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The DNA Damage Response Gene DDR48 Regulates Sterol Synthesis Genes to Confer Antifungal Resistance to the Pathogenic Fungus *Histoplasma capsulatum*

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The DNA Damage Response Gene *DDR48* Regulates Sterol Synthesis Genes to Confer Antifungal Resistance to the Pathogenic Fungus *Histoplasma capsulatum*

by

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Abstract

Histoplasma capsulatum is a dimorphic fungal pathogen that is endemic to the Ohio, Missouri, and Mississippi river valley regions. The fungus grows as a mold at environmental temperatures (25°C) and transforms into a unicellular yeast upon inhalation by a mammalian host (37°C). The mold to yeast shift is required for pathogenicity in host organisms, where the potentially fatal disease, histoplasmosis, can present. This study aims to characterize the DNA damage response protein *DDR48* and the role that it plays in combating cellular stressors in *H. capsulatum.* We found that *DDR48* is expressed strongly in the mold phase but expressed only at basal levels in the yeast phase. However, *DDR48* expression is significantly upregulated in the yeast phase under stressful conditions, like antifungal stress. We found that wild-type *H. capsulatum* yeast exposed to the antifungal drugs ketoconazole or Amphotericin-B upregulated *DDR48* expression (assayed via qRT-PCR) at least 4-fold. The mold growth form, however, showed no significant change in expression of *DDR48* under these conditions. Additionally, the *DDR48* knockout mutant previously created in our lab was significantly more sensitive to these antifungal drugs. The knockout cells also experienced dysregulation of their membrane sterol synthesis genes, indicating a potential role of *DDR48* in conferring antifungal resistance to *H. capsulatum* by acting as a regulator of membrane maintenance and integrity. Research is ongoing to further elucidate the role of *DDR48* in the stress response pathway of this common human pathogen.

Key Words: *Histoplasma capsulatum*, *DDR48*, Amphotericin-B, ketoconazole, antifungal resistance, ergosterol

Dedication

Mom, Dad, and Michael:

Thank you for your unwavering support and encouragement of every single one of my goals. Even at the toughest times, you all helped me see the light and make it through every trial. I could not have done this without all of you!

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List of Abbreviations

C. albicans	*Candida albicans*
DDR48	DNA damage-response gene 48
DNA	Deoxyribonucleic acid
H. capsulatum	*Histoplasma capsulatum*
HMM	*Histoplasma* macrophage medium
IC_{50}	Half-maximal inhibitory concentration
MIC	Minimum inhibitory concentration
qRT-PCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
S. cerevisiae	*Saccharomyces cerevisiae*

Chapter I: Introduction

The aim of this study is to investigate the role that the DNA response protein, *DDR48,* plays within the fungal pathogen, *Histoplasma capsulatum,* by quantifying its expression levels in response to antifungal drug exposure, as well as observing expression changes in other genes within the cell when *DDR48* is deleted from the genome. Because of the ability of *H. capsulatum* to infect humans and cause the potentially fatal pulmonary disease, histoplasmosis, there is an interest in determining the function of *DDR48* and the role that it may possibly play in conferring antifungal resistance to the organism. The findings may contribute to further developing antifungal treatments to combat the organism. This study examines how *DDR48* expression levels change in response to antifungal drugs, the doses of antifungal drugs needed to inhibit growth of wild-type and *DDR48* deletion mutant cells, and, lastly, expression changes in ergosterol synthesis genes in a non-*DDR48* expressing strain of *H. capsulatum.*

Chapter II: Literature Review

Kingdom Fungi is one of the most diverse kingdoms of life on earth, encompassing approximately 1.5 million species, each with its own morphology, life cycle, and metabolism (Lutzoni et al., 2004). Fungi are capable of both parasitic and symbiotic relationships with plants and insects, as demonstrated by the ability of *Metarhizium*, for example, to parasitically digest insects while also transferring nitrogen and other nutrients to the plant(s) with which it associates (Behie, Zelisko, & Bidochka, 2012). The four major groups that have been established in Kingdom Fungi are the Chytridiomycota, Zygomycota, Ascomycota, and Basidiomycota, though more recent proteome studies suggest that Chytridiomycota and Zygomycota may fall under the larger Monokarya clade (Choi & Kim, 2017). Chytridiomycota consists of unicellular or filamentous fungi that feature flagellated cells and normally reside in aquatic environments. Zygomycota comprises a wide array of filamentous species that include soil saprobes, and symbionts of arthropods and plants. Zygomycetes lack flagella, as do Ascomycetes and Basidiomycetes, while the latter two kingdoms also exhibit the unique morphology of both single-celled growth and multi-celled, mycelial growth (Lutzoni et al., 2004). Basidiomycota are characterized as fungi that produce fruiting bodies, such as the "pedestal" structure of a typical mushroom. Ascomycota fungi are very diverse in morphology and produce spores that reside inside sacs called "asci," that are released during reproductive cycles (Lutzoni et al., 2004).

Ascomycota is the largest phylum of fungi, consisting of over 64,000 species and nearly 40% of all discovered fungi. They are found in a broad range of ecosystems across the globe, ranging from the inside of frozen rock in Antarctica to the depths of the sea in fallen wood fragments (Schoch et al., 2009). They are utilized in many industrial and agricultural settings, producing many foodstuff products on which humans have become reliant. *Saccharomyces*

cerevisiae has become widely used to make fermented food and beverage products including beers, wines, cheeses, and breads, due to its ability to ferment different carbohydrate sources (Bokulich & Bamforth, 2013). While Ascomycota pose many benefits to humans externally in the manufacturing industry, some species can also reside internally as part of a commensal relationship with the human host, possibly resulting in a parasitic relationship under certain conditions.

The spores found within the asci of Ascomycetes are major contributors to the spread of the organism and permit inhalation by host organisms. *Candida albicans* is a well-known opportunistic fungal pathogen that exists as a member of the normal human flora and maintains a commensal relationship with its host. Environmental changes, however, can trigger the activity of virulence factors, thereby allowing the organism to invade and infect host tissue. Another pathogenic Ascomycete is *Histoplasma capsulatum*, whose pathogenicity is similarly activated when subjected to certain virulence factors (Maresca & Kobayashi, 1989).

Histoplasma capsulatum is endemic to the Ohio, Mississippi, and Missouri River valley regions and is thermally dimorphic, meaning it exists in both mold and yeast forms depending on the temperature in which the organism is cultured. Studies have demonstrated that at environmental temperatures (~25°C), multicellular mold growth will occur, whereas at the average mammalian host temperature (37°C), the unicellular yeast form will instead occur. This mold-to-yeast shift, primarily triggered by the temperature increase, is essential for the pathogenicity of the organism (Maresca & Kobayashi, 1989). Once in the yeast form, *H. capsulatum* utilizes several mechanisms to evade the host immune response. One such mechanism is the concealment of its cell wall β-glucans, which are easily recognized by the Dectin-1 receptor on host macrophages. The pathogen achieves this feat by producing a layer of

α 1-3-glucan molecules to mask the β-glucans (Domer, 1971). Utilizing this virulence factor, the pathogenic organism may then colonize within the host macrophage and replicate within the phagolysosome to eventually infect lung tissue. Invasion of the lungs spurs the onset of histoplasmosis, the most common pulmonary mycosis in the United States (Benedict & Mody, 2016).

Micro- and macroconidia of *H. capsulatum* are regularly inhaled by natives of the organism's endemic regions due to environmental disruptions of the soil. In fact, it is believed that more than 40 million people in the United States have been infected with *H. capsulatum* (Zarnowski, Miyazaki, Dobrzyn, Ntambi, & Woods, 2007). This organism is a primary fungal pathogen, meaning it has the potential to infect any individual, regardless of their health status. Patients need not be immunosuppressed to experience symptoms of disease, as is the case with some other opportunistic fungi, such as *Cryptococcus neoformans* (Walsh & Dixon, 1996). However, infected individuals often remain asymptomatic, unaware of the self-limiting disease. The majority of those infected show mild symptoms such that an estimated 95% of histoplasmosis cases are not diagnosed (Zarnowski et al., 2007). For those experiencing mild symptoms of acute pulmonary histoplasmosis, fever, headaches, dry cough, and general lethargy may present, which the individual may mistakenly attribute to the common cold. This self-limited disease has the ability to resolve itself, unlike the more severe, disseminated form of histoplasmosis. Specifically, patients who are immunocompromised, such as those infected with HIV, transplant recipients, those undergoing cancer treatments or receiving any other immunosuppressive treatments are at the highest risk for severe infections (Assi et al., 2013). *Histoplasma capsulatum* also possesses the ability to survive within macrophages, which then spread throughout the body via the lymphatic system, granting the organism access to every

organ system in the body (Kauffman, 2007). As modern medical advances produce new therapies to combat histoplasmosis, awareness of the disease is becoming more prevalent, thereby stressing the importance of understanding the mechanisms that *H. capsulatum* uses to survive in the harsh host environment.

When under stress-inducing or exposed to DNA damaging agents, various stress response pathways in *H. capsulatum* may be activated. One such mechanism in fungi involves a family of proteins called the DNA damage response (DDR) protein group, which responds to damage caused by various stressors acting upon the cell. When there are breaks or other mutations in the DNA, *DDR* genes and proteins are recruited to initiate the signaling and repair process of the damage (Polo & Jackson, 2011). One specific protein in this family, DDR48p, has been shown to be upregulated during filamentation and biofilm formation in *C. albicans*, but its true function still remains unknown. *DDR48* has also shown increased transcription in response to DNA lesion-producing agents, osmotic stress, and heat shock in *S. cerevisiae* (Cleary, MacGregor, Saville, & Thomas, 2012). A homologous gene has been isolated in *H. capsulatum, DDR48,* and has been shown to respond to stressors including DNA damage. This gene has not been extensively studied in *H. capsulatum,* so understanding more about the role it plays in the stress response pathway of the organism is of particular interest.

Though much research has been conducted on *H. capsulatum,* there is still far more to learn regarding its gene and protein expression, virulence, and survival mechanisms. On the molecular level, there is considerable focus on the signaling mechanisms that the organism uses to combat stress and survive. As mentioned previously, *DDR48* in *H. capsulatum* shares sequence homology with *DDR48* in *C. albicans* and *S. cerevisiae,* where the protein has been shown to respond to stress-inducing agents. DDR48p has been found to be a stress response

protein in these organisms that is utilized when the cell faces oxidative stress, cell wall-disturbing agents, and antifungal drugs, but to be nonessential for survival (Dib, Hayek, Sadek, Beyrouthy, & Khalaf, 2008). In *C. albicans,* DDR48p has even been suggested to be involved in conferring resistance to antifungal drugs, which, along with assisting in biofilm formation, increases the pathogenicity of the fungus (Cleary et al., 2012). Furthermore, if DDR48p is able to sense changes in the environment, then it may function farther beyond the general stress response and contribute to the overall pathogenicity of the organism.

The aim of this study is to investigate the role that *DDR48* plays within *H. capsulatum* by quantifying its expression levels in response to antifungal drug exposure, as well as observing the effects on other genes within the cell when *DDR48* is deleted from the genome. The drugs used in this study are two that are commonly used in the clinical setting to treat histoplasmosis: Amphotericin-B and ketoconazole (Assi et al., 2013; Hage et al., 2009). Amphotericin-B is an intravenously administered antifungal medication that binds irreversibly to the ergosterol found in the cell membranes of *H. capsulatum,* forming pores and disrupting membrane integrity by allowing the leakage of ions out of the cell. Its mechanism of action is illustrated in **Figure 1.** Ketoconazole is an imidazole-based antifungal agent that is administered orally as a tablet and interferes with the synthesis of ergosterol by inhibiting the enzyme, cytochrome P450 14α-demethylase. This enzyme is pivotal in converting lanosterol to another precursor of ergosterol in the ergosterol biosynthesis pathway, ultimately contributing to membrane maintenance and integrity for *H. capsulatum.*

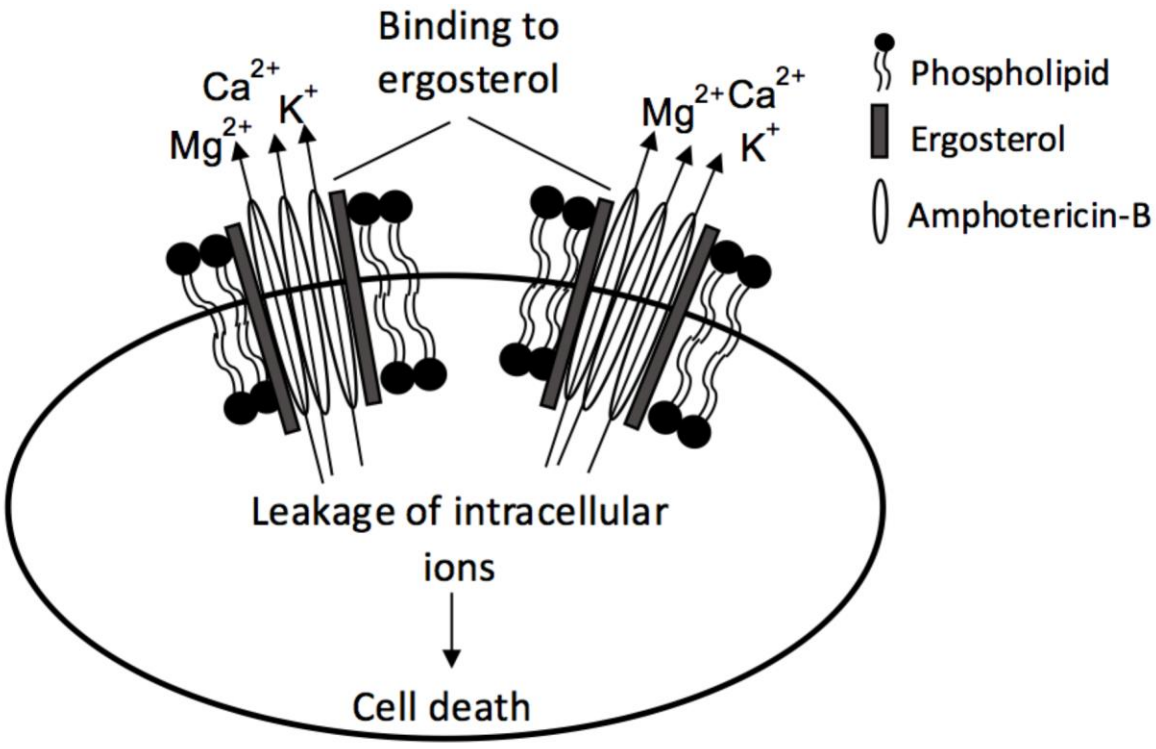


Figure 1. The mechanism of Amphotericin-B acting on a yeast cell.

By subjecting yeast phase *H. capsulatum* cells to these antifungals and observing the subsequent transcriptional responses, more may be uncovered about the function of *DDR48*. Additionally, since the two chosen antifungals interfere with the synthesis of ergosterol, certain genes in the ergosterol synthesis pathway were selected so that their expression levels may be observed when *DDR48* is and is not present in the cell. In doing so, the role of *DDR48* as a possible regulator of the ergosterol synthesis pathway may be investigated.

Chapter III: Methodology

Cell Culturing

H. capsulatum cells were cultured in *Histoplasma* Macrophage Medium (HMM), a modified tissue culture medium designed to mimic growth *in vivo* (Worsham & Goldman, 1988). The components of this medium consist of the following, per liter: 10.7 g F12 Nutrient Mix

(Sigma-Aldrich Chemical Company, St. Louis, MO #N6760), 18.2 g D-glucose, 1.0 g L-glutamic acid, 5.96 g HEPES buffer, and 84 mg L-cysteine. The pH was adjusted to 7.5, and the medium was filter sterilized and stored at 4°C. The *H. capsulatum* clinical isolate G186A (ATCC 26027) was the wild-type background strain used in this study. WU27 was constructed from this strain, which features a spontaneous smooth phenotype and *ura5Δ*-deletion (Klimpel & Goldman, 1987). **Table 1** lists the strains constructed from WU27. Both mold and yeast cells were cultured in HMM that was supplemented with 50 μg/mL ampicillin and 100 μg/mL streptomycin to reduce bacterial contamination. Cultures for uracil auxotrophs were also supplemented with 100 μg/mL uracil. All yeast cultures were grown at 37°C in 5% CO_2/95% room air with shaking at 200 rpm. Mold phase cultures were grown at 25°C in room air with shaking at 200 rpm.

Strain	Genotype	Other Designation
G186A	wild-type (ATTC# 26027)	
WU27	*ura5-D32*	WT, *DDR48(+)*
USM10	*ura5-D32 / ddr48-D1::hph*	*ddr48*Δ
USM13	*ura5-D32 / ddr48-D1::hph + pLE4 [URA5, DDR48]*	*ddr48*Δ *+ DDR48*

Table 1. Strains constructed from WU27.

Nucleic Acid Extraction

Total RNA from mold phase cells was extracted as follows: 150 mL mycelial cultures were grown for 10 days at 25ºC and harvested by vacuum filtration onto a Whatman, Grade No. 1, 11 μm filter paper. Cells were washed with cold 1X PBS (0.137 M NaCl, 0.0027 M KCl, 0.01 M Na_2HPO_4, 0.0018 M KH_2PO_4) (pH 7.4) and removed with a sterile cell lifter. Yeast cells were

grown to exponential phase (OD_{600} 1.2-1.8) and collected via centrifugation at 500 x g and resuspended in cold, sterile H_2O. Cells were recovered again via centrifugation at 500 x g and resuspended in the RNA extraction cocktail described below.

To extract the RNA from mold and yeast phase cells, glass beads were used in acid phenol to gently lyse the cell and release its contents. Yeast or mold cells were resuspended in a 2-ml screw-top microcentrifuge tube in 300 μL of RNA extraction buffer (0.1 M Na acetate, 0.2 M NaCl (pH 5.0), 0.2% SDS), 300 μL phenol: chloroform (5:1, pH 4.5), and 200 μL 0.5 mm acid-washed glass beads. RNase inhibitor and DNase I were also added to this mixture, in quantities of 0.25 μL each. The tubes were then placed on a MP Fast Prep-24 vortexer (MP Biomedicals) to shake at 4.0 m/s in 20-second intervals with 1 minute of resting on ice between each interval. For yeast, 4 cycles were used, and 6-8 cycles were used for mold. The samples were then centrifuged at 10,000 x g for 10 minutes at 4°C to pellet the cell debris and separate the aqueous phase that would contain the RNA from the organic phase. The aqueous phase was recovered and transferred into a new 1.5-mL microcentrifuge tube containing 1 mL of cold 100% ethanol. Samples were stored at -80°C overnight to precipitate the RNA.

RNA was purified for use in qPCR as follows: RNA samples were removed from -80°C storage and centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatants were removed, and the RNA was allowed to air dry before being resuspended in 50 μL of Tris-EDTA (TE) buffer (10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA). The NanoDrop™ spectrophotometer (ThermoFisher Scientific) was used to quantify the concentration of RNA in each sample and assess quality via calculating the 260 nm/280 nm absorbance ratio. To remove harmful DNases, the following components were then added to a 0.6 ml centrifuge tube: 10 μg of RNA, 5 μL 10X TURBO DNase I Buffer (Ambion), 1 μL TURBO DNase (Ambion), and sterile H_2O to bring the

final volume to 50 μL. The samples were incubated at 37°C for 30 minutes. Then, 5 μL of DNase Inactivation Reagent (Ambion) were added to each sample, and the samples incubated at 25°C for 2 minutes. They were then centrifuged at 10,000 x g for 1.5 minutes to pellet the inactivation reagent. The resulting DNA-free RNA was used to synthesize cDNA with the Maxima First Strand cDNA Synthesis Kit (ThermoFisher Scientific) according to the manufacturer's directions.

The purified RNA was quantitated with the NanoDrop™ spectrophotometer (A_{260} and A_{280} measurements) and 500 ng of purified RNA was added to a new 0.6 mL centrifuge tube with the following components: 1 μL 10X dsDNase Buffer, 1 μL dsDNase, and sterile H_2O to bring the final volume to 10 μL. The samples were incubated for 2 minutes at 37°C. Then, to the same tubes, 4 μL 5X Reaction Mix, 2 μL Maxima Enzyme Mix, and 4 μL H_2O were added. The samples were incubated at 25°C for 10 minutes and 50°C for 30 minutes, then the reaction was terminated by heating at 85°C for 5 minutes. Synthesized cDNA was then stored at 4°C until needed.

Quantitative Real Time-Polymerase Chain Reaction

The cDNA was quantitated with the NanoDrop™ and sterile type 1 H_2O was added to yield a final concentration of 250 ng/μL. One μL (250 ng) was used as template for quantitative real-time PCR (qRT-PCR) analysis. Reactions were performed in triplicate for each gene of interest. A SYBR Green-based Master Mix (Maxima) was utilized, and a master mix was created containing the SYBR, cDNA, forward and reverse primers, and nuclease-free H_2O for each gene. For four reactions per gene (three replicates and one additional reaction to account for error), the master mix contained 4 μL cDNA (250 ng/reaction), 4 μL forward primer, 4 μL reverse primer, 38 μL nuclease free H_2O, and 50 μL SYBR. 25 μL of this mix was then pipetted into 3 sterile

tubes on an 8-tube PCR strip for each gene being studied. The tubes were placed in a CFX96 Touch™ Real-Time PCR Detection System (BioRad) to undergo the following steps: initial denaturation at 95°C for 10 minutes; 30 repeats of denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. The integrity of each cycle was measured by melt-curve analysis. Relative expression was determined using the *ΔΔCt* method after normalizing to levels of *Histone H3* transcript.

Validation of *DDR48* Mutant Strains

Previously, the laboratory created several supplemental variants of *Histoplasma capsulatum* to study *DDR48.* The first strain utilized for this project was a "knockout" strain (*ddr48Δ*) featuring allelic replacement of *DDR48* with the hygromycin resistance gene (*hph*), and the second was a "complement" strain (*ddr48Δ + DDR48*), in which *DDR48* was returned to *ddr48Δ* cells on a plasmid to confirm that any changes seen in the deletion mutant were restored when *DDR48* was returned and expressed in the cell.

These strains were confirmed at the genomic and transcriptional level. RNA was extracted from WU27 (*DDR48(+)*), USM10 (*ddr48Δ*), and USM13 (*ddr48Δ + DDR48*) *H. capsulatum* cells as described above. Purified RNA underwent reverse transcription, and the cDNA was used in qRT-PCR to determine if *DDR48* was functionally transcribed in these strains (results depicted in **Figure 2b**).

Antifungal Susceptibility

To further elucidate the function of *DDR48* in *H. capsulatum, WU27,* USM10, and USM13 cells were stressed with two antifungal agents: Amphotericin-B (5 μg/mL) and ketoconazole (50 μg/mL). The purpose of these assays was to examine the potential role of DDR48 in the stress-response of the cell. That is, following the addition of antifungal drugs,

DDR48 was analyzed for upregulation, which would indicate a potential role in conferring antifungal resistance to the organism. RNA was extracted from mold and yeast cells at different timepoints following the addition of the drugs at 15, 30, 60, and 120 minutes utilizing the previously described methods. RNA purification and cDNA synthesis similarly followed as described. qRT-PCR yielded the results depicted in **Figure 4**. Each concentration was tested with three biological replicates of wild-type *H. capsulatum* cells.

To further investigate *DDR48*'s potential role in cell wall regulation, ergosterol synthesis genes were studied in cells with and without a functional copy of *DDR48* once again using qRT-PCR. WU27, USM10, and USM13 cells were grown to mid-log phase, Amphotericin-B (5 μg/mL) and ketoconazole (50 μg/mL) were added, and RNA extraction, purification, and cDNA synthesis were performed as detailed above. For qRT-PCR, the primers listed in **Table 2** were used to elucidate differential expression of genes in the ergosterol synthesis pathway.

Susceptibility to Amphotericin-B and Ketoconazole

To examine how the growth of *H. capsulatum* cells was affected by the presence of Amphotericin-B, a microplate dose-response assay was performed, adapted from the methods of Goughenour, Balada-Llasat, & Rappleye (2015). Cells were grown to mid-log phase (OD_{600} 1.4-1.9), enumerated in a hemocytometer, and diluted to 4×10^6 cells/mL in 2X HMM. Cells were added to the wells of a 96-well, flat-bottomed microplate. Amphotericin-B concentrations were prepared at twice the desired concentrations (two-fold dilutions from 32 μg/mL to 0.02 μg/mL final concentrations). Then, 50 μL of HMM medium with or without Amphotericin-B were added to each well, for a total volume of 100 μL. A multi-channel micropipette was used to gently mix the volume in each well. The microplates were covered with their lids and sealed with Blenderm™ (3M) breathable tape to be placed in the 37°C incubator for 4 days. Twice daily, the

plates were aerated by rocking on a benchtop rocker for 30 minutes. Growth was then quantified by the measurement of turbidity of each well at OD_{600} with a microplate reader. GraphPad Prism software was then utilized to calculate an IC_{50} curve from the dose-response data by nonlinear regression. This protocol was repeated using ketoconazole as the stressor. Final concentrations ranged from 32 μg/mL to 0.02 μg/mL, as well.

Primer	Sequence
Erg2	F – TTCAGCAACCACGGAAAC – (T_m = 57.5) R – CGCCATGCAGTATCGTAAA – (T_m =57.2)
Erg3	F – GGATTATGCCAAGCCCTTAC – (T_m = 57.8) R – CAGGACAGTCCAGATGTTAATG – (T_m = 57.8)
Erg4	F – GTACATCGTCTATCTGTTTGTTTAC – (T_m = 60.0) R – GCATATCCATACCACCCATC – (T_m = 59.9)
Erg5	F – CCACCATCTTCACCATCTTG – (T_m = 60.5) R – CGACGAACTTGTGGAAGAC – (T_m = 60.4)
Erg6	F – CTCTTACGCGACATTACTACAA – (T_m = 60.1) R – CCACAGCCTACATCAAGAAC – (T_m = 60.4)
Erg7	F – GGCACCTGTATGAACTACAC– (T_m = 60.1) R – GGAAGCAACCAGAGTTCAG – (T_m = 60.2)
Erg11a	F – TTCTTGGAACAAAAGGCAACG – (T_m = 59.2) R – CGAGGTTAGCCCGTATTTGAC – (T_m = 60.1)
Erg11b	F – CTATGGAACCGACCCGTATAAG – (T_m = 59.1) R – TCGTTGCCCTTTATGCCTAG – (T_m = 59.3)
Erg24	F – CCTTCTACTCTTGTGCTTGATAC – (T_m = 60.6) R – AGTGGTAAGAAAGGTGTTGAAT – (T_m = 60.4)
Erg25	F – GCAATAAAATCCCTAGCCTGAAG – (T_m = 58.8) R – TTTGATAAGTCATAGTCCACGGG – (T_m = 59.4)
Erg26	F – TATACAGAGACGAAGGCCCAA – (T_m = 62.7) R – GAGGACGAATGGAGAGGATTTG – (T_m = 62.4)
Srb1	F – GTAGCAGCCGAACAACATCTG – (T_m = 60.8) R – AATGAGACCTTGGGCGATACG – (T_m = 61.7)
H3	F – TGGTAAGGTCCCTCGTAAGC – (T_m = 60.6) R – GGAGTTTGCGGATGAGGAG – (T_m = 59.5)
DDR48	F – GACAATACTACCACCTATGGGTCTAA – (T_m = 60.8) R – CTTATCAGCGATGGTTTCCTTCTG – (T_m = 60.8)

Table 2. Primers used for qRT-PCR with melting temperatures (T_m) shown. All PCR products were approximately 200 bp in length.

Chapter IV: Results and Discussion

To analyze basal expression levels of *DDR48* in *H. capsulatum* mold and yeast cells under optimal growth conditions, RNA was extracted from wild-type strains and analyzed via qRT-PCR, as described above. Northern blot analyses were performed on the RNA to visualize the abundance of *DDR48* transcripts. The results are depicted in **Figure 2a.** It can be seen that expression of *DDR48* in the mold morphotype is nearly 6-fold greater than that in the yeast morphotype under normal conditions.

Allelic replacement of the *DDR48* gene yielded the strain USM10, also referred to as the knockout strain or *ddr48*Δ. Constructing a plasmid containing *DDR48* and adding it to the knockout strain yielded USM13, also referred to as the complement strain or *ddr48*Δ + *DDR48.* The purpose of creating these strains was to ensure that changes in subsequent assays seen between the wild-type and knockout strains were truly due to the absence of *DDR48* by ensuring that the phenotype in question was restored in the complement strain. These constructs were created previously in the lab, but qRT-PCR and Northern blotting techniques (**Figure 2b)** validated that the strains were created successfully. *DDR48* is absent in *ddr48*Δ, as seen by the lack of expression in the qRT-PCR assays and the lack of transcripts in the northern blot, while expression is restored in both instances in the complement strain.

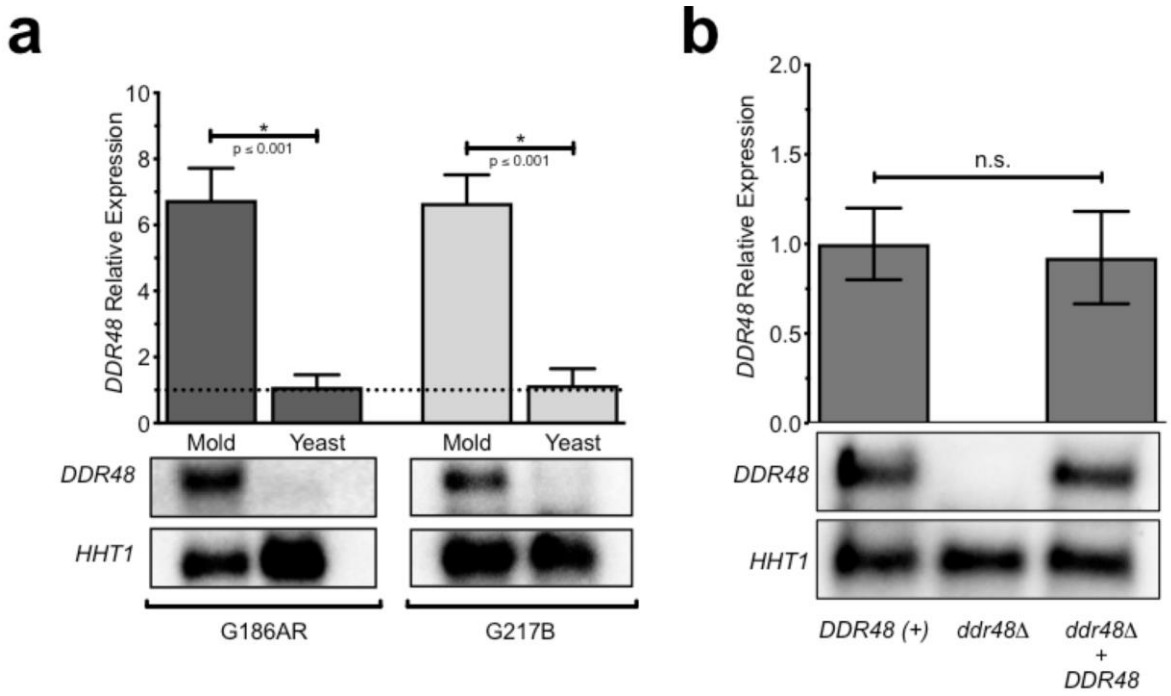


Figure 2. **DDR48 expression in mold vs. yeast morphotypes**. **a**. qRT-PCR assays and Northern blot analysis were performed on mold and yeast from two wild-type, *DDR48*-expressing strains. *Histone H3* was used as a normalizer gene for Northern blot loading. **b**. qRT-PCR assays and Northern blot analysis were performed on wild-type, *ddr48*Δ allelic replacement mutant, and *ddr48*Δ + *DDR48* complement strains. *Histone H3* was used as a normalizer gene using the ΔΔct method to normalize to wild-type *DDR48 (+)* expression. All experiments were performed with at least three biological replicates. Data from replicates are graphed as mean ± standard deviation.

Since *DDR48* gene expression is known to respond to antifungals in *C. albicans*, we exposed yeast phase *H. capsulatum* cells to Amphotericin-B and ketoconazole to determine if similar results would be achieved in this fungus. Dose-response analyses demonstrated susceptibility of yeast phase *H. capsulatum* cells to these antifungals (**Figure 3**). This data was then used to identify the minimum inhibitory concentration (MIC) of each drug for each strain, or the lowest concentration of drug that inhibited visible *H. capsulatum* growth. The half maximal inhibitory concentrations (IC_{50}), doses required to inhibit the growth of *H. capsulatum* by 50%, were also calculated. It was clear that wild-type cells could tolerate the additional stress

imparted by higher drug concentrations better than could knockout cells, which were more sensitive. Specifically, knockout cells subjected to Amphotericin-B stress were inhibited by drug concentrations that were three times lower than those required to inhibit wild-type growth. For ketoconazole, the MIC for knockout cells was approximately 50% that for wild-type cells. Additionally, the IC_{50} value for knockout cells under Amphotericin-B stress was 0.06 μg/mL compared to that for wild-type cells at 0.24 μg/mL. Ketoconazole stress resulted in an IC_{50} value of 0.48 μg/mL in knockout cells compared to 1.56 μg/mL in wild-type cells. For each experiment, resistance was restored in the complement strain; thus, knockout cells were more susceptible to antifungal drugs due to the loss of *DDR48*.

Next, we chose to use the same antifungals to compare *DDR48* expression levels between mold and yeast *H. capsulatum* cells. Recalling that basal transcriptional expression levels of *DDR48* in mold phase cells were nearly 6-fold higher than in yeast cells (**Figure 2a**), the aim was to determine how *DDR48* expression in each morphotype changed under antifungal stressors. RNA was extracted from mold and yeast cells at 0, 0.25, 0.5, 1, and 2 hour timepoints, respectively, and analyzed via qRT-PCR (**Figure 4**). Interestingly, we found that even under antifungal stress, *DDR48* was constitutively expressed in the mold phase. The yeast morphotype, however, exhibited significant up-regulation of *DDR48* expression in response to these stressors. Due to the expressional response observed, it appears that *DDR48* may contribute to combating the stress inflicted on the yeast morphotype and may help confer antifungal resistance to the organism.

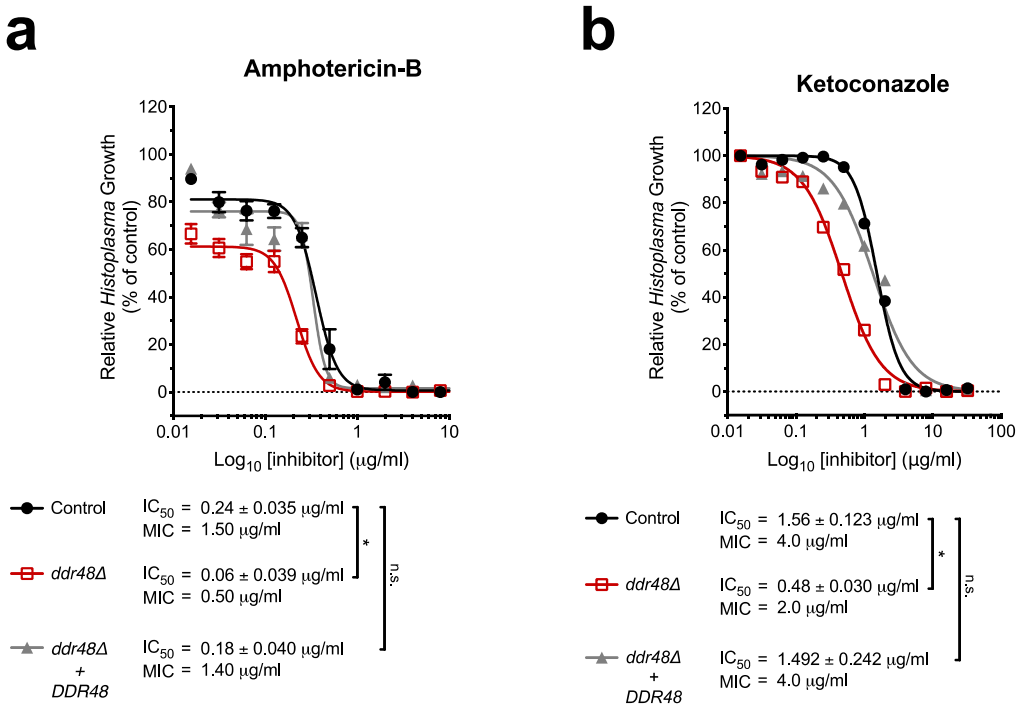


Figure 3. **The *DDR48* deletion mutant is more susceptible to antifungal drugs than the wild-type strain.** Dose-response curves for Amphotericin-B (**a**) and ketoconazole (**b**) for wild-type, knockout, and complement strains. Half maximal inhibitory concentrations (IC_{50}) and minimum inhibitory concentrations (MIC) of Amphotericin-B (**a**) and ketoconazole (**b**) for wild-type, knockout, and complement strains of *H. capsulatum*. All data generated were performed with at least two biological replicates. Data from seven replicates are graphed as mean ± standard error of the mean. All calculations were performed in GraphPad Prism.

Since the chosen antifungals inhibit ergosterol synthesis as previously described, we chose to investigate any potential changes to the ergosterol synthesis pathway when *DDR48* expression was absent. **Figure 5** illustrates the differential expression of several ergosterol synthesis genes in response to *DDR48* knockout (all genes analyzed are presented in **Supplemental Figures**) under optimal conditions. Interestingly, even in basal HMM conditions, the absence of *DDR48* resulted in dysregulation of some ergosterol synthesis genes, as seen with *Erg11a*, *Erg11b*, and *Erg6* (**Figure 5a-c**). Other genes in the pathway, like *Erg25*, exhibited statistically insignificant changes in their transcriptional expression despite this gene knockout (**Figure 5d**).

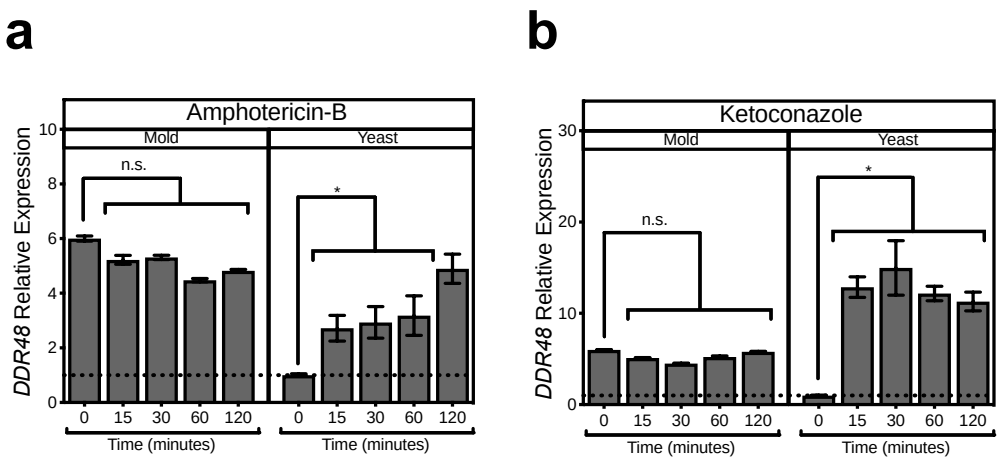


Figure 4. ***DDR48*** **expression levels in the yeast morphotype are significantly upregulated in response to antifungal stress.** qRT-PCR assays were performed on mold and yeast phase wild-type (DDR48 (+)) cells under optimal conditions (control) and 0.25 hour, 0.5 hour, 1 hour, and 2 hours after the addition of 5 μg/ml Amphotericin-B (**a**) or 50 μg/ml ketoconazole (**b**) to observe the expression of DDR48. Histone H3 was used as a normalizer gene using the ΔΔct method to normalize to the no Amphotericin- b (dashed lines) control. Data from triplicate samples are graphed as mean ± standard error of the mean.

This led us to determine if *DDR48* is acting as a regulator of the ergosterol synthesis pathway and helping combat environmental stressors by helping maintain membrane integrity.

We treated wild-type, knockout, and complemented mutant cells with Amphotericin-B to observe the effect on expression levels of ergosterol synthesis genes. **Figure 6** shows several genes in the ergosterol pathway that experienced dysregulation upon addition of the antifungal drug for different treatment times before extraction. It is evident that different patterns occurred; *Erg11a* experienced up-regulation upon deletion of *DDR48, Erg2* experienced no significant change in expression in knockout cells, and *Erg25* and *Erg26* were down-regulated in the knockout. Additional qRT-PCR assays for other ergosterol genes analyzed can be found in the **Supplemental Figures** section.

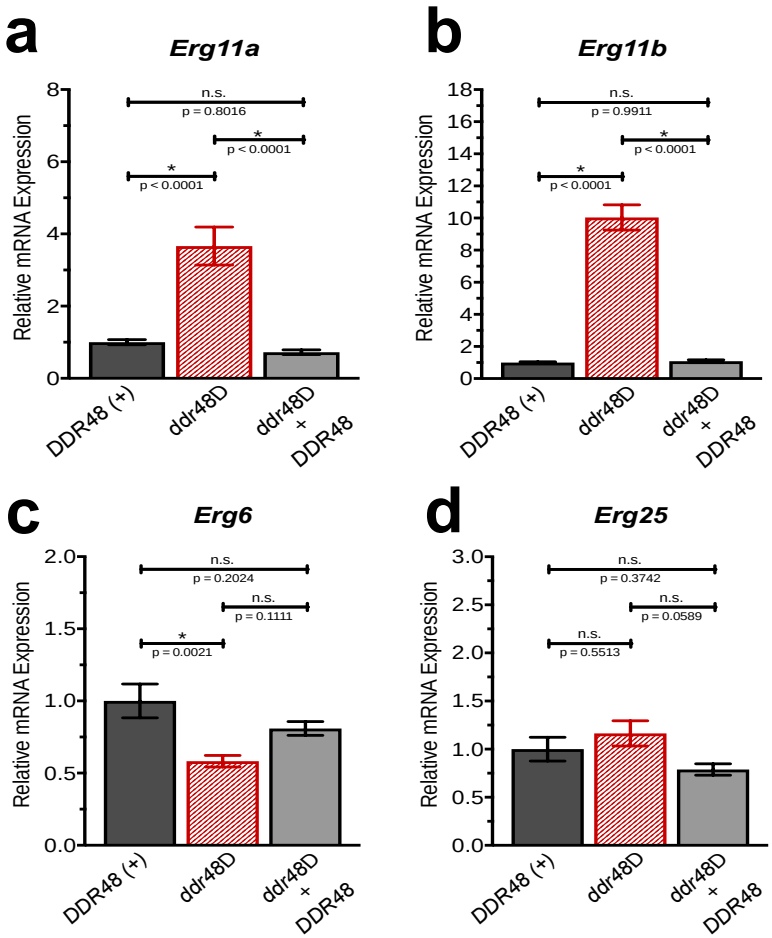


Figure 5. Under optimal conditions, yeast phase *H. capsulatum* cells show dysregulation of ergosterol synthesis genes in response to *DDR48* deletion. qRT-PCR assays were performed on yeast phase wild type, knockout, and complement cells under optimal conditions to observe the expression of several membrane sterol genes. *Histone H3* was used as a normalizer gene using the ΔΔct method to normalize to the no Amphotericin-B control (dashed lines). Data from triplicate samples are graphed as mean ± standard error of the mean.

Under Amphotericin-B stress in knockout cells, it appears that the genes analyzed lost their ability to be transcribed, for the most part. This is evident by looking at *Erg6*, for example, and visualizing how knockout cells failed to "turn on" this gene; that is, expression resembled that of control wild-type cells throughout the stress assay. This was the case for most other genes in the pathway, with the exception of *Erg11a*, which experienced significant up-regulation when *DDR48* was knocked out.

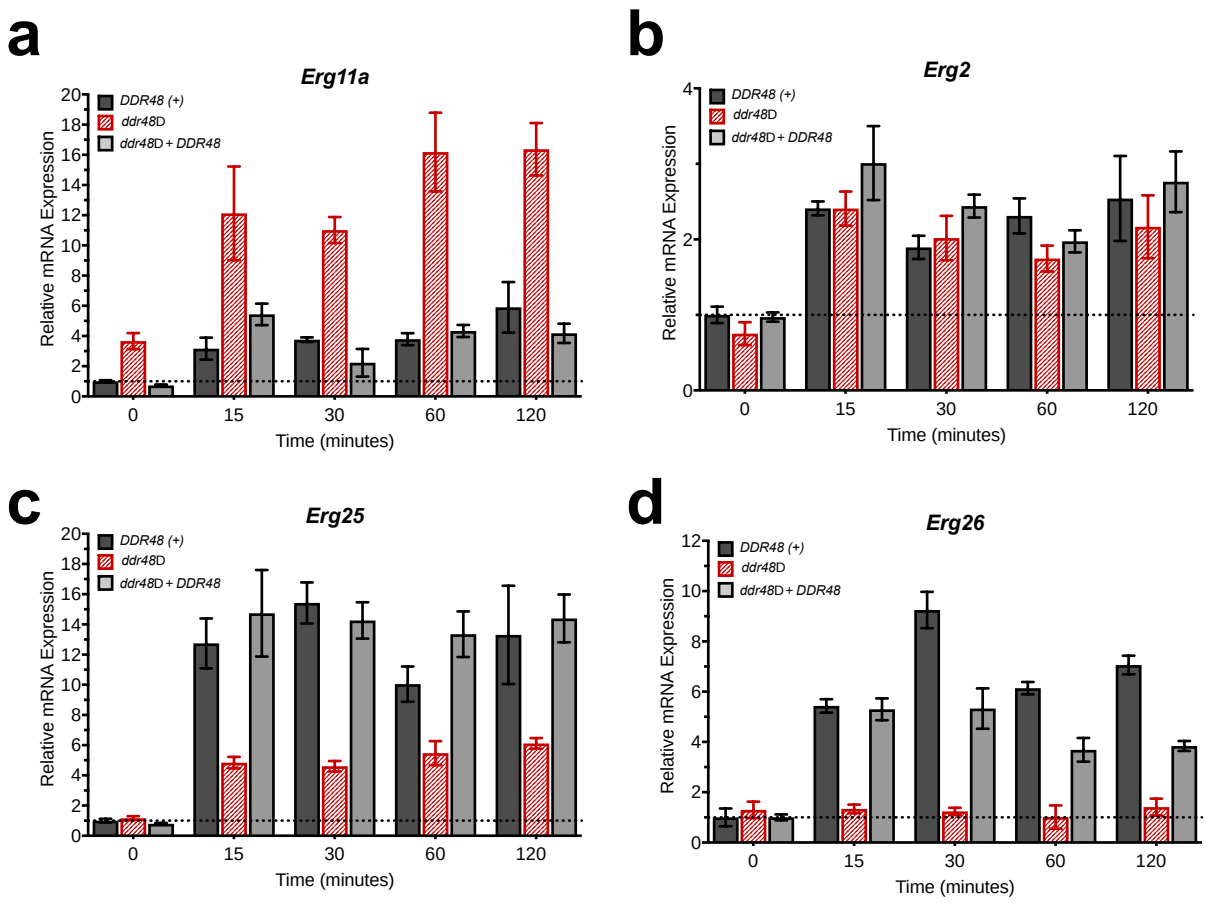


Figure 6. **Under Amphotericin-B stress, the deletion of *DDR48* from *H. capsulatum* alters expression of membrane sterol synthesis genes.** qRT-PCR assays were performed on yeast phase wild-type cells under optimal conditions (control) and 0.25 hour, 0.5 hour, 1 hour, and 2 hours after the addition of 5 ug/mL Amphotericin-B to observe the expression of several membrane sterol genes. *Histone H3* was used as a normalizer gene using the ΔΔct method to normalize to the no Amphotericin-B control (dashed lines). Data from triplicate samples are graphed as mean ± standard error of the mean.

Figure 7 shows the ergosterol synthesis pathway with the results of our qRT-PCR analyses (under Amphotericin-B stress) depicted as color-coded genes for the patterns of expression changes observed. Genes shown in red font indicate those that experienced loss of function or significant down-regulation as a result of *DDR48* deletion. Genes shown in green font indicate those that experienced up-regulation or gain of function. Genes shown in yellow

font indicate those that experienced no significant change in expression compared to wild-type expression.

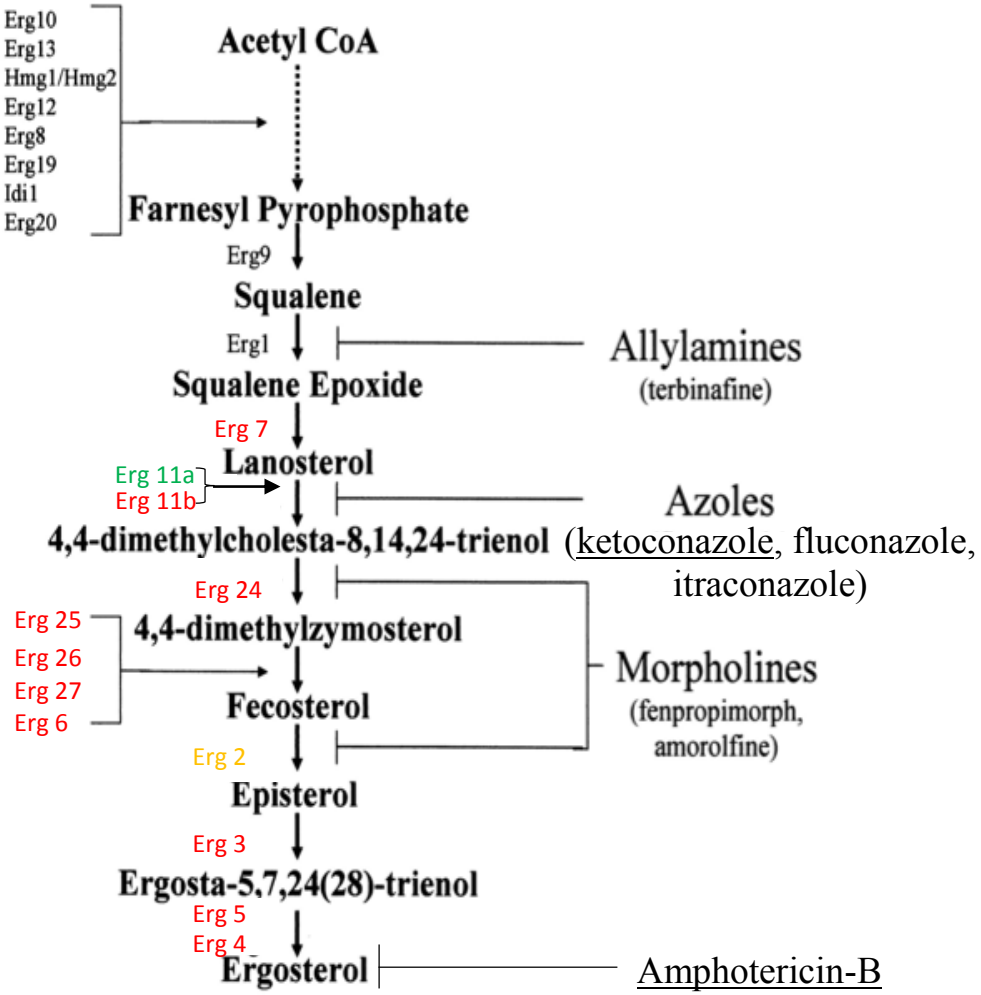


Figure 7. The ergosterol biosynthesis pathway showing the inhibitory mechanisms of azole and Amphotericin-B therapies. Color coded genes depict expression changes observed in *ddr48*Δ after treatment with Amphotericin-B. Genes that were up-regulated are coded green, those that experienced no significant change are shown in yellow, and down-regulated genes are indicated in red font.

Chapter V: Conclusion

Through creation of a *DDR48* deletion mutant strain, previous studies demonstrated that *DDR48*-deficient *C. albicans* was susceptible to antifungal drugs including ketoconazole (Dib et al., 2008). For this study, we chose to similarly investigate the impact of *DDR48* on cell viability under antifungal stressors in *H. capsulatum* and found that cells were more susceptible to Amphotericin-B and ketoconazole when the gene was absent (**Figure 3**). Drug resistance was restored in the complemented strain as demonstrated when MIC and IC_{50} values returned to wild-type levels.

In addition to combating cell stress in *C. albicans, DDR48* has shown increased transcription in *S. cerevisiae* in response to heat shock, osmotic stress, and DNA lesion-producing agents (Treger & McEntee, 1990). Similarly, in *H. capsulatum,* the up-regulation of *DDR48* after antifungal drug exposure confirmed our hypothesis that the gene plays some role in combating cell stress in the organism. The yeast morphotype, specifically, saw an increase in *DDR48* expression in response to these stressors, which is of particular interest since this phase is predominant in pathogenesis. One hypothesis we propose to distinguish the basal expression patterns of *DDR48* in the mold and yeast phases involves the natural environment of the mold phase; since this phase is found in nature where there are many stressors acting on the cells at all times, the increased basal-level transcription seen in **Figure 1a** may possibly be a result. The yeast phase, grown *in vitro*, is not exposed to the same level of stress and may therefore lack the number of *DDR48* transcripts that the mold phase exhibits. However, when stressed with antifungals, *DDR48* expression increased to combat the stress, while the gene is already "turned on" in the mold phase to combat natural, environmental stressors (**Figure 4)**.

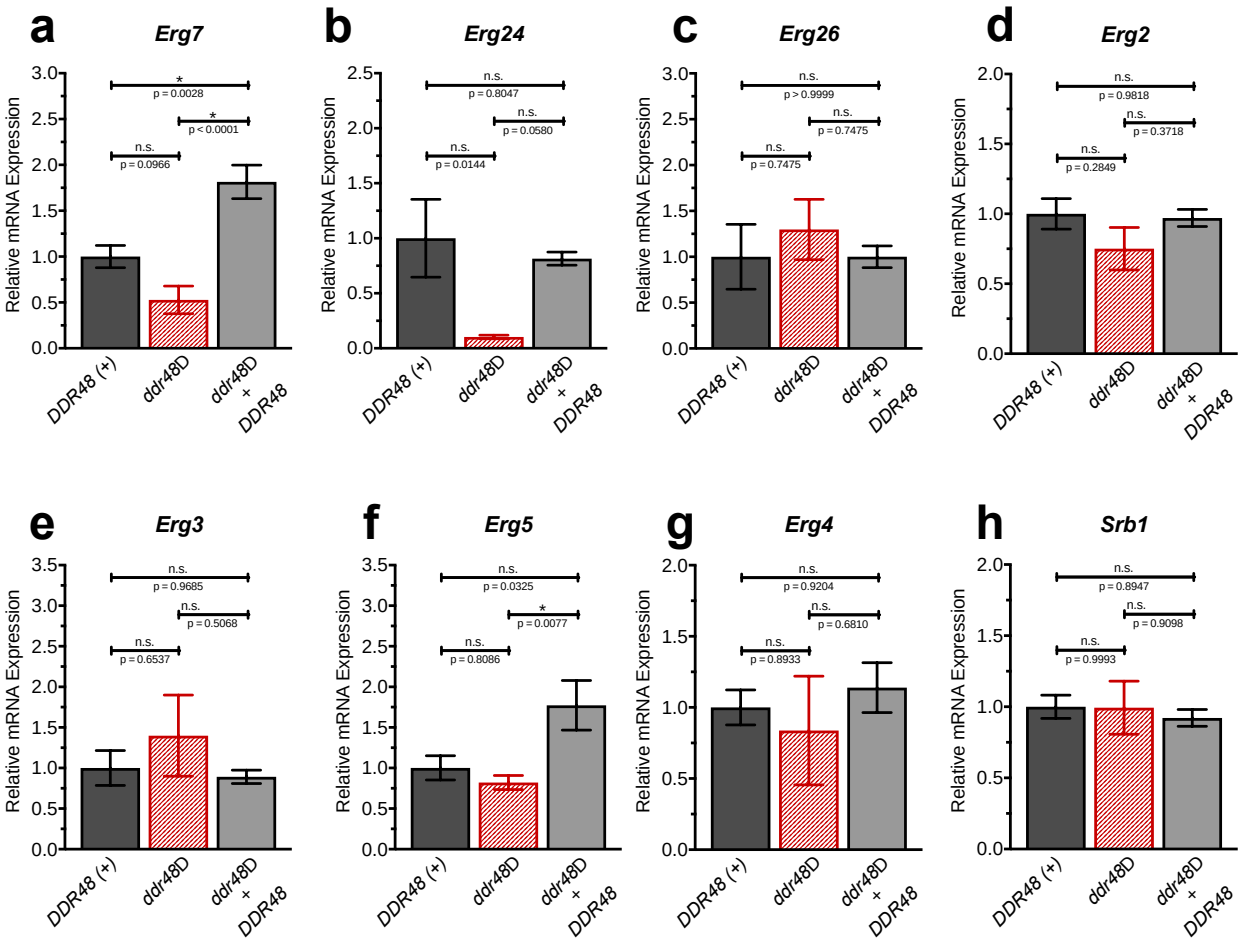
Our study of transcriptional changes in ergosterol synthesis genes in response to *DDR48* deletion resulted in novel discoveries regarding *DDR48*'s potential role in membrane maintenance in *H. capsulatum*. When knockout cells were stressed with Amphotericin-B, differential regulation of the sterol synthesis genes occurred (**Figure 6**). As noted previously, Amphotericin-B stress acts at the end of the ergosterol synthesis pathway (**Figure 7**) by binding to ergosterol to induce cell death (**Figure 1**). By depleting ergosterol, the cell might attempt to produce more of the sterol to maintain membrane integrity and promote viability. However, when *DDR48* was deleted from the genome, it appeared that the majority of the genes in the pathway, with the exception of *Erg11a,* could not be transcribed to wild-type levels. Thus, it appears that *DDR48* is acting somewhere upstream of the ergosterol synthesis pathway as a regulatory gene, thereby resulting in transcriptional issues downstream when it is deleted from the cell.

A study by DuBois and Smulian in 2016 showed that the sterol regulatory element binding protein (SREBP), *Srb1* is required for maximum transcription efficiency of several ergosterol synthesis genes including *Erg2, Erg3, Erg11a,* and *Erg25*. Our data showed that in *DDR48*-deficient cells under Amphotericin-B stress, *Erg2* was expressed at nearly wild-type levels, indicating that *DDR48* may act even upstream of *Srb1* to affect ergosterol synthesis genes. This further indicates *DDR48* as a gene potentially responsible for more global regulatory mechanisms of the stress response in *H. capsulatum* if it operates upstream of *Srb1*.

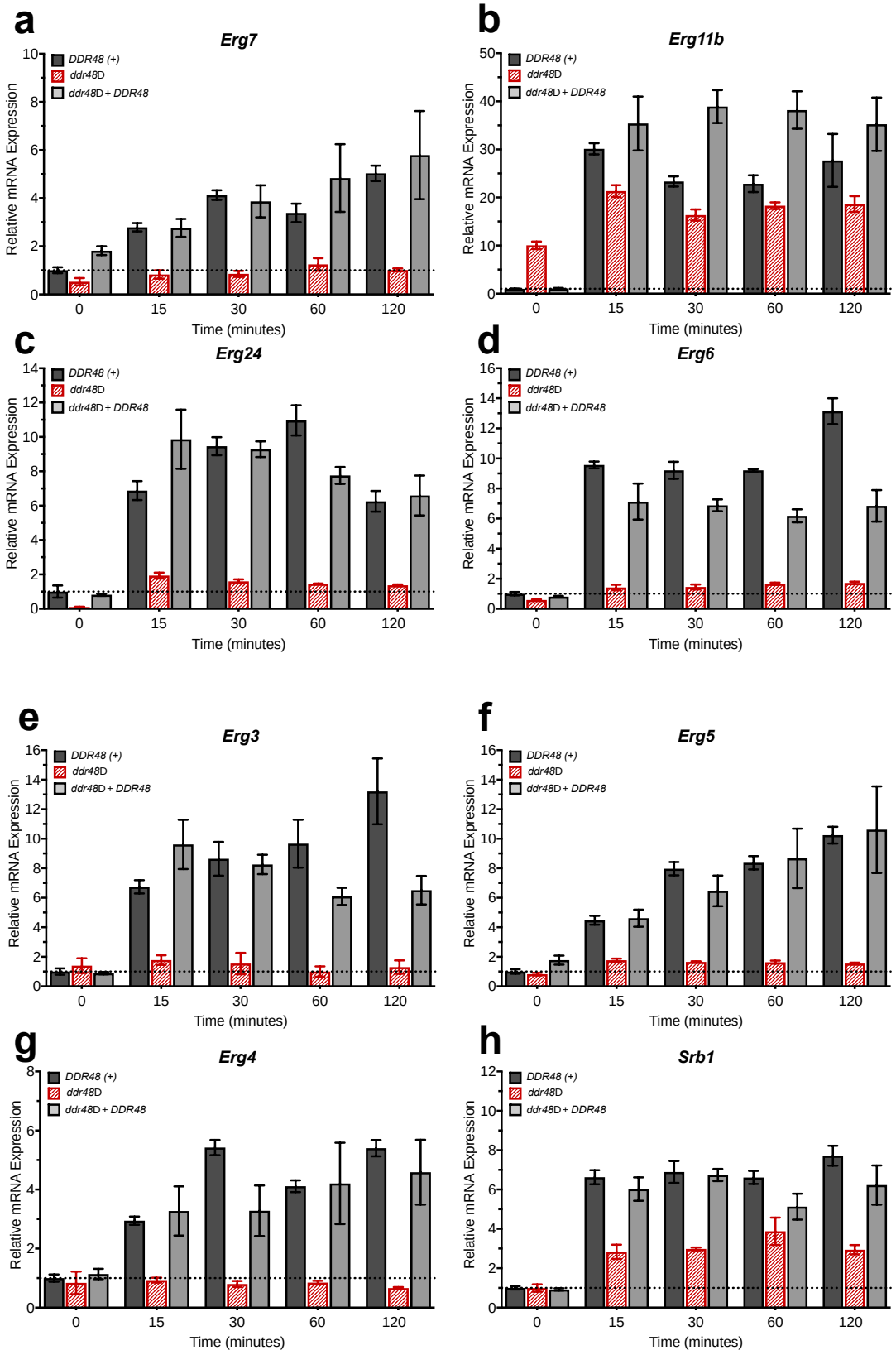
The presented findings suggest that *DDR48* may be responsible for viability of *H. capsulatum* cells under stress-inducing agents. This indicates the gene as a potential target for antifungal drug design in the future. By targeting *DDR48,* future antifungals may dampen the stress response of the cell enough to allow macrophages to engulf and successfully degrade *H.*

capsulatum cells. Thus, severe cases of histoplasmosis may be prevented, especially in immunocompromised patients. Additional studies may aim to elucidate further the identity and mechanism of action of the *DDR48* protein or even visualize its location in the cell. However, this study successfully verified that the gene is involved in combating cellular stressors, in part by regulating membrane sterol genes by some mechanism upstream of the biosynthesis pathway.

Chapter VI: Supplemental Figures



S1. (a-h) Additional ergosterol synthesis genes analyzed under optimal conditions in response to *DDR48* deletion.



S2. (a-h) Additional ergosterol synthesis genes exhibiting dysregulation under Amphotericin-B stress.

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