cell Biol 3:2287-97.

320. Tangen, K. L., W. H. Jung, A. P. Sham, T. Lian, and J. W. Kronstad. 2007. The iron- and cAMP-regulated gene SIT1 influences ferrioxamine B utilization, melanization and cell wall structure in Cryptococcus neoformans. Microbiology 153:29-41.

321. Tekaia, F., and J. P. Latge. 2005. Aspergillus fumigatus: saprophyte or pathogen? Curr Opin Microbiol 8:385-92.

322. Tenover, F. C. 2006. Mechanisms of antimicrobial resistance in bacteria. Am J Med 119:S3-10; discussion S62-70.

323. Todd, B. L., E. V. Stewart, J. S. Burg, A. L. Hughes, and P. J. Espenshade. 2006. Sterol regulatory element binding protein is a principal regulator of anaerobic gene expression in fission yeast. Mol Cell Biol 26:2817-31.

324. Tomee, J. F., and H. F. Kauffman. 2000. Putative virulence factors of Aspergillus fumigatus. Clin Exp Allergy 30:476-84.

325. Tsai, H. F., R. G. Washburn, Y. C. Chang, and K. J. Kwon-Chung. 1997. Aspergillus fumigatus arp1 modulates conidial pigmentation and complement deposition. Mol Microbiol 26:175-83.

326. Tsai, H. F., M. H. Wheeler, Y. C. Chang, and K. J. Kwon-Chung. 1999. A developmentally regulated gene cluster involved in conidial pigment biosynthesis in Aspergillus fumigatus. J Bacteriol 181:6469-77.

327. Tsiodras, S., G. Samonis, D. T. Boumpas, and D. P. Kontoyiannis. 2008. Fungal infections complicating tumor necrosis factor alpha blockade therapy. Mayo Clin Proc 83:181-94.

328. Urbani, L., and R. D. Simoni. 1990. Cholesterol and vesicular stomatitis virus G protein take separate routes from the endoplasmic reticulum to the plasma membrane. J Biol Chem 265:1919-23.

329. Valiante, V., T. Heinekamp, R. Jain, A. Hartl, and A. A. Brakhage. 2008. The mitogen-activated protein kinase MpkA of Aspergillus fumigatus regulates cell wall signaling and oxidative stress response. Fungal Genet Biol 45:618-27.

330. Valiante, V., R. Jain, T. Heinekamp, and A. A. Brakhage. 2009. The MpkA MAP kinase module regulates cell wall integrity signaling and pyomelanin formation in Aspergillus fumigatus. Fungal Genet Biol 46:909-18.

331. van Burik, J. A., S. L. Carter, A. G. Freifeld, K. P. High, K. T. Godder, G. A. Papanicolaou, A. M. Mendizabal, J. E. Wagner, S. Yanovich, and N. A. Kernan. 2007. Higher risk of cytomegalovirus and aspergillus infections in recipients of T cell-depleted unrelated bone marrow: analysis of infectious complications in patients treated with T cell depletion versus immunosuppressive therapy to prevent graft-versus-host disease. Biol Blood Marrow Transplant 13:1487-98.

332. Van Leeuwen, M. R., W. Smant, W. de Boer, and J. Dijksterhuis. 2008. Filipin is a reliable in situ marker of ergosterol in the plasma membrane of germinating conidia (spores) of Penicillium discolor and stains intensively at the site of germ tube formation. J Microbiol Methods 74:64-73.

333. Vandiviere, H. M., W. E. Loring, I. Melvin, and S. Willis. 1956. The treated pulmonary lesion and its tubercle bacillus. II. The death and resurrection. Am J Med Sci 232:30-7; passim.

334. Venkatramesh, M., and W. D. Nes. 1995. Novel sterol transformations promoted by Saccharomyces cerevisiae strain GL7: evidence for 9 beta, 19-cyclopropyl to 9(11)-isomerization and for 14-demethylation to 8(14)-sterols. Arch Biochem Biophys 324:189-99.

335. Verweij, P. E., S. J. Howard, W. J. Melchers, and D. W. Denning. 2009. Azole-resistance in Aspergillus: proposed nomenclature and breakpoints. Drug Resist Updat 12:141-7.

336. Verweij, P. E., E. Mellado, and W. J. Melchers. 2007. Multiple-triazole-resistant aspergillosis. N Engl J Med 356:1481-3.

337. Vik, A., and J. Rine. 2001. Upc2p and Ecm22p, dual regulators of sterol biosynthesis in Saccharomyces cerevisiae. Mol Cell Biol 21:6395-405.

338. Vinci, G., X. Xia, and R. A. Veitia. 2008. Preservation of genes involved in sterol metabolism in cholesterol auxotrophs: facts and hypotheses. PLoS One 3:e2883.

339. Vodisch, M., K. Scherlach, R. Winkler, C. Hertweck, H. P. Braun, M. Roth, H. Haas, E. R. Werner, A. A. Brakhage, and O. Kniemeyer. 2011. Analysis of the Aspergillus fumigatus proteome reveals metabolic changes and the activation of the pseurotin A biosynthesis gene cluster in response to hypoxia. J Proteome Res 10:2508-24.

340. Vogel, J. P., J. N. Lee, D. R. Kirsch, M. D. Rose, and E. S. Sztul. 1993. Brefeldin A causes a defect in secretion in Saccharomyces cerevisiae. J Biol Chem 268:3040-3.

341. Wakabayashi, S., H. Matsubara, and D. A. Webster. 1986. Primary sequence of a dimeric bacterial haemoglobin from Vitreoscilla. Nature 322:481-3.

342. Walfridsson, M., J. Hallborn, M. Penttila, S. Keranen, and B. Hahn-Hagerdal. 1995. Xylose-metabolizing Saccharomyces cerevisiae strains overexpressing the TKL1 and TAL1 genes encoding the pentose phosphate pathway enzymes transketolase and transaldolase. Appl Environ Microbiol 61:4184-90.

343. Wallis, R. S., M. Broder, J. Wong, and D. Beenhouwer. 2004. Granulomatous infections due to tumor necrosis factor blockade: correction. Clin Infect Dis 39:1254-5.

344. Wallis, R. S., M. S. Broder, J. Y. Wong, M. E. Hanson, and D. O. Beenhouwer. 2004. Granulomatous infectious diseases associated with tumor necrosis factor antagonists. Clin Infect Dis 38:1261-5.

345. Wallner, A., M. Blatzer, M. Schrettl, B. Sarg, H. Lindner, and H. Haas. 2009. Ferricrocin, a siderophore involved in intra- and transcellular iron distribution in Aspergillus fumigatus. Appl Environ Microbiol 75:4194-6.

346. Walsh, T. J., E. J. Anaissie, D. W. Denning, R. Herbrecht, D. P. Kontoyiannis, K. A. Marr, V. A. Morrison, B. H. Segal, W. J. Steinbach, D. A. Stevens, J. A. van Burik, J. R. Wingard, and T. F. Patterson. 2008. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. Clin Infect Dis 46:327-60.

347. Walsh, T. J., V. Petraitis, R. Petraitiene, A. Field-Ridley, D. Sutton, M. Ghannoum, T. Sein, R. Schaufele, J. Peter, J. Bacher, H. Casler, D. Armstrong, A. Espinel-Ingroff, M. G. Rinaldi, and C. A. Lyman. 2003. Experimental pulmonary aspergillosis due to Aspergillus terreus: pathogenesis and treatment of an emerging fungal pathogen resistant to amphotericin B. J Infect Dis 188:305-19.

348. Wang, Y., P. M. Rogers, C. Su, G. Varga, K. R. Stayrook, and T. P. Burris. 2008. Regulation of cholesterologenesis by the oxysterol receptor, LXRalpha. J Biol Chem 283:26332-9.

349. Warrilow, A. G., N. Melo, C. M. Martel, J. E. Parker, W. D. Nes, S. L. Kelly, and D. E. Kelly. 2010. Expression, purification, and characterization of Aspergillus fumigatus sterol 14-alpha demethylase (CYP51) isoenzymes A and B. Antimicrob Agents Chemother 54:4225-34.

350. Warris, A., A. Bjorneklett, and P. Gaustad. 2001. Invasive pulmonary aspergillosis associated with infliximab therapy. N Engl J Med 344:1099-100.

351. Weete, J. D., M. Abril, and M. Blackwell. 2010. Phylogenetic distribution of fungal sterols. PLoS One 5:e10899.

352. Weinberg, E. D. 1999. The role of iron in protozoan and fungal infectious diseases. J Eukaryot Microbiol 46:231-8.

353. Welihinda, A. A., A. D. Beavis, and R. J. Trumbly. 1994. Mutations in LIS1 (ERG6) gene confer increased sodium and lithium uptake in Saccharomyces cerevisiae. Biochim Biophys Acta 1193:107-17.

354. Wheat, L. J., and T. J. Walsh. 2008. Diagnosis of invasive aspergillosis by galactomannan antigenemia detection using an enzyme immunoassay. Eur J Clin Microbiol Infect Dis 27:245-51.

355. White, P. L., C. J. Linton, M. D. Perry, E. M. Johnson, and R. A. Barnes. 2006. The evolution and evaluation of a whole blood polymerase chain reaction assay for the detection of invasive aspergillosis in hematology patients in a routine clinical setting. Clin Infect Dis 42:479-86.

356. White, T. C., and P. M. Silver. 2005. Regulation of sterol metabolism in Candida albicans by the UPC2 gene. Biochem Soc Trans 33:1215-8.

357. Wilcox, L. J., D. A. Balderes, B. Wharton, A. H. Tinkelenberg, G. Rao, and S. L. Sturley. 2002. Transcriptional profiling identifies two members of the ATP-binding cassette transporter superfamily required for sterol uptake in yeast. J Biol Chem 277:32466-72.

358. Willger, S. D., E. J. Cornish, D. Chung, B. A. Fleming, M. M. Lehmann, S. Puttikamonkul, and R. A. Cramer. 2012. Dsc orthologs are required for

hypoxia adaptation, triazole drug responses, and fungal virulence in Aspergillus fumigatus. Eukaryot Cell 11:1557-67.

359. Willger, S. D., S. Puttikamonkul, K. H. Kim, J. B. Burritt, N. Grahl, L. J. Metzler, R. Barbuch, M. Bard, C. B. Lawrence, and R. A. Cramer, Jr. 2008. A sterol-regulatory element binding protein is required for cell polarity, hypoxia adaptation, azole drug resistance, and virulence in Aspergillus fumigatus. PLoS Pathog 4:e1000200.

360. Williamson, P. R. 1994. Biochemical and molecular characterization of the diphenol oxidase of Cryptococcus neoformans: identification as a laccase. J Bacteriol 176:656-64.

361. Williamson, P. R., K. Wakamatsu, and S. Ito. 1998. Melanin biosynthesis in Cryptococcus neoformans. J Bacteriol 180:1570-2.

362. Wingard, J. R., S. L. Carter, T. J. Walsh, J. Kurtzberg, T. N. Small, L. R. Baden, I. D. Gersten, A. M. Mendizabal, H. L. Leather, D. L. Confer, R. T. Maziarz, E. A. Stadtmauer, J. Bolanos-Meade, J. Brown, J. F. Dipersio, M. Boeckh, and K. A. Marr. 2010. Randomized, double-blind trial of fluconazole versus voriconazole for prevention of invasive fungal infection after allogeneic hematopoietic cell transplantation. Blood 116:5111-8.

363. Worlitzsch, D., R. Tarran, M. Ulrich, U. Schwab, A. Cekici, K. C. Meyer, P. Birrer, G. Bellon, J. Berger, T. Weiss, K. Botzenhart, J. R. Yankaskas, S. Randell, R. C. Boucher, and G. Doring. 2002. Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients. J Clin Invest 109:317-25.

364. Xue, T., C. K. Nguyen, A. Romans, and G. S. May. 2004. A mitogen-activated protein kinase that senses nitrogen regulates conidial germination and growth in Aspergillus fumigatus. Eukaryot Cell 3:557-60.

365. Yang, C., J. G. McDonald, A. Patel, Y. Zhang, M. Umetani, F. Xu, E. J. Westover, D. F. Covey, D. J. Mangelsdorf, J. C. Cohen, and H. H. Hobbs. 2006. Sterol intermediates from cholesterol biosynthetic pathway as liver X receptor ligands. J Biol Chem 281:27816-26.

366. Yasmin, S., L. Alcazar-Fuoli, M. Grundlinger, T. Puempel, T. Cairns, M. Blatzer, J. F. Lopez, J. O. Grimalt, E. Bignell, and H. Haas. 2012. Mevalonate governs

interdependency of ergosterol and siderophore biosyntheses in the fungal pathogen Aspergillus fumigatus. Proc Natl Acad Sci U S A 109:E497-504.

367. Young, L. Y., C. M. Hull, and J. Heitman. 2003. Disruption of ergosterol biosynthesis confers resistance to amphotericin B in Candida lusitaniae. Antimicrob Agents Chemother 47:2717-24.

368. Yu, J. H., Z. Hamari, K. H. Han, J. A. Seo, Y. Reyes-Dominguez, and C. Scazzocchio. 2004. Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. Fungal Genet Biol 41:973-81.

369. Zamora, M. R. 2001. Use of cytomegalovirus immune globulin and ganciclovir for the prevention of cytomegalovirus disease in lung transplantation. Transpl Infect Dis 3 Suppl 2:49-56.

370. Zarember, K. A., J. A. Sugui, Y. C. Chang, K. J. Kwon-Chung, and J. I. Gallin. 2007. Human polymorphonuclear leukocytes inhibit Aspergillus fumigatus conidial growth by lactoferrin-mediated iron depletion. J Immunol 178:6367-73.

371. Zhang, Y. Q., S. Gamarra, G. Garcia-Effron, S. Park, D. S. Perlin, and R. Rao. 2010. Requirement for Ergosterol in V-ATPase Function Underlies Antifungal Activity of Azole Drugs. Plos Pathogens 6.

372. Zhou, S., S. Fushinobu, S. W. Kim, Y. Nakanishi, J. Maruyama, K. Kitamoto, T. Wakagi, and H. Shoun. 2011. Functional analysis and subcellular location of two flavohemoglobins from Aspergillus oryzae. Fungal Genet Biol 48:200-7.

373. Zhou, S., S. Fushinobu, Y. Nakanishi, S. W. Kim, T. Wakagi, and H. Shoun. 2009. Cloning and characterization of two flavohemoglobins from Aspergillus oryzae. Biochem Biophys Res Commun 381:7-11.

374. Zinkernagel, A. S., R. S. Johnson, and V. Nizet. 2007. Hypoxia inducible factor (HIF) function in innate immunity and infection. J Mol Med (Berl) 85:1339-46.

375. Znaidi, S., X. De Deken, S. Weber, T. Rigby, A. Nantel, and M. Raymond. 2007. The zinc cluster transcription factor Tac1p regulates PDR16 expression in Candida albicans. Mol Microbiol 66:440-52.

376. Znaidi, S., S. Weber, O. Z. Al-Abdin, P. Bomme, S. Saidane, S. Drouin, S. Lemieux, X. De Deken, F. Robert, and M. Raymond. 2008. Genomewide location analysis of Candida albicans Upc2p, a regulator of sterol metabolism and azole drug resistance. Eukaryot Cell 7:836-47.

APPENDICES

APPENDIX A

ABBREVIATIONS UTILIZED IN THIS DISSERTATION

ABC	ATP-binding cassette
ABPA	allergic bronchopulmonary aspergillosis
AIDS	acquired immunodeficiency syndrome
AmB	amphotericin B
AML	acute myeloid leukemia
BAL	bronchioalveolar lavage
BiFC	bimolecular fluorescence complementation
BFSTA	*N,O-bis*(trimethylsilyl)trifluoroacetamide
cAMP	cyclic adenosine monophosphate
CDC	Centers for Disease Control
CF	cystic fibrosis
CGD	chronic granulomatous disease
CHiP	chromatin immunoprecipitation
CMV	cytomegalovirus
COPD	chronic obstructive pulmonary disease
CR	Congo Red
CWI	cell-wall integrity
DTT	dithiothreitol
EGFR	epidermal growth factor receptor
EI	electron ionization
EIA	enzyme immunoassay
EMSA	electrophoretic mobility shift assay
ER	endoplasmic reticulum
FDA	Food and Drug Administration
FF-MAS	follicular-fluid meiosis-activating sterol
FLC	Fluconazole
GC-MS	gas chromatography mass spectrometry
GMM	glucose minimal media
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
HMG	3-hydroxyl-3-methyl-glutaryl
IA	Invasive Aspergillosis
IFI	invasive fungal infection
ITZ	Itraconazole
L-AmB	liposome-associated Amphotericin B
LGMM	liquid glucose minimal media
LXR	liver X receptor
MAP	mitogen activated protein
MAPK	MAP-kinase
MAS	meiosis activating sterols
MDR	multidrug resistance protein
MIC	minimum inhibitory concentration

MFS	major facilitator superfamily
MOPS	morpholinepropanesulfonic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NET	neutrophil extracellular trap
NO	nitric oxide
ORF	open reading frame
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKA	protein kinase A
qRT-PCR	quantitative real time polymerase chain reaction
ROS	reactive oxygen species
RPKM	reads per kilobase per million mapped reads
S1P	site-1 protease
S2P	site-2 protease
SMO	sterol methyl oxidase
SOT	solid organ transplant
SRE	sterol response element
SREBP	sterol regulatory element binding protein
SRD	sterol rich domains
TCA	tricarboxylic acid cycle
T-MAS	testis meiosis-activating sterol
TNF	tumor necrosis factor
UPR	unfolded protein response
VCZ	Voriconazole
UPGMA	unweighted pair group method with arithmetic mean

APPENDIX B

ORGANISMS REFERENCED IN THIS DISSERTATION

Abbreviation	*Genus species*	Classification	Other:
A. clavatus	*Aspergillus clavatus*	a filamentous fungus	an allergen; can occasionally cause Invasive Aspergillosis
A. flavus	*Aspergillus flavus*	a filamentous fungus	a major agricultural contaminant; produces aflatoxin, which is carcinogenic; can occasionally cause Invasive Aspergillosis
A. fumigatus	*Aspergillus fumigatus*	a filamentous fungus	causative agent for Invasive Aspergillosis
A. niger	*Aspergillus niger*	a filamentous fungus	used the commercial production of citric acid; can occasionally cause Invasive Aspergillosis
A. nidulans	*Aspergillus nidulans*	a filamentous fungus	a model organism; can occasionally cause Invasive Aspergillosis, especially in patients with CGD
A. oryzae	*Aspergillus oryzae*	a filamentous fungus	used in the commercial production of fermented products; can occasionally cause Invasive Aspergillosis
A. terreus	*Aspergillus terreus*	a filamentous fungus	can cause Invasive Aspergillosis
A. thaliana	*Arabidopsis thaliana*	a plant	model organism
B. dermatitidis	*Blastomyces dermatitidis*	a dimorphic fungus	Causative agent of blastomycoses
C. albicans	*Candida albicans*	a hemi-ascomycete yeast	Causative agent for vaginal and invasive Candidiasis
C. dubliniensis	*Candida dubliniensis*	a yeast	can cause invasive Candidiasis
C. lusitaniae	*Candida lusitaniae*	a yeast	can cause invasive Candidiasis
C. elegans	*Caenorhabditis elegans*	a nematode	model organism
C. neoformans	*Cryptococcus neoformans*	a basidomycete yeast	Causative agent for Cryptococcus, fungal meningitis
D. melanogaster	*Drosophila melanogaster*	fruit fly	model organism
E. coli	*Escherichia coli*	a bacteria	model organism
H. capsulatum	*Histoplasma capsulatum*	a dimorphic fungus	Causative agent for Histoplasmosis
H. sapiens	*Homo sapiens*	Human	
L. amazonensis	*Leismania amazonensis*	a trypanosome parasite	one of the causitive agents of mucocutaneous

			leismaniasis
M. musculus	*Mus musculus*	mouse	
M. tuberculosis	*Mycobacterium tuberculosis*	a mycobacterium	causative agent of tuberculosis
N. benthamiana	*Nicotiana benthamiana*	a plant	a model organism
P. brasiliensis	*Paracoccidoties brasiliensis*	a dimorphic fungus	causative agent of paracoccidiodomycosis
P. marneffei	*Penicillium marneffei*	a dimorphic fungus	causitive agent of penicilliosis
P. vivax	*Plasmodium vivax*	a protozoal parasite	one of the causitive agents of malaria
P. carnii	*Pneumoncystic carnii*	a yeast-like fungus	the causative agent of *pneumocystis* pneumonia; also known as *Pneumocystis jirovecii*
P. aeruginosa	*Pseudomonas aeruginosa*	a gram-negative bacteria	causative agent of *Pseudomonas* infection
S. cerevisiae	*Saccharomyces cerevisiae*	a yeast	model organism
S. pombe	*Schizosaccharomyces pombe*	a fission yeast	model organism
S. schenckii	*Sporothrix schenckii*	a dimorphic fungus	causative agent of sporotrichosis
S. aureus	*Staphylococcus aureus*	a gram positive bacteria	causative agent for “staph” infection
S. pyogenes	*Streptococcus pyogenes*	a gram-positive bacteria	causative agent of “strep throat,” localized skin infection, and toxic shock syndrome
T. rubrum	*Trichophyton rubrum*	a dermatophyte	causative agent for athletes foot, ringworm, etc.

APPENDIX C

GENE/PROTEIN NAMES & FUNCTIONS REFERENCED IN THIS DISSERTATION

Name	Organism	Function
aspF1	*Aspergillus spp.*	Allergen Asp f 1; ribonuclease mitogillin family of cytotoxins; similar to restrictocin; inhibits protein synthesis in mammalian cells; 18kD IgE-binding protein; expression increases in vivo; biofilm-induced vs planktonic cells
bcr1	*C. albicans*	C2H2 zinc finger transcription factor; master regulator of biofilm formation; mutation affects filamentous growth, adherence; regulates cell-surface-associated genes; filament induced; Tup1p-, Tec1p-, Mnl1p-regulated; mRNA binds to She3p
cap1	*C. albicans*	AP-1 bZIP transcription; oxidative stress response, resistance, multidrug resistance; oxidative stress regulates nuclear localization; rescues complements S. cerevisiae yap1 mutant; oralpharyngeal candidasis-, human neutrophil-induced
cph2	*C. albicans*	Myc-bHLH family transcriptional activator of hyphal growth; directly regulates Tec1p, which regulates hypha-specific genes; probably homodimeric, phosphorylated; required for wild-type levels of Candida colonization of the mouse GI tract
cyp51	Metazoans	Another name for *erg11*
ecm22	*S. cerevisiae & C. albicans*	Sterol regulatory element binding protein; regulates transcription of sterol biosynthetic genes; contains Zn[2]-Cys[6] binuclear cluster; relocates from intracellular membranes to perinuclear foci on sterol depletion; ECM22 has a paralog, UPC2, that arose from the whole genome duplication (transcription factor)
erg1	Metazoans	squalene monooxygenase with a role in ergosterol biosynthesis
erg3	Metazoans	sterol delta 5,6-desaturase with a role in ergosterol biosynthesis
erg4	Metazoans	C-24(28) sterol reductase with a role in ergosterol biosynthesis
erg5	Metazoans	Cytochrome P450, sterol C22-desaturase with a role in ergosterol biosynthesis
erg6	Metazoans	sterol 24-C-methyltransferase with a role in ergosterol biosynthesis
erg7	Metazoans	oxidosqualene:lanosterol cyclase; involved in ergosterol biosynthesis
erg11	Metazoans	14-α demethylase with a role in ergosterol biosynthesis
erg24	Metazoans	C-14 sterol reductase with a role in ergosterol biosynthesis
erg25	Metazoans	C-4 methyl sterol oxidase with a role in ergosterol biosynthesis
erg26	Metazoans	C-3 sterol dehydrogenase/C-4 decarboxylase with a role in ergosterol biosynthesis
erg27	Metazoans	3-ketosteroid reductase with a role in ergosterol biosynthesis
erg28	Metazoans	Putative ergosterol biosynthesis protein
flavA	*A. fumigatus*	Putative flavohemoprotein; protein induced by heat shock and hypoxia
gpdA	*Aspergillus spp.*	Glyceraldehyde-3-phosphate dehydrogenase; predicted gene pair with AFUA_5G01030; protein induced by hydrogen

		peroxide; reacts with rabbit immunosera exposed to A. fumigatus conidia: induced by L-tyrosine
hacA	*Aspergillus spp.*	bZIP transcription factor, major regulator of the unfolded protein response (UPR)
hapB	*Aspergillus spp.*	Sequence-specific CCAAT DNA binding transcription factor
hapC	*Aspergillus spp.*	Component of AnCP/AnCF CCAAT-binding complex; can act as both a positive and negative regulator of transcription
hapE	*Aspergillus spp.*	CCAAT-binding factor complex subunit
hapX	*Aspergillus spp.*	bZIP transcription factor required for adaption to iron starvation and for transcriptional activation of the siderophore system; transcript repressed by iron
hmg1	*Aspergillus spp.*	Hydroxymethylglutaryl-CoA (HMG-CoA) reductase
hms1	*S. cerevisiae & C. albicans*	Transcriptional regulator required for morphogenesis induced by elevated temperature or Hsp90 compromise; acts downstream of Pcl1p
IFNA	*H. sapiens & M. musculus*	The protein encoded by this gene is produced by macrophages and has antiviral activity. This gene is intron less and the encoded protein is secreted.
INSIG	*H. sapiens*	Oxysterols regulate cholesterol homeostasis through the liver X receptor (LXR)- and sterol regulatory element-binding protein (SREBP)-mediated signaling pathways. This gene is an insulin-induced gene. It encodes an endoplasmic reticulum (ER) membrane protein that plays a critical role in regulating cholesterol concentrations in cells. This protein binds to the sterol-sensing domains of SREBP cleavage-activating protein (SCAP) and HMG CoA reductase, and is essential for the sterol-mediated trafficking of the two proteins. Alternatively spliced transcript variants encoding distinct isoforms have been observed
ireA	*Aspergillus spp.*	Transmembrane protein kinase and ribonuclease involved in the unfolded protein response (UPR); under ER stress, activates the main UPR regulator HacA by splicing its mRNA; required for normal virulence
LXR	*H. sapiens*	The protein encoded by this gene belongs to the NR1 subfamily of the nuclear receptor superfamily. The NR1 family members are key regulators of macrophage function, controlling transcriptional programs involved in lipid homeostasis and inflammation. This protein is highly expressed in visceral organs, including liver, kidney and intestine. It forms a heterodimer with retinoid X receptor (RXR), and regulates expression of target genes containing retinoid response elements. Studies in mice lacking this gene suggest that it may play an important role in the regulation of cholesterol homeostasis. Alternatively spliced transcript variants encoding different isoforms have been found for this gene.
mpkA	*Aspergillus spp.*	Mitogen-activated protein kinase; activated by phosphorylation; role in cell wall signaling and the oxidative stress response; mpkA(p)-lacZ expression increased by cell wall disturbing compounds; required for adaptation to iron starvation
mpkB	*Aspergillus spp.*	Putative mitogen-activated protein kinase (MAPK)

mpkC	*Aspergillus spp.*	Putative mitogen activated protein kinase (MAPK); involved in the oxidative stress response; transcript abundance increases in response to carbon source and oxidative stress
mrr1	*C. albicans*	Zinc-finger protein; regulator of MDR1 transcription; gain-of-function mutations cause upregulation of MDR1 (a plasma membrane multidrug efflux pump) and consequent multidrug resistance
MSMO1	*H. sapiens*	Sterol-C4-methyl oxidase-like protein was isolated based on its similarity to the yeast ERG25 protein. It contains a set of putative metal binding motifs with similarity to that seen in a family of membrane desaturases-hydroxylases. The protein is localized to the endoplasmic reticulum membrane and is believed to function in cholesterol biosynthesis. Alternatively spliced transcript variants encoding distinct isoforms have been found for this gene
niiA	*Aspergillus spp.*	nitrite reductase; nitrogen-regulated gene, used as a conditional promoter
PXR	*H. sapiens*	This gene product belongs to the nuclear receptor superfamily, members of which are transcription factors characterized by a ligand-binding domain and a DNA-binding domain. The encoded protein is a transcriptional regulator of the cytochrome P450 gene CYP3A4, binding to the response element of the CYP3A4 promoter as a heterodimer with the 9-cis retinoic acid receptor RXR. It is activated by a range of compounds that induce CYP3A4, including dexamethasone and rifampicin. Several alternatively spliced transcripts encoding different isoforms, some of which use non-AUG (CUG) translation initiation codon, have been described for this gene. Additional transcript variants exist, however, they have not been fully characterized.
pyrG	*Aspergillus spp.*	Orotidine 5'-monophosphate decarboxylase
rox1	*S. cerevisiae*	Heme-dependent repressor of hypoxic genes; mediates aerobic transcriptional repression of hypoxia induced genes such as COX5b and CYC7; repressor function regulated through decreased promoter occupancy in response to oxidative stress; contains an HMG domain that is responsible for DNA bending activity; involved in the hyperosmotic stress resistance
rpe1	*S. cerevisiae*	D-ribulose-5-phosphate 3-epimerase, catalyzes a reaction in the non-oxidative part of the pentose-phosphate pathway; mutants are sensitive to oxidative stress
RXR	*H. sapiens*	Retinoid X receptors (RXRs) and retinoic acid receptors (RARs), are nuclear receptors that mediate the biological effects of retinoids by their involvement in retinoic acid-mediated gene activation. These receptors exert their action by binding, as homodimers or heterodimers, to specific sequences in the promoters of target genes and regulating their transcription. The protein encoded by this gene is a member of the steroid and thyroid hormone receptor superfamily of transcriptional regulators
sakA	*Aspergillus spp.*	Putative mitogen-activated protein kinase (MAPK) with predicted roles in the osmotic and oxidative stress responses;

		involved in sensing nitrogen in the medium
SC4MOL	*H. sapiens*	Alternative name for MSMO1
Scap	*H. sapiens* & *S. pombe*	This gene encodes a protein with a sterol sensing domain (SSD) and seven WD domains. In the presence of cholesterol, this protein binds to sterol regulatory element binding proteins (SREBPs) and mediates their transport from the ER to the Golgi. The SREBPs are then proteolytically cleaved and regulate sterol biosynthesis.
SMO1	*A. thaliana*	A member of the SMO1 family of sterol 4alpha-methyl oxidases. More specifically functions as a 4,4-dimethyl-9beta,19-cyclopropylsterol-4alpha- methyl oxidase
SMO2	*A. thaliana*	A sterol 4-alpha-methyl-oxidase, specifically a 4-alpha-methyl-delta-7-sterol-4alpha-methyl-oxidase.
srbA	*A. fumigatus*	Sterol regulatory element binding protein (SREBP); basic helix-loop-helix leucine zipper DNA binding domain; role in the hypoxia response, iron homeostasis, ergosterol biosynthesis, resistance to azole drugs, maintenance of cell polarity (transcription factor)
sre1	*C. neoformans*	Sterol regulatory element binding protein homolog
srbB	*A. fumigatus*	Basic helix-loop-helix leucine zipper DNA binding domain
sreA	*Aspergillus spp.*	GATA transcription factor that regulates iron uptake; represses of iron-regulated genes; represses siderophore biosynthesis genes in high-iron; mutants accumulate increased amounts of iron and ferricrocin
SREBP	*H. sapiens*	This gene encodes a transcription factor that binds to the sterol regulatory element-1 (SRE1), which is a decamer flanking the low-density lipoprotein receptor gene and some genes involved in sterol biosynthesis. The protein is synthesized as a precursor that is attached to the nuclear membrane and endoplasmic reticulum. Following cleavage, the mature protein translocate to the nucleus and activates transcription by binding to the SRE1. Sterols inhibit the cleavage of the precursor, and the mature nuclear form is rapidly catabolized, thereby reducing transcription. The protein is a member of the basic helix-loop-helix-leucine zipper (bHLH-Zip) transcription factor family. This gene is located within the Smith-Magenis syndrome region on chromosome 17. Two transcript variants encoding different isoforms have been found for this gene.
tac1	*C. albicans*	Transcriptional activator of drug-responsive genes including CDR1 and CDR2; has Zn(2)-Cys(6) binuclear cluster; binds DRE element; gene in zinc cluster region near MTL locus
tec1	*S. cerevisiae & C. albicans*	TEA/ATTS transcription factor; involved in white cell pheromone response, regulates hypha-specific genes, wild-type biofilm formation; regulates BCR1; transcription regulated by Cph2p in some conditions; alkaline-, and biofilm-induced
tefA	*Aspergillus spp.*	Putative translation elongation factor EF-1 alpha subunit; protein abundant in conidia; protein induced by heat shock
tkl1	*S. cerevisiae*	Transketolase; catalyzes conversion of xylulose-5-phosphate and ribose-5-phosphate to sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate in the pentose phosphate pathway; needed for synthesis of aromatic amino acids;

		TKL1 has a paralog, TKL2, that arose from the whole genome duplication
TLR4	*H. sapiens & Mus musculus*	The protein encoded by this gene is a member of the Toll-like receptor (TLR) family which plays a fundamental role in pathogen recognition and activation of innate immunity. TLRs are highly conserved from Drosophila to humans and share structural and functional similarities. They recognize pathogen-associated molecular patterns that are expressed on infectious agents, and mediate the production of cytokines necessary for the development of effective immunity. The various TLRs exhibit different patterns of expression. This receptor has been implicated in signal transduction events induced by lipopolysaccharide (LPS) found in most gram-negative bacteria. Mutations in this gene have been associated with differences in LPS responsiveness. Multiple transcript variants encoding different isoforms have been found for this gene.
tye7	*S. cerevisiae & C. albicans*	bHLH transcription factor involved in control of glycolysis; required for biofilm formation; hyphally regulated by Cph1p, Cyr1p; flucytosine, Hog1p induced; amphotericin B, caspofungin repressed; induced in biofilm and planktonic growth
upc2	*S. cerevisiae & C. albicans*	Sterol regulatory element binding protein; induces transcription of sterol biosynthetic genes and of DAN/TIR gene products; relocates from intracellular membranes to perinuclear foci on sterol depletion; UPC2 has a paralog, ECM22, that arose from the whole genome duplication (transcription factor)
yap1	*Aspergillus spp.*	bZIP family transcription factor; required for resistance to oxidative stress; expression repressed by dexamethasone

Gene/Protein names and functions can be additionally located in their various genome databases. *Aspergillus:* AspGD, *Candida*: CGD, H. *sapiens:* NCBI, *Saccharomyces:* SGD, *Schizosaccharomyces:* PomBase.

APPENDIX D

ASPERGILLUS FUMIGATUS STRAINS USED IN THIS DISSERTATION

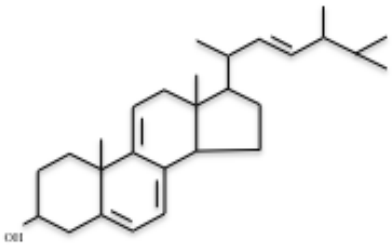
Strain Name	Type	Marker	Background	Construction	Constructed By:
CBS 144.89/CEA10	Wild type				
CEA17	Wild type, uracil auxotroph	pyrG (-)	CEA10	(67b)	C. d'Enfert
Δ*erg11A*	Gene replacement	pyrG	CEA17	(138)	T. Roemer
Δ*erg25A*	Gene replacement	pyrG	CEA17	This study, Chapter 3	B. Hendrickson
Δ*erg25B*	Gene replacement	pyrG	CEA17	This study, Chapter 3	B. Hendrickson
*pniiA-erg11A-*Δ*erg11B*	Promoter replacement	pyrG	*-pyrG*Δ*erg11B*	(138)	T. Roemer
*pniiA-erg11A-*Δ*srbA*	Promoter replacement	pyrG	*-pyrG*Δ*srbA*	This study, Chapter 2	S.J. Blosser
*pniiA-erg25A-*Δ*erg25B*	Promoter replacement	pyrG	*-pyrG*Δ*erg25B*	This study, Chapter 3	B. Hendrickson
*pniiA-erg25B-*Δ*erg25A*	Promoter replacement	pyrG	*-pyrG*Δ*erg25A*	This study, Chapter 3	S.J. Blosser
*pflavA-erg25A-*Δ*srbA*	Promoter replacement	pyrG	*-pyrG*Δ*srbA*	This study, Chapter 4	S.J. Blosser
Δ*srbA*	Gene replacement	pyrG	CEA17	(359)	S.D. Willger

APPENDIX E

FRAGMENTATION PATTERNS & STRUCTURES OF STEROL INTERMEDIATES

Nickname:	**9(11)-Dehydroergosterol**
IUPAC Name:	24-methyl-cholesta-5,7,9(11),22-tetraen-3β-ol
Alternative Name:	ergosta-5,7,9(11),22-tetraenol
Molecular Weight:	394.63g/mol
CAS:	516-85-8

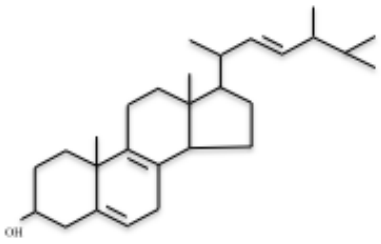
Primary Ion:
251 m/z
Important Secondary Ions:
209 m/z
376 m/z



Reference:
Griffiths, K.M. *et al.* (2003). Phytochemistry,62:147-153.

Nickname:	**lichesterol**
IUPAC Name:	24-methyl cholesta-5,8,22-trien-3b-ol
Alternative Name:	ergosta-5,8,22-trien-3b-ol
Molecular Weight:	396.65g/mol
CAS:	50657-31-3

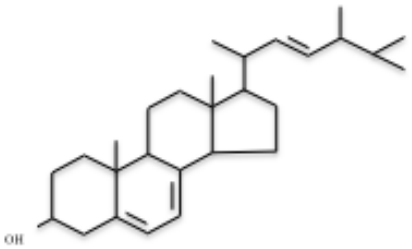
Primary Ion:
363 m/z
Important Secondary Ions:
468 m/z
327 m/z



Reference:
Anastasia and Fiecchi, 1981; JCS Perkin 1, 2125

Nickname:	**ergosterol**
IUPAC Name:	24-methyl-cholesta-5,7,22-trien-3β-ol
Alternative Name:	
Molecular Weight:	396.65g/mol
CAS:	57-87-4

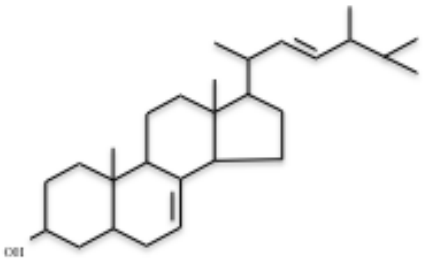
Primary Ion:
363 m/z
Important Secondary Ions:
468 m/z
327 m/z



Reference:
Weete & Gandhi, Lipids (1997), 32(12)

Nickname:	**5-Dihydroergosterol**
IUPAC Name:	24-methyl-cholesta-7,22-dien-3β-ol
Alternative Name:	ergosta-7,22-dienol
Molecular Weight:	398.671g/mol
CAS:	2465-11-4

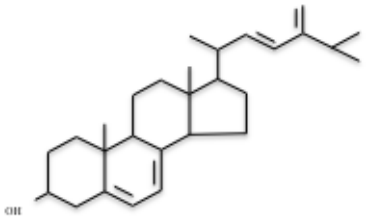
Primary Ion:
255 m/z
Important Secondary Ions:
470 m/z
455 m/z
380 m/z
343 m/z



Reference:
Weete & Gandhi, Lipids (1997), 32(12)

Nickname: **ergostatetraenol**
IUPAC Name: 24-methyl cholesta-5,7,22,24(28)-tetraen-3b-ol
Alternative Name:
Molecular Weight: 394.32g/mol
CAS:

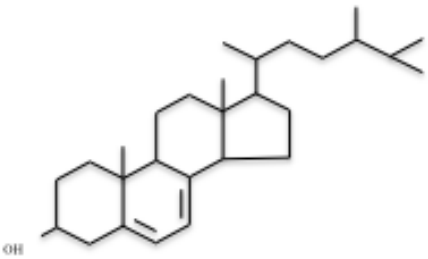
Primary Ion:
394 m/z
Important Secondary Ions:
361 m/z
253 m/z
251 m/z
123 m/z



Reference:
Goad, L.J., Holz, G.G., and D.H. Beach, (1984). Mol. Biochem Parasitol, 10:161-170.
Rahman, M.D. and R.A. Pascal, Jr. (1990). J Biol Chem, 265(9):4989-4996.

Nickname: **22-dihydroergosterol**
IUPAC Name: 24-methylcholesta-5,7-dien-3b-ol
Alternative Name:
Molecular Weight: 398.66g/mol
CAS: 516-79-0

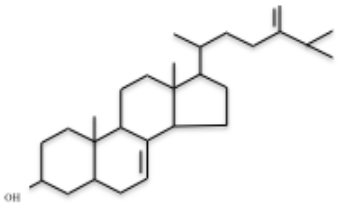
Primary Ion:
363m/z
Important Secondary Ions:
337 m/z*
468 m/z
378 m/z
253 m/z



Reference:
Weete & Gandhi, Lipids (1997), 32(12)

Nickname: **episterol**
IUPAC Name: 24-methyl cholesta-7,24(28)-dien-3b-ol
Alternative Name:
Molecular Weight: 398.66g/mol
CAS: 57-87-4

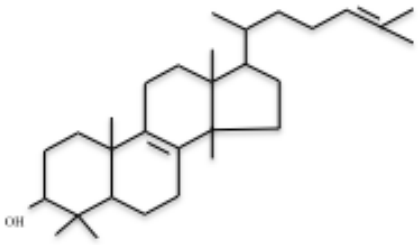
Primary Ion:
343m/z
Important Secondary Ions:
470 m/z (~24%)
455 m/z (~26%)
365 m/z (~14%)
255 m/z (~47%)
253 m/z (~24%)



Reference:
Weete & Gandhi, Lipids (1997), 32(12)
Goad, L.J. & T. Akihisa, Analysis of Sterols, Chapman & Hall, 1997

Nickname: **lanosterol**
IUPAC Name: 4,4,14-trimethyl cholesta-8,24-dien-3b-ol
Alternative Name:
Molecular Weight: 426.71g/mol
CAS: 79-63-0

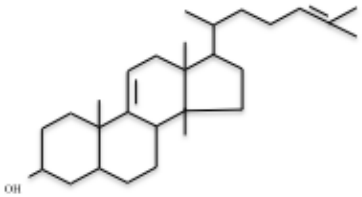
Primary Ion:
393m/z
Important Secondary Ions:
498 m/z (~41%)
483 m/z (~31%)



Reference:
Goad, L.J. & T. Akihisa, Analysis of Sterols, Chapman & Hall, 1997

Nickname: **parkeol**
IUPAC Name: 4,4,14-trimethyl cholesta-9(11),24-dien-3b-ol
Alternative Name:
Molecular Weight: 426.71g/mol
CAS: 28032-52-2

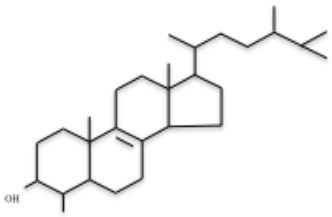
Primary Ion:
424m/z
Important Secondary Ions:
393 m/z
376 m/z
299 m/z
251 m/z



Reference:
Goad, L.J. & T. Akihisa, Analysis of Sterols, Chapman & Hall, 1997
Personal Communication with M. Bard

Nickname: **4-methyl fecosterol**
IUPAC Name: 4,24-dimethyl cholesta-8,24(28)-dien-3b-ol
Alternative Name:
Molecular Weight: 412.37g/mol
CAS: 131723-74-5

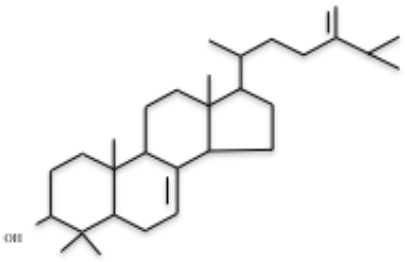
Primary Ion:
484m/z
Important Secondary Ions:
469 m/z
394 m/z
379 m/z
337 m/z



Reference:
Griffiths, K.M. *et al*. (2003). Phytochemistry,62:147-153.

Nickname: **4,4-dimethyl fecosterol**
IUPAC Name: 4,4,24-trimethyl cholesta-8,24(28)-dien-3b-ol
Alternative Name:
Molecular Weight:
CAS:

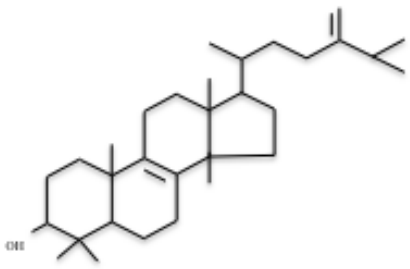
Primary Ion:
498m/z
Important Secondary Ions:
394 m/z
393 m/z



Reference:
Goad and Akihisa, Analysis of Sterols 1997.

Nickname: **eburicol**
IUPAC Name: 4,4,14,24-tetramethyl cholesta-8,24(28)-dien-3b-ol
Alternative Name:
Molecular Weight: 440.8 [512 (first peak) – 71.3]
CAS: 6890-88-6

Primary Ion:
407m/z
Important Secondary Ions:
512 m/z
497 m/z



Reference:
Griffiths, K.M. *et al*. (2003). Phytochemistry,62:147-153.