**The *C.albicans* Ddr48 Stress Response Protein
is Essential for Filamentation, Virulence, and
Confers Partial Antifungal Drug Resistance**

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by

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A handwritten signature in cursive script, appearing to read "Leila", is written over a horizontal line.

*To my Dad & Mom:
Mohammed & Nada*

*To my brother and sisters:
Bassem, Abeer, Dalia ,& Dana*

Acknowledgment

As I close a chapter in my life and I open up a new one, I realized that I will always cherish the good and bad memories in LAU forever and ever and ever. I will always remember faces, names, and even though those that I never got along with. Although the ending of this chapter in my life was not an heroic ending, after all I am satisfied of how things turned out to be.

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Abstract

Candida albicans is a pathogenic yeast that causes mucosal and systemic infections. *C.albicans* pathogenicity is attributed to its ability to exist in different morphological states and to respond to stress by up regulating several genes. Many genes involved in stress response have been partially characterized. *DDR48* is a stress associated gene involved in DNA repair and in response to antifungal drug exposure. The aim of the present study is to investigate any possible role of *DDR48* *in vitro* filamentation and *in vivo* virulence in a mouse systemic infection model by generating a homozygous null mutant strain. Furthermore, this study attempts to assess the antifungal drug susceptibilities of the mutant strain against azoles, amphotericin B, and caspofungin drugs *in vitro*.

It was observed that *DDR48* was haploid insufficient and essential, since only a heterozygote, but not a homozygous null mutant was generated. On corn meal agar, the heterozygote strain caused a delay of chlamydospore and hyphae formation with respect to the parental BWP17 strain. However, the heterozygote *DDR48/ddr48* strain, unlike the parental strain, was non filamentous on potato dextrose agar plus 50% fetal bovine serum, and on 50% fetal bovine serum medium. Furthermore, the heterozygote mutant showed additional defects in filamentation formation on Lee's medium, and on M199 pH7.5 as BWP17 formed an extensive network of filaments whereby the heterozygous strain exhibited defective filamentation formation. When cultured in fetal bovine serum, the heterozygote strain formed short aberrant germ tubes within 12 hours whereas the

parental strain formed an extensive hyphal network within 6 hours. *In vivo*, the mice survival rate was 66 % after 33 days of post infection with the heterozygous strain, as opposed to rapid death within 10 days with the parental strain.

DDR48/ddr48 was also susceptible dose dependent to itraconazoles and fluconazoles compared with the wildtype parental strain and susceptible to ketoconazoles. However, both the heterozygote and parental strain were sensitive to caspofungin, voriconazoles, and resistant to amphotericin B. In sum, the diminished virulence phenotype of the heterozygous mutant reflects the essential role of the *DDR48* in filamentation and pathogenesis.

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Abbreviations

ADE: Adenine

AP: Amphotericin B

ARG4 : Arginine

Benomyl: Benzimidazole Carbamate

Ca: *Candida albicans*

CS: Caspofungin

FBS: Fetal Bovine Serum

FL: Fluconazole

GCN : General Amino Acid Control Pathway

HIS1: Histidine

Itraconazole: IT

Ketoconazole : KE

MIC: Minimum Inhibitory Concentrations

MNC: Mononuclear cell leukocytes

Mops: Morpholine propanesulfonic acid

NCBI: National Center Biotechnology Information

PDA: Potato Dextrose Agar

PDB: Potato Dextrose Broth

PCR: Polymerase Chain Reaction

PMN: Polymorphonuclear cell leukocytes

PKA: Protein Kinase A

RT-PCR: Reverse Transcriptase Polymerase Chain Reaction

SAB: Sabourad Dextrose Agar

SRE: Stress Response Elements

Sc: *S.cerevisiae*

VO: Voriconazole

Chapter I

Introduction

Candida consists of approximately 200 extremely diverse yeast species, a fraction of which are human pathogens (Kirsch et al., 1990). However, at least 80% of *Candida* clinical isolates are *Candida albicans* (Hopfer, 1985). In most healthy humans, *C.albicans* occurs as benign commensal colonizer of mucosal membranes (Cheng et al., 2003). However, when the environment of the host is disturbed, this pathogenic yeast is able to cause mucosal infections, like acute pseudomembranous oral candidias (thrush), vaginal yeast infection, and systemic infections. Systemic candidiasis occurs where *C.albicans* is disseminated in the bloodstream without or with tissue invasion (Kirsch et al., 1990). Moreover, disseminated candidiasis is seen primarily among HIV infected patients and vulnerable intensive care patient, especially those undergoing immunosuppressive therapy following organ or bone marrow transplants and chemotherapy (Sudbury et al., 2004). The United States spends annually between \$1 and \$2 billion to treat *C.albicans* blood stream infections (Wilson et al., 2000). Despite that, mortality approaches 50% (Vindues et al., 2002).

The specific pathway by which *C.albicans* transforms itself into an opportunistic pathogen requires further elucidation (Cheng et al., 2003). Although disruption of *C.albicans* genes encoding secreted aspartyl proteinases (*Saps4-6*), glycosylphosphatidylinositol (GPI)-anchored superoxide dismutase (*SOD5*), and cell surface hydrophobicity (*CSH1*) reduced virulence in a mouse model of disseminated infection, no individual gene is the determinate

of virulence (Calderone and Fonzi, 2001). Rather, pathogenesis depends upon the *C.albicans* ability to coordinate the expression of multiple genes to adapt to changes in the local host environment (Mahan et al., 2000; Staib et al., 2000).

An interesting feature of *C.albicans* is its ability to exhibit dimorphism. Dimorphism, which refers to *C.albicans* ability to switch between yeast, pseudohyphae, and hyphae, is often considered to be essential for virulence. Hypha develops from germ tubes-unconstricted cylindrical projection from blastospores-that form during the first cell cycle (Sudbery et al., 2004). It has parallel sides along its length and no constriction at the neck of the mother cell (Calderone, 2002). The hyphal form can be induced in fetal bovine serum (FBS), hyphal inducing serum, or hyphal inducing medium at a growth temperature of about 37°C or at neutral pH. The hyphal form helps evade the host's defense mechanism and penetrate tissue, whereas the yeast form can disseminate more thoroughly than the hyphal form (Sherwood et al., 1992).

Pseudohyphae are often considered to be the intermediate state between yeast and hyphae. Therefore, mutations in the pseudohyphal state block the yeast to hyphae transition and cause loss of virulence. Pseudohyphal cells have a constriction at every subsequent septal junction neck and at the neck of the mother cell with no initial germ tube formation (Sudbery et al., 2004). The term filamentous is usually used to describe both the hyphal and pseudohyphal form and it is important for virulence (Kumamoto and Vines, 2005).

In response to various environmental signals, *C.albicans* utilizes converging pathways to regulate its yeast to hyphae transition. These signal transduction cascades include a Cph1-mediated MAP kinase pathway, an Efg1-mediated cAMP/PKA pathway, and a Cph2 pathway (Lane et al., 2001). The cAMP and the MAP kinase pathways are induced by

CaRas1p, a GTPase that is required for filamentation in response to hyphal inducing serum. In the cAMP signaling pathway, CaRas1p through Cap1, adenylate cyclase associated protein, stimulates CaCdc35, adenylate cyclase, which in turn successively stimulates the intracellular secondary messenger cAMP (Feng et al., 1999). In turn, cAMP binds to CaTpk1 and CaTpk 2, catalytic subunits of Protein Kinase A (PKA), and Bcy1, a putative regulatory subunit (Fig.1) (Sonneborn et al., 2000). Eventually, Bcy1 stimulates Efg1, a basic helix loop helix transcription factor, that influences morphogenesis, metabolism, and cell wall dynamics. Also, in the MAP kinase pathway CaRas1p carry's the signal from Cacdc42p, a small Rho-like G protein, to the MAP kinase Cst20 that successively stimulates CaSte17, Hst7, and Cek1 (Fig1). Eventually, Cek1 stimulates the transcription factor Cph1p (Fig.1). Cph1p has an important role in morphogenesis and virulence (Dhillon et al., 2003).

Cph2, a basic helix loop helix protein, has also been identified by its ability to promote yeast to hyphae transition. Cph2 activity is mediated through Tec1, a transcription factor (Fig.1). Moreover, Cph2 is involved in the transcriptional activation of hypha specific genes that contain E-box motifs (consensus sequence, 5'-CANNTG-3') in the upstream sequences or stress response element (SRE) elements. Cph1 and Efg1 overexpressions are able to suppress the defects in hyphal development in the *cph2/cph2* strain, while Cph2 overexpression overcomes the defects of filamentation in each *cph1/cph1* and *efg1/efg1* mutant strains (Dhillon et al., 2003). Furthermore, the *efg1/efg1 cph1/cph1* double mutant has a greater defect in virulence and filamentation than either single mutant (Lane et al., 2001).

Fig 1: Converging pathways involved in regulating filamentation and morphological switching in *Candida albicans*. Light shaded background: kinases and the circles: transcription factors (Dhillon et al., 2003).

On the other hand, transcriptional repression also plays an important role in controlling virulence and filamentation in *C. albicans*. Filament specific genes become active via inactivation of transcriptional repressors, such as Tup1, Nrg1, Ssn6, and Rfg1. Tup1, a global transcriptional repressor, mediate the repression of genes through the recruitment of Nrg1 and Rfg1, promoter sequence specific DNA binding proteins (Fig 2). Moreover, Ssn6 interacts with Tup1 forming a corepressor that is targeted to specific promoters by Nrg1 and Rfg1 (Murad et al., 2001). The corepressor recruits histone deacetylases to the promoter and eventually remodels the chromatin on yeast promoters (Smith and Johnson,

2000). Therefore, the assembly of the transcriptional apparatus is inhibited (Murad et al., 2001).

Moreover, CaMig1, similar to Nrg1 is a DNA binding protein, recruits CaSsn6/CaTup1 complex to the promoter to repress transcription of genes involved in stress response, carbon metabolism, and morphogenesis. *C.albicans* strains lacking any one of these transcriptional repressors are constitutively filamentous under non-filamentous inducing conditions. Alternatively, certain virulence genes are repressed by neither thus an unknown protein might mediate repression (Murad et al., 2001).

Fig 2: Schematic representation of *C.albicans* virulence and filamentous growth. Under hyphal inducing serum and at 37°C, transcriptional repressors are repressed. Under non filament inducing conditions, Nrg1, Rfg1 or an unknown protein might bind to the promoters of filament specific genes via recruitment of the corepressor Tup1 (Kadosh and Johnson, 2005).

Availability of amino acids is important in *C.albicans* morphogenesis. In response to starvation, *C.albicans* depends on the general amino acid control (GCN) pathway. Gcn2,

histidyl-tRNA synthetase, which accelerates the accumulation of the transcription factor Gcn4, enhances the GCN response. As a result, amino acid biosynthetic genes are activated. However, Gcn2 has a relatively minor role in the GCN response pathway in *C.albicans*, because the inactivation of Gcn2 only partially reduced growth under amino acid starvation conditions, and thus other activators of *GCN4* are probably present (Tournu et al., 2005).

Adhesion is an initial step in the establishment of *C.albicans* infections (Chaffin et al., 1998). *C.albicans* adhere to inert material, endothelial cells, and epithelial cells by forming biofilms, which are dense mycelial networks of yeast cells and hyphal elements encased within a matrix of exopolymeric material. In the biofilm form, *C.albicans* exhibit increased resistance to the host's immune defense mechanisms, antifungal drugs, and environmental stress. Furthermore, the *C.albicans* cell wall is essential for pathogenicity, adhesion, and virulence (Thomas et al., 2006). The cell wall constituents, including mannoproteins, chitin, and glucan are able to regulate the actions of the host's immune response (Chaffin et al., 1998).

A striking feature of *C.albicans* is its ability to produce chlamydospores. This property is shared only with *Candida dubliniensis*, a closely related species (Nobel et al., 2003). Chlamydospores are thick walled terminal or intercalary asexual spores that form without or with suspensor cells attached to pseudohyphae or hyphae (Odds, 1988). *C.albicans* produces chlamydospores under unfavorable conditions of nutrient, dryness, temperature, and upon aging. Hence, chlamydospores function as a storage form or dormant growth under non-favorable conditions, such as at low temperature or under nutrient poor oxygen limited conditions (Calderone, 2002). The production of chlamydospores is enhanced when

C.albicans is cultivated in a poorly utilized carbon source media, such as corn meal tween 80 agar (Kirsch et al., 1990). Moreover, *C.albicans* might have a functional role in infection, although this role has yet to be elucidated (Nobel et al., 2003).

C.albicans has developed different mechanisms to adapt to changes in the environment. One form of response to dramatic and sudden changes such as stress involves up regulation of the expression of key genes, such as *DDR48* (Ruis and Schuller, 1995).

1.1 Introduction to *CaDDR48*

DDR48 is an immunogenic stress associated protein located on chromosome 2. *DDR48* is also known as a heat shock and flocculent specific protein. Flocculation, a result of the distinctive cell to cell interactions, is the rapid sedimentation of clumps from a medium containing Ca^{2+} (Odds, 1988). *DDR48* contains multiple repeats of the amino acid sequence NNDSYGS (Harcus et al., 2004) that might be involved in drug resistance, since differences in drug resistance of various *C.albicans* isolates has been attributed to mutations in regions surrounding this amino acid sequence (Gupta et al., 1998). *DDR48* has a rich GC sequence element in the upstream promoter region that has been shown to be essential for its proper regulation and function (Treger and Mcentee, 1990).

Moreover, *DDR48* is an important immunogenic component of the cell surface of *C.albicans* cell wall and it has a significant role in virulence and immunomodulation. Recently, *DDR48* and other cell wall proteins have been used in vaccine formulations against disseminated candidiasis in animal models (Thomas et al., 2006). *DDR48* was also identified in the cell surface extracts of mature biofilms and planktonic cultures. During biofilm formation *DDR48* is up regulated (Garcia-Sanchez et al., 2005 a), therefore

enabling *C.albicans* to adapt to different environmental niches in the host (Thomas et al., 2006).

1.2 Virulence

Escape from the host's immune system and penetration into tissues are critical steps for *C.albicans* to cause disseminated infections. During systemic infections, the expression of an abundant number of genes is altered. To assess the influence of *DDR48* on *C.albicans* transcript profile and morphology during systemic infections, *C.albicans* strain was exposed to blood fractions enriched in macrophages, plasma, mononuclear leukocytes (MNC), and polymorphonuclear leukocytes (PMN). Transcript profiling revealed that *C.albicans* exposed to PMN, MNC, and plasma led to the up regulation of *DDR48* 1.3, 1.2, and 0.73 fold respectively. Despite the fact that *DDR48* was only induced 0.73 fold in plasma, *C.albicans* activated the transcriptional pathway and yeast cells changed to hyphal form (Fradin et al., 2003). Furthermore, exposure of *C.albicans* cells to macrophages led to the up regulation of *DDR48*, 8.0 fold, suggesting that *DDR48* is predominantly induced in response to macrophages (Singh et al., 2005). Thus, *C.albicans* engulfed by macrophages can filament, lyse the phage, counterattack neutrophils, and cause systemic infections (Fradin et al., 2003).

1.3 Environmental stress

Nitric oxides and reactive oxygen intermediates are important in controlling *C.albicans* infections (Hromatka et al., 2005). Nitric oxide inhibits the cytochrome oxidase enzyme, therefore blocking cellular respiration (Carreras et al., 2004), and eventually increasing the

production of O_2 and H_2O_2 (Poderose et al., 1996). O_2 and H_2O_2 rapidly diffuse across the cell membrane causing DNA, lipid, and protein damage. However, *C.albicans* detoxifies nitric oxides by flavohemoglobin, *YHB1*, which reacts with oxygen to form ferric flavohemoglobin and nitrate (Hromatka et al., 2005).

Using *C.albicans* genomic DNA microarrays, Hromatka et al. (2005) showed that the exposure of *C.albicans* cells to nitric oxides for 10 minutes led to the up regulation of *DDR48*, 1.9 fold, suggesting that *DDR48* was involved in combating the effects of nitric oxide. Moreover, *DDR48* was persistently induced, 57.1 fold, in the *yhb1/yhb1* null mutant *C.albican* strain providing evidence that *DDR48* is involved in DNA repair (Hromatka et al., 2005).

1.4 Amino acid starvation

Under amino acid starving conditions, the expression of *DDR48* is dependent on the amino acid pathway. Transcription profiling revealed that the expression of *DDR48* was significantly enhanced, 2.1 fold, in the presence of the transcription factors Gcn4 and Gcn2. However, *DDR48* was repressed in the presence of Gcn4 alone, indicating that in response to starvation the expression of *DDR48* is dependent on both the transcription factors Gcn4 and Gcn2 (Tournu et al., 2005).

1.5 Filamentation pathway

1.5.1 Cph1, Cph2, and Efg1 pathway

On hyphal inducing medium, the expression of *DDR48* is dependent on the Cph1, Cph2, and Efg1 filamentation pathways. Northern blotting analysis of *DDR48* in a *cph1/cph1* null

mutant strain indicated that *DDR48* was repressed on hyphal inducing medium, at 37°C as compared to the *Cph1* overexpression strain. Similarly, the expression of *DDR48* was not significant in *cph2/cph2* and *efg1/efg1* null mutants on YPD, rich non filamentous yeast extract peptone dextrose inducing medium for cultivating *C.albicans* cells, and YPD plus serum at 37°C (Lane et al., 2001). Lee's medium at 37°C, an amino acid liquid synthetic medium for cultivating mycelial and yeast forms of *Candida albicans* (Lee et al., 1975), derepressed *DDR48* in the *cph2/cph2* null mutant strain suggesting that the combination of activation and repression from different *Efg1* and *Cph1* pathways is complex and can lead to a dependent pattern of expression (Lane et al., 2001).

Using DNA microarrays and serum growth, Nantel et al. (2002) showed that in FBS at 37°C, *efg1* up regulated the expression of *DDR48* slightly. Also, *DDR48* in the double mutant *efg1/cph1* strain had a very similar profile to the *efg1* disruption strain (Nantel et al., 2002). Hence, *Efg1* more than *Cph1* regulates the expression of *DDR48* in serum.

1.5.2 Transcriptional Repressors

Transcriptional repressors play differential roles in the regulation of *DDR48* (Murad et al., 2001). Transcriptional profiling performed on *C.albicans* in a *tup1/tup1* and *nrg1/nrg1* null mutant strain, revealed the over representation of *DDR48* by more than 15 fold. These results are interesting bearing in mind that *Tup1* and *Nrg1* are repressors of many filamentation and virulence required genes, and thus suggest a possible role in filamentation and virulence for *Ddr48*. On the other hand, no decrease in *DDR48* expression was found in each of the *rfg1/rfg1* and *ssn6/ssn6* null mutants (Kadosh and Johnson, 2005), suggesting that the transcriptional repressors *Ssn6* and *Rfg1* don't

contribute to the regulation of *DDR48*. Moreover, Mig1 is not essential for Tup1 repression of *DDR48*, which a high degree of transcript profiling similarity would have been observed between the Tup1 and Mig1 regulon (Garcia-sanchez et al., 2005 b). Hence, Tup1 and Nrg1 can act independently of Rfg1, Mig1, and Ssn6 to repress *DDR48*.

1.6 Hyphal induction in medium and serum

By studying 6850 ORFs defined in *C.albicans*, Nantel et al. (2002) showed that when cells are cultured in FBS at 37°C, hyphae formation was correlated with the increased expression of Ddr48, putative cell wall protein Rbt1, and hyphal cell wall protein Hwp1 by 2.9, 3.01, and 6.5 fold respectively. Even more, in FBS at 25°C, Ddr48, Hwp1, and Rbt1 were persistently induced suggesting that temperature shift has slight induction on *C.albicans* elongation (Nantel et al., 2002). Nantel et al. (2002) identified several other hyphal inducing conditions, such as growth on Lee's medium. In addition to *DDR48*, other genes up regulated under the serum conditions were repressed on Lee's medium. Singh et al. (2005), using RT-PCR, showed that the expression of *DDR48* was increased in yeast extract peptone dextrose (YPD) medium, YPD plus 10% FBS and Spider's medium, hyphal inducing medium containing nutrient broth, mannitol, and phosphate buffer (Kohler and Fink, 1996).

As far as pH regulation of *DDR48* is concerned, microarray analysis revealed that at pH 8 *DDR48* was significantly enhanced, 2.68 fold. However, at pH 4 transcription of *DDR48* was repressed 1.544 fold, suggesting that *DDR48* has no significant role in the acidic response pathway. The results are interesting bearing in mind that *C.albicans* does not filament at low acidic pH but at slightly alkaline pH (Bensen et al., 2004).

1.7 Antifungal agents

1.7.1 Amphotericin B

1.7.1.1 Mechanism of action

Amphotericin B, a polyene, is the standard therapy for disseminated fungal infections. In the membrane of cells, there is a close relationship between the presence of ergosterols and polyene susceptibility. It has been suggested that amphotericin B molecules link to membrane sterols as a result of the interaction of amphotericin B with ergosterols. Therefore, the polyene hydroxyl residue faces inward eventually leading to the leakage of vital cytoplasmic components, as a result of the altered permeability, and eventually death of *C.albicans* (Fig 3) (Kerridge, 1985).

Fig 3: Model of the interaction between cholesterol and amphotericin B in the phospholipids bilayer. The dotted lines represent short range London Vander Waals Forces between the hydrocarbon chains of the phospholipids (Ghannoum and Rice, 1999).

1.7.1.2 Mechanism of Resistance

Hamilton-Miller (1972) suggested that qualitative or quantitative *C.albicans* resistance to amphotericin B occurs due to changes in the ergosterol content of the cells. As a result of

reducing the ergosterol content, smaller amounts of amphotericin B bind to the altered ergosterol. Furthermore, replacement of ergosterol with 3-oxosterol or 3-hydroxy lead to binding of amphotericin B less well (Ghannoum and Rice, 1999). Recently, by examining genome wide expression profiles of *C.albicans* in response to amphotericin B, Liu et al. (2005) observed an up regulation of *DDR48*, 3.4 folds, which was due to reduced ergosterol biosynthesis in the *C.albicans* strain.

1.7.2 Azoles

1.7.2.1 Mechanism of action

C.albicans has developed different mechanisms of resistances to Azole and its derivatives, Fluconazole, Itraconazole, Ketoconazole and Voriconazole. However, azole derivatives inhibit cytochrome p-450, which leads to the accumulation of sterol precursors, such as 4-14 dimethylzymosterol, 24-methylenedihydrolanosterol, and lanosterol (Fig 4). Therefore, depletion of ergosterol eventually leads to the formation of an altered *C.albicans* plasma membrane function and structure (Ghannoum and Rice, 1999).

Fig 4: Ergosterol synthetic pathway. Antifungal agents exert their inhibitory activates on different sterol precursors. FLU: Fluconazole, ITRA: itraconazole, VOR: voriconazole (Ghannoum and Rice, 1999).

1.7.2.2 Mechanism of Resistance

Studies have suggested that the reduced susceptibility of *C. albicans* to azoles and its derivatives was due to differences in cytochrome p-450 enzyme. Elevated levels of cytochrome P-450 and low binding affinity for azoles confer resistance to azole. Moreover, the efflux pump is an important mechanism of resistance to azoles (Ghannoum and Rice, 1999). *C. albicans* posses at least 2 drug efflux pumps, Major Facilitator Super family (MFS) and the ATP-binding cassette super family (ABC) (Karababa et al., 2004). In resistant isolates, mRNA levels of the *C. albicans* Multidrug resistance gene (*CaMDR*), a member of the MFS family, and two of the ABC transporters, *C. albicans* Drug Resistant 1 (*CDR1*) and *C. albicans* Drug Resistant 2 (*CDR2*) were overexpressed. Overexpression of

the efflux pumps prevents accumulation of the azoles in *C.albicans*. Gene expression profiles of azole resistant clinical isolates of *C.albicans* revealed that the up regulation of *DDR48* was not correlated with the expression of either *CDR1* or *CDR2* (Rogers and Barker, 2003). On the other hand, upon exposure to fluconazole and benomyl *DDR48* was up regulated 6.6 fold by inducing the *C.albicans* *CaMDR1* pathway (Karababa et al., 2004).

1.7.3 Caspofungin

1.7.3.1 Mechanism of Action

Caspofungin, an echinocandin antifungal agent, is an active agent against *C.albicans*. It is a noncompetitive inhibitor of the enzyme β -(1,3)-glucan synthetase, an essential component of the *C.albicans*'s cell wall (Fig 5). Caspofungin inhibits the GSL1, catalytic membrane subunit of D-glucan, from binding to the intracellular UDP-glucose. Caspofungin inhibits the GSL1, catalytic membrane subunit of D-glucan, from binding to the intracellular UDP-glucose. (Letscher-Bru and Herbrecht, 2003). Hence, disruption of the cell wall will eventually lead to osmotic stress, lysis, and death of the *C.albicans* (Fung et al., 2002).

Fig 5: Caspofungin inhibits the synthesis of the fungal cell wall (Fung et al., 2002).

1.7.3.2 Mechanism of Resistance

In response to Caspofungin, *C.albicans* upregulates the transcription factors CaCas5, CaRlm1, and CaAda2. CaCas5, CaRlm1, and CaAda2, which are activators of many cell wall protein genes, are required for resistance to numerous antifungal drugs. CaAda2, a subunit of the transcriptional coactivator complex, functions in conjunction with CaRlm1 and CaCas5. CaCas5, through the recruitment of CaAda2, activates transcription of genes involved in cell wall damage (Bruno et al., 2006). Recently, Bruno et al. (2006) determined whether caspofungin sensitive mutants, *cas5*, *rlm1*, and *ada2*, were defective in regulation of cell wall protein genes. By microarray comparisons of the reference strain and of each mutant, the expression profile revealed that, upon exposure to caspofungin, *ada2/ada2*, *cas5/cas5*, and *rlm1/rlm1* null mutants repressed the expression of *DDR48* 4.27, 1.8, and 0.42 fold respectively (Bruno et al., 2006). Hence, it is possible that *DDR48* plays a role in caspofungin resistance.

1.8 Homologue genes to *CaDDR48*

1.8.1 Homologue genes in the NCBI database

Homologue genes to *DDR48* were identified in the National Center Biotechnology Information (NCBI) database by performing a BLAST P, protein Blast, search using the Ddr48 ORF. As observed in the NCBI database, the most similar gene to *CaDDR48* is the *Saccharomyces cerevisiae* (*Sc*)*DDR48*. The small Expectation (E)-Value of *ScDDR48*, 1E-20, indicates that both evolved from a common ancestor, and that similarity is not due to chance. Furthermore, the high score bit of 102, which measures alignment using statistical significance, supports the fact that the *ScDDR48* sequence is similar to *CaDDR48*.

Whereas, the high E-values of XP_386868.1, hypothetical protein FG06692.1 in *Gibberella zeae* PH-1, BAE62408.1, unnamed protein product in *Aspergillus oryzae*, and XP_501947.1, hypothetical protein in *Yarrowia lipolytica*, suggest the lower the probability that they are similar to *CaDDR48*.

ScDdr48 is an extremely hydrophilic protein composed of multiple repeated peptide sequences of Ser-Asn-Asn-x-Asp-Ser-Tyr-Gly, x is either Asp or Asn. Moreover, and similar to *CaDDR48* it has a relatively rich GC sequence element in the upstream region. *ScDDR48* is not only similar to *CaDDR48* in sequence only but it is similar to it in function too. *ScDDR48* displayed increased transcription response to heat shock stress and to treatments that produced DNA lesions, such as 4 nitroquinoline-1-oxides. At high concentrations of 4 nitroquinoline-1-oxides, *ddr48* null mutant showed a slightly greater survival curve than the wild type strain. Furthermore, *ddr48* null mutant was more resistant to exposure to methyl methanesulfonate and UV radiation. Thus, in response to heat shock and DNA damage *DDR48* represents a unique set of stress response elements (Treger and Mcentee, 1990).

In *S.cerevisiae*, *DDR48* is involved in at least one pathway of mutagenesis (Treger and Mcentee, 1990). By examining induced mutation frequencies at *HIS4* and *ADE2* loci, it was revealed that the *ddr48* null mutant resulted in 2 to 3 fold decrease in the spontaneous mutation frequency at the *ADE2* loci and 10-fold reduction at the *HIS4* locus. Thus, as a result of *DDR48* inactivation mutagenesis is reduced (Whelan et al., 1979). Moreover, the *ddr48* strain resulted in 6 to 14 fold reduction in the spontaneous mutation rate of the reversion of *his4* mutant (Whelan et al., 1979). This is rather an unexpected result and

somehow conflicts with the *CaDDR48* data that suggest a positive role in the nitric oxide DNA repair pathway (Hromatka et al., 2005).

1.8.2 Homologue genes in the *Candida albicans* Database

Homologue genes to *CaDDR48* were identified in the *Candida albicans* database by BLASTP, protein blast, search using the CaDdr48 ORF. The *C.albicans* Database revealed that Asr1, putative heat shock proteins, is the most similar protein to Ddr48. The small probability (N) value of Asr1, 9.9e-05, suggest that Asr1 is probably similar in function to CaDdr48. Actually, Asr1, similar to Ddr48, is transcriptionally regulated by cAMP pathway, since blocking the cAMP pathway repressed the transcription of Asr1 (Harcus, et al, 2004). Moreover, the transcription repressors Tup1 and Nrg1 also regulate the expression of Asr1 (Garcia-Sanchez et al., 2005 b).

Interestingly *IHD1*, *HYR1*, and *ALS7* showed up in this search and all three genes are expressed under hyphal conditions (Nantel et al., 2002; Nobel et al., 2003). The deletion of *IHD1* and *ALS7* resulted in attenuated virulence (Nobel et al., 2003), suggesting a possible role for *CaDDR48* in filamentation and possibly virulence. Nevertheless, their Probability (N) values are quite elevated implying low homology.

1.9 Purpose of the project

To date, no studies have been done reporting the creation of a *Caddr48* null mutant strain. Therefore, the present study attempts to knock out *DDR48* and characterize any possible role in filamentation in hyphal inducing and non inducing synthetic medium and in serum. Furthermore, the purpose of this study is to characterize the role of the *DDR48* in

chlamydospore formation and in virulence through a mouse model of disseminated infection. Finally, since *DDR48* expression is up regulated in response to antifungal drugs, this study attempts to assess any differences in resistance between the *ddr48* mutant and parental strain to 6 widely used antifungal drugs: Fluconazole, Ketoconazole, Itraconazole, Voriconazole, Amphotericin B, and Caspofungin.

Chapter II

Materials and Methods

2.1 Construction of *DDR48 HIS* fragment

2.1.1. PCR

The heterozygous *DDR48* strain was generated by transforming cells with PCR fragments. The *HIS1* deletion allele fragment was generated by using one synthetic forward primer that contained *DDR48* sequences from nucleotide position +1 to +50 5' and *HIS1* sequences from nucleotide position -322 to -343 3', and a second reverse primer that contained *DDR48* sequences from nucleotide position +651 to +558 5' and *HIS1* sequences from nucleotide position +1035 to +1012, 3'.

For amplification, typical PCR mixtures contained: 50 ng of CAI12 DNA (*ura3Δ::imm434/URA3*; Fonzi & Calderone, 1999) CAI12 was offered as a gift by Dr. William Fonzi (Georgetown university, Washington D.C, USA), 2 µl of 10mM dNTP, 3µl of 25mM MgCl₂, 1µl each of the 60mM forward and reverse *DDR48* primers (the primer sequences are found in Table 1), 6µl of the 10x Taq buffer (NH₄)₂SO₄ and 1µl of 5 units of Taq polymerase (Fermentas Life Sciences, USA), and 46µl of distilled water. PCR was performed in a Gene Amp PCR system 9700 (Applied Biosystems) using the following PCR conditions: initial denaturation at 94° C for 5 min, followed by 30 cycles of 1 min denaturation at 94°C, 50 sec annealing at 52°C, 1.45 min elongation at 72°C, followed by a final extension step at 72°C for 10 min and a termination step at 4°C for ∞.

2.1.2 Gel electrophoresis

The reaction mixtures were visualized by electrophoresis (Pharmacia Biotech Gel Electrophoresis Apparatus GNA-100, Sweden) on a 1.5 % agarose gel along with marker XVI (250-3000bp; Roche, Manheim, Germany) to confirm the presence of a product with the expected size.

Table 1: PCR primers used for amplification and verification

Primer name	Primer sequence
1. <i>DDR48</i> <i>HIS</i> Deletion Upstream F	5' ATGGTTTTTCGGTTTCGGTAAAGACGAC ACAAAGACAAGAACGACAGAAGAGAT CAGAAGTTAGTAGTAACAATTTG 3'
2. <i>DDR48</i> <i>HIS</i> Deletion Downstream R	5' ATGGTTTTTCGGTTTCGGTAAAGACGACG ACAAAGACAAGAACGACAGAAGAGAT CAGAAGTTAGTAGTAACAATTTG 3'
3. <i>DDR48</i> Integration Verification primer	5' CTGTGGATTATTCTCTTTGAA 3'
4. <i>HIS1</i> Internal primer	5' CGGTCTGGTAAATGATTGACTAAATCC TCC 3'
5. Actin F primer	5' GTTGCTGCTTTAATTATCGATAACGGT TCTGG 3'
6. Actin R primer	5' GGTGAACAATGGATGGACCAGATTCC TC 3'
7. <i>DDR48</i> Internal F primer	5' AGATAACGATTCTTACGGATC 3'

F: forward, R: reverse. (The following primers were designed by Dr. Roy Khalaf)

2.2 Verification of integration by PCR

Verification of integration was confirmed by PCR analysis of the *DDR48/ddr48* heterozygous colonies. Verification involved the use of 3 sets of primers. The first set involved *DDR48* internal forward primer that hybridized to sequences internal to the heterozygous allele at nucleotide position +271 to +293, and *DDR48* internal reverse primer that hybridized to sequences internal to the heterozygous allele at nucleotide

position +51 to +73. For the second set of primers, *DDR48* verification primer hybridized outside the *DDR48* integration site and the *HIS1* primer hybridized to one end of the *HIS1* sequence at nucleotide position +1 to +30. Finally, actin primers were used as a control for the quality of the DNA preparations (Khalaf and Zitomer, 2001).

PCR samples were prepared as follows: the first mixture sample contained 50 ng of the presumed heterozygous *DDR48/ddr48* DNA, 2 μ l of 10mM dNTP, 3 μ l of 25mM $MgCl_2$, 1 μ l of the 60mM *DDR48* Integration verification primer, 1 μ l of the 60 mM *HIS1* primer (the primer sequences are found in Table 1), 6 μ l of the 10x Taq buffer $(NH_4)_2SO_4$, and 1 μ l of 5 units of Taq polymerase (Fermentas Life Sciences, USA), and 46 μ l of distilled water. The second and the third PCR mixtures were similar to the first, except that in the second mixture 1 μ l of 60mM *DDR48* internal forward and reverse primers (the primer sequences are found in Table 1) were used instead of the integration primers to confirm that only one copy of *DDR48* was deleted. As for the third mixture, it contained 1 μ l of 60 mM Actin forward and reverse primer (the primer sequences are found in Table 1), as a control of DNA quality in the presumed heterozygote.

As for the fourth and fifth samples, conditions used were similar to the above but one contained the internal and actin control in one sample using the presumed heterozygote DNA, and the other the internal and actin control using the CAI12 control strain DNA. The following PCR conditions were used: initial denaturation at 94°C for 5 min, followed by 30 cycles of 30 sec denaturation at 94°C, 50 sec annealing at 52°C, and 1.22 min elongation at 72°C, followed by a final extension at 72°C for 10 min and a termination step at 4°C for ∞ .

2.3 DNA extraction from agarose gel

DNA was extracted from the agarose gel by the Nucleotrap PCR Kit and following the manufacture's protocol (Macherey-Nagel, Germany).

2.4 Transformation Protocol

C.albicans strain BWP17 (*ura3Δ/ura3Δ*, *his1Δ/his1Δ*, *arg4Δ/arg4Δ*; Wilson *et al*, 1999), offered as a gift from Dr. Richard Zitomer (University of Albany, New York, USA), was transformed with the *HIS1-DDR48* fragment using the Alkali Cation Yeast Transformation kit (Q-Biogene, Germany). Transformation was performed following the protocol modified by Dr. Khalaf (Khalaf & Zitomer,2001).

2.5 DNA Extraction

Heterozygous *DDR48/ddr48* DNA was extracted following the procedure described by Yazbeck et al. (2007).

2.6 Growth on corn meal tween 80 agar

The *DDR48/ddr48* heterozygous and BWP17 strain were grown on corn meal tween 80 agar. A sterile blade was used to cut the corn meal agar plate in the center gently. Bwp17 was pasted along the edges of the opening and was covered completely with a glass cover slip to create an anaerobic condition. This procedure was repeated again using the *DDR48/ddr48* heterozygous strain. The two plates were incubated in a dark room at room temperature and they were monitored microscopically over 7 days for the formation of hyphae and chlamydospores.

2.7 Filamentation on hyphal inducing media and FBS

2.7.1 Filamentation on hyphal inducing media

From an overnight BWP17 culture grown in potato dextrose broth (PDB), cells were diluted to an OD₆₀₀ 0.1 in 10 ml of PDB and grown to OD 0.75 at 30°C. 10 ml of the culture was centrifuged at maximum speed for 5 minutes. The supernatant was discarded and the pellet was suspended in 10 ml of distilled water. Then, 2 µl of the solution was spotted on Lee's medium, Medium199 pH 4, Medium 199 pH 7.5, 50% FBS solid medium, and PDB plus 25% FBS medium. The protocol was repeated again using the heterozygous *DDR48/ddr48* strain. The plates were then incubated at 37°C and monitored over 7 days for the formation of filaments.

2.7.2 Filamentation in FBS

The heterozygous and parent strain were each inoculated in 10 ml of FBS (Biowhittaker, Belgium) and incubated at 37°C with a shaking speed of 265 rpm. 3 µl of each aliquot was examined microscopically every 2-3 hours over a period of 12 hours for the presence of germ tubes.

2.8 Murine model of Disseminated Candidiasis

Bwp17 and *DDR48/ddr48* heterozygous cultures at OD₆₀₀ 0.1 were each inoculated in 10 ml of PDB and incubated at 30°C to OD 0.75. Cells were counted using a hemacytometer, diluted to 5×10^{10} cells/ml, harvested, and washed with 200µl of Phosphate buffered Saline solution (PBS). 3 female mice BALB/C (Lebanese American university stock) weighing 20g to 30g were injected via the lateral tail vein with 200µl of 5×10^{10} cells/ml of BWP17, while 3 others were injected with *DDR48/ddr48*. Mice had free access to water and mice

chow food and were housed in a controlled environment (22±2°C, with 12 hours of light and 12 hours of dark). The mice were monitored for survival 3 times daily, over a period of 33 days.

2.9 Antifungal agents

The E-test (AB Biodisk, Solna, Sweden) was used to assess the BWP17 and *DDR48/ddr-48* heterozygous strains' antifungal susceptibility on both Sabourad Dextrose agar (SAB) and RPMI-1640 media. The reference strain *C. parapsilosis* ATCC 22019, offered as a gift by Dr George Araj (American university of Beirut, Lebanon), was used as for quality control purposes. The Etest was performed following the manufacture's suggestion (AB Biodisk, Solna, Sweden).

2.10 Identification of Homologue genes to *CaDDR48*

Homologue genes to *CaDDR48* were identified in the NCBI and *Candida albicans* database. ORF 19.4082 of *DDR48* was obtained from the *C.albicans* website at <http://genolist.pasteur.fr/CandidaDB/genome> (Table 2). A BLASTP, protein blast, comparison of the ORF 19.4082 of *DDR48* with the *C.albicans* genome database (www.candidagenome.org) and the NCBI database was performed (<http://www.ncbi.nlm.nih.gov>).

Table 2: ORF19.4082 of *C.albicans* used in BLAST P

<p>><i>C. albicans</i> fungus CA4336 <i>DDR48</i>: 213 aa - stress protein (by homology) MVFGFGKDDDKDKNDRRDND SYGSSNRRNND SYGSSSFGSSNDDDNSYGSSNKR SND SYGSSNTD SYGSSNRRDND SYGSSNND SYGSSKTD SYGSSNRSGND SYGSSNN DSYGSSNRKGND SYGSSNTD SYGSSNTD SYGSSNTD SYGSSNKRGNDSYGSSNDDS YGSSNDDSYGSSNRRGND SYGSSNDDSYGSSNRRGNSDSYGSSDY*</p>
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Chapter III

Results

3.1 Verification of integration

DDR48/ddr48 heterozygous strain was created by transforming a DNA cassette containing a *HIS1* marker flanked by 50 bps of upstream and downstream sequences of the *DDR48* ORF (Fig 6a,b). PCR confirmed that the *HIS1* marker was flanked by 50 bps of upstream and downstream sequences of the *DDR48* (Fig 7). Further, homologous recombination displaced the wild type *DDR48* allele creating a heterozygote *DDR48/ddr48:HIS1* strain (Fig 6c,d). The integration was verified by amplifying a 750 bps fragment with the *DDR48* Integration Verification primer that hybridized upstream of the integration site and the *HIS1* Internal primer that hybridized within the *HIS1* locus (Fig 8a; Fig 9a lane 3). To confirm that the transforming fragment did not integrate at both *DDR48* loci, generating a homozygous *ddr48* mutant strain, internal *DDR48* primers were used to amplify a 500 bps fragment (Fig 8b; Fig 9 b lane2, lane 3).

3.2 Growth on cornmeal agar media

On corn meal agar, Bwp17 formed an abundant number of hyphae and chlamydospores (Fig10A-B) within 3 days of incubation, whereas *DDR48/ddr48* heterozygote strain produced fewer chlamydospores and hyphae (Fig10C). However, by day 7 *DDR48/ddr48* formed an extensive number of chlamydospores (arrow) at the ends of the long hyphae (Fig10D), comparable to BWP17 (Fig10B).

Fig 6: Generation and integration of *HIS 1* cassette. a) primers used to generate integration cassette b) PCR product of c) a homologous recombination at the 5' and 3' flanking regions should occur generating d) integration of cassette at *DDR48* locus.

Fig 7: PCR confirming *HIS 1* flanked by *DDR48* sequences. Lane 1 and Lane 2: *DDR48::HIS1* DNA at 1.0 kb band. Lane 3: Molecular weight marker XVI.

Fig 8: Verification of integration. a) *HIS 1* internal and *DDR48* integration verification primers confirming integration of the *HIS* cassette. b) *DDR48* internal primers verifying *HIS1* integration.

Fig 9: PCR confirming *HIS1* integration. a) Lane 1: Molecular weight marker XVI. Lane 2: actin *DDR48/ddr48* control band at 1.1 kb. Lane 3: 750 bps *DDR48/ddr48* fragment verifying *HIS 1* integration. b) Lane 1: Molecular weight marker XVI. Lane 2: 500 bps *DDR48/ddr48* internal band confirming heterozygosity. Lane 3: upper band, actin *DDR48/ddr48* control band at 1.1 kb; lower band, 500 bps internal *DDR48* band. Lane 4: upper band, actin *CAI12* control band at 1.1 kb; lower band, 500 bps *CAI12* internal band.

Fig 10: Phenotype of BWP17 and *DDR48/ddr48* on corn meal tween 80 agar. A-B: Bwp17; A: day 3, extensive hyphae (arrow). B: day 7, chlamydospores (arrow) at the end of the long hyphae. C-D: *DDR48/ddr48*; C: day 3, short hyphae and reduced number of chlamydospores (arrow) formed. D: day 7, extensive number of chlamydospores (arrow) and hyphae. 40X magnification.

3.3 Filamentation on hyphal inducing medium and serum

3.3.1 Agar plus 50% FBS

When plated on agar plus 50% FBS, BWP17 produced peripheral filamentation within 3 days (Fig11B). On the other hand, the heterozygous strain failed to develop filamentous

colonies (Fig11 F). By day 7, BWP17 displayed extensive filamentation (Fig11C), whereas filaments extending from the *DDR48/ddr48* colony were significantly reduced in both length and number (Fig11G).

Fig 11: Filamentation on Agar plus 50% FBS medium. A-C: Bwp17; A: day 1, colony was smooth and round. B: day 3, short filaments emerged. C: day 5, extensive filamentation. D-G: *DDR48/ddr48*; after day 1 (D), day 3 (E), and day 5 (F) respectively the colony remained smooth and round. G: day 7, short filaments emerged from the periphery of the colony. 40X magnification.

3.3.2 PDA plus 25% FBS

On PDA plus 25% FBS, BWP17 produced highly filamentous colonies within 5 days (Fig12C). In contrast, the heterozygous colony produced a small fringe of filamentous cells at the periphery of a small centered area (Fig12G).

Fig 12: Filamentation on PDA plus 25% FBS medium. A-C: Bwp17; A: day 1 short filaments formed. B: day 3, more filamentation formed. C: day 5, extensive filamentation. D-G: *DDR48/ddr48*; after day 1 (D) and day 3 (E) respectively the colony remained smooth and round. On day 5 (F) and day 7 (G) respectively, short filaments emerged from the edge of the colony. 40X magnification.

3.3.3 Lee's medium

The capacity of the heterozygous and parent strain to produce filaments was examined on Lee's medium. The parent strain, BWP 17, produced an abundant number of filaments at the periphery of its colony within 3 days of incubation (Fig13B). By day 5, the colony became completely filamentous (Fig13C). In contrast, short filaments were observed around the edge of the *DDR48/ddr48* colony after 7 days of incubation (Fig13G).

Fig 13: Filamentation on Lee's medium. A-C: Bwp17; A: day 1, the colony was smooth and round. B: day 3, radial filaments emerged. C: day 5, colony was completely filamentous. D-G: *DDR48/ddr48*; after day 1 (D), day 3 (E), and day 5 (F) respectively *DDR48/ddr48* remained smooth and round. G: day 7, short filaments emerged from the colony. 40X magnification.

3.3.4 Medium 199 buffered at pH 4.5 and pH 7.5

The heterozygote, *DDR48/ddr48*, and the parent strain, Bwp17, showed suppressed filament formation on Medium 199 at pH 4 (Fig14A-H). On the other hand, short filaments emerged from the edge of the Bwp17 colony after 3 days of incubation on Medium199 buffered at pH 7.5 (Fig15B), whereas the *DDR48/ddr48* strain remained smooth (Fig 15 D-F). By day 5, radial filaments grew extensively from the BWP17 colony (Fig15 C), even though within 7 days the *DDR48/ddr48* strain showed a severally reduced number of these filaments (Fig15 G).

Fig 14: Filamentation on M199 at pH: 4. A-D: Bwp17; after day 1 (A), day 3 (B), day 5 (C), and day 7 (D) respectively Bwp17 was smooth and round. E-H: *DDR48/ddr48*, after day 1 (E), day 3 (F), day 5 (G), and day 7 (H) respectively *DDR48/ddr48* was smooth and round. 40x magnification.

Fig 15: Filamentation on M199 at pH 7.5. A-C: BWP17; after day1 (A), and day 3 (B) respectively radial filaments started to emerge from the edge of the colony. C: day 5, extensive filamentation was observed. D-G: *DDR48/ddr48*; after day1 (D), day 3 (E), and day 5 (F) respectively *DDR48/ddr48* remained smooth and round. G: day 7, short filaments emerged from the edge of the colony. 40X magnification.

3.3.5 FBS

Each strain, *DDR48/ddr48* and BWP17, was incubated in FBS at 37°C to induce filamentation. Under these conditions, BWP17 formed uniform and rapid induction of germ tubes after 3 hours (Fig16C). By 6 hours, the majority of the germ tubes elongated to form a mycelial network (Fig16D). In contrast, no germ formation was observed in the *DDR48/ddr48* strain within 6 hours (Fig16E-G). However, few cells exhibited aberrant short projections that failed to extend into true germ tubes and hyphae (Fig16H), even after 72 hours of growth in serum.

Fig 16: Germ tube formation in FBS at 37°C. A-D: BWP17; A: 0 hour, B: 1 hour, initial stages of germ tubes formation. C: 3 hours, long germ tubes (arrow) were observed. D: 6 hours, an extensive hyphae network (arrow) formed. E-H: *DDR48/ddr48*; E: 0 hours, F: 1 hour, G: 3 hours, respectively no germ tube formation. H: 12 hours, initial stages of germ tubes formation (arrow) and it remained the same way after 72 hours of monitoring. 40 x magnifications.

3.4 Murine model of Disseminated Infection

Using the mouse systemic candidiasis model, it was observed that 66% of the mice infected with 5×10^{10} cells of *DDR48/ddr48* remained alive until the experiment was terminated at

day 33 (Fig 17). Whereas, the same inoculum of the wild type parent strain, Bwp17, killed 33% of the mice within 3 days, 66% within 6 days, and eventually all mice within 10 days (Fig 17).

Fig 17: Survival curves of mice following intravenous injection of 5×10^{10} cells/ml of BWP 17 and DDR48/ddr48.

3.5 Antifungal drugs

The antifungal susceptibility E-test was done to determine sensitivity of the heterozygous and parent strain to azoles, amphotericin B, and caspofungin on both RPMI 1640 and SAB medium. After 48 hours of incubation, the MIC values were read. The inhibition ellipses were sharply defined as seen in Figure 18. The MIC values were determined for BWP17, DDR48/ddr48, and the reference strain *C.parapsilosis* ATCC 22019 (Table 3). Data presented in Table 3 indicate that the MIC values of the *C.parapsilosis* ATCC 22019 were within the control limits.

Fig 18: Etest of different antifungal drugs. A: BWP 17 on RPMI medium, caspofungin strip (right) and amphotericin B (left). B: DDR48/ddr48 on SAB medium, itraconazole (right) and voriconazole (left). Note the clearly defined zone of inhibition. 40x magnification.

Table 3: MIC determination of Bwp17, DDR48/ddr48, *C.parapsilosis* on SAB and RPMI 1640 medium.

Antifungal agents	Bwp17		DDR48/ddr48		<i>C.parapsilosis</i>	
	SAB	RPMI	SAB	RPMI	SAB	RPMI
Fluconazole	64µg/ml	64µg/ml	32µg/ml	32µg/ml	8µg/ml	1µg/ml
Ketoconazole	1µg/ml	1µg/ml	0.38µg/ml	0.25µg/ml	0.125µg/ml	0.064µg/ml
Itraconazole	1µg/ml	1µg/ml	0.047µg/ml	0.023µg/ml	0.064µg/ml	0.064µg/ml
Voriconazole	0.094µg/ml	0.016µg/ml	0.032µg/ml	0.08µg/ml	0.032µg/ml	0.032µg/ml
Amphotericin B	1µg/ml	0.5µg/ml	0.5µg/ml	0.5µg/ml	1µg/ml	0.25µg/ml
Caspofungin	1µg/ml	0.5µg/ml	0.5µg/ml	0.38 µg/ml	1µg/ml	0.38µg/ml

Chapter IV

Discussion and Conclusion

Due to the diploid nature of *C.albicans*, molecular analysis of the *DDR48* function requires knocking out both alleles. In this study, 50 bps of PCR primer homology were sufficient to create the *DDR48/ddr48::HIS1* strain. In fact, two heterozygote isolates were obtained with such primers, even though a recent study demonstrated that flanking homology regions up to 60 bps yield correct integration of the *HIS1* cassette with an efficiency of less than 1% in *C.albicans* strain BWP17. Increasing the homology region to 100 bps increases the rate of correctly targeted mutants to 20-80 % (Gola et al., 2003). Primers with 100 bps *DDR48* homology were designed and utilized to amplify a *URA3* deletion cassette, but upon transformation and screening of 15 *HIS^r/URA⁺* transformants we were unable to obtain a *ddr48/ddr48* homozygous isolate. Thus, the present finding indicates that the *DDR48* is possibly an essential gene. In an essential gene, one allele can be deleted but the second can not (Enloe et al., 2000). Hence, it is possible that *DDR48* is essential for the viability of *C.albicans*, since it has a crucial role in DNA repair and stress response (Hromatka et al., 2005; Harcus et al., 2004).

4.1 Virulence

This study provides clear evidence that *DDR48* has a critical role in filamentation and virulence. An unexpected feature was that *DDR48/ddr48* exhibited a homozygous mutant phenotype suggesting that *DDR48* is haploid insufficient, which it was unable to maintain a wildtype phenotype. When grown on PDB plus 25% FBS and 50% FBS medium the

heterozygote strain was filament deficient compared to the BWP17 strain. Likewise, on minimal amino acid synthetic Lee's medium the heterozygote strain exhibited reduced filamentous growth. On medium 199 at pH4, the acidic conditions abolished filamentation production by the parent and heterozygous strain, whereas at pH 7.5, the heterozygous strain produced fewer filaments than the parent strain. Hence, a *DDR48/ddr48* mutant grown at a neutral pH has the same filamentation defect as a *DDR48/ddr48* mutant grown at an acidic, non filamentation inducing pH. Altered filamentous growth is correlated to a defect in pH sensing.

In the presence of fetal bovine serum, no germ tube formation was detected in the heterozygote mutant strain. Inhibition of germ tube formation was consistent with the weakly induced filamentation phenotype on hyphal inducing medium. On corn meal agar, the *DDR48/ddr48* strain formed hyphae and chlamydospores more slowly than the parental strain, but ultimately the extent of chlamydospore and hyphae formation was similar in each. This observation suggests that *DDR48* has a profound affect on chlamydospore formation and filamentation. The *DDR48/ddr48* strain also caused a significant reduction in virulence in mice whereas the BWP17 resulted in rapid death. Even though 5×10^{10} cells/ml is considered to be a lethal high dose that normally elicits an acute infection and eventually death (Molero et al., 1999), only one out of three mice carrying the *DDR48* heterozygote strain died while all 3 control mice were died by day 10. The experiment was initially done using 5×10^6 cells/ml, a dose considered to be lethal (Molero et al., 1999), but no death occurred in the heterozygote strain so the concentration was progressively increased to 5×10^{10} cells/ml. Recently, Thewes et al. (2007) demonstrated that *DDR48* was expressed at high levels at all time points (attachment, superficial invasion, and deep

invasion) during tissue invasion of a mouse's and pig's liver regardless of the degree of invasiveness of the strain utilized. Hence, it is possible that the reduction in virulence of *DDR48/ddr48* is attributed to the reduction in the capacity to form filaments, as well as a decrease in the ability to adhere to host cells. These findings indicate that *in vitro* conditions elicited the same response as was induced *in vivo*.

4.2 MIC profiles

DDR48/ddr48 heterozygote strain generated in this study was susceptible dose dependent to fluconazoles and itraconazoles compared to the parent strain, BWP17, on both RPMI 1640 medium and SAB agar. This was expected since data show that the exposure to fluconazoles and itraconazoles lead to the increased expression of Ddr48 RNA (Rogers and Barker, 2003). Although interpretive breakpoints have not been well established, *DDR48/ddr48* seemed to be sensitive to ketoconazole with respect to the parent strain on RPMI 1640 and SAB media. In contrary, both the heterozygous and the parent strain were resistant to amphotericin B but sensitive to voriconazole and caspofungin on both media.

Even though the MIC values of the reference strain *C.parapsilosis* ATCC 22019 were within the control limits, the results obtained in this study can provide an indication of the *C.albicans in vitro* susceptibility only. Since determinations of susceptibility endpoints and MIC breakpoints combined with technical aspects of plate preparation and inoculation influence antifungal susceptibility determinations, *in vivo* susceptibility might differ (Odds, 1988).

4.3 Haploid insufficient genes

Uhl et al. (2003) proposed that haploinsufficiency occurs in *C.albicans* as a consequence of the evolution and subsequent slight differentiation of two alleles of a gene. Hence, the inactivation of one of the alleles would produce a phenotype since the other allele cannot complement it anymore. Moreover, *C.albicans* has been found to be highly sensitive to gene dosage and since heterozygotes express only 50% of a particular gene product a haploinsufficiency phenotype insues (Uhl et al., 2003). In addition to *DDR48*, there are numerous numbers of haploid insufficient genes in the *C.albicans* database. For instance, *RBT4*, which encodes a pathogenesis related protein, shows haploinsufficiency phenotype as reduced levels of infection in a rabbit's cornea and a virulence in a mouse model (Braun et al., 2000). *INT1*, an integrin like protein, also displays haploinsufficiency since the heterozygote strain shows a similar phenotype to the homozygote null mutant by decreased murine intestinal colonization with respect to the wild type (Braun et al., 2000). Finally, both the *CSH3* heterozygote and homozygote mutant strains, required for wild-type amino-acid responsive hyphal growth, exhibited attenuated viurulence defects in a mouse model compared with the wildtype (Martinez and Ljungdahl, 2004).

4.4 *ScDDR48*

Saccharomyces cerevisiae is not a pathogen and thus does not exhibit any virulence in mice or even true hyphal formation, making any comparison between the phenotypes of a *S.cerevisiae ddr48* deletion and our mutant unfeasible. However *S. cerevisiae* in its diploid state, and under certain growth conditions, can produce filaments in the form of pseudohyphae (Gimeno et al., 1992). Giaever et al. (2002) tested for the formation of

pseudohyphae on SLAD medium, nitrogen limiting medium, in both *ScDDR48/ddr48* heterozygote strain and *Scddr48* deletion strain. Both strains, however, were not defective in pseudohyphal growth and even exhibited normal pseudohyphal cell morphology suggesting that the *ddr48* mutation did not affect DNA synthesis, cell division, or cell growth. Thus even though both *DDR48* genes converged on stress response, the *CaDDR48* gene has sufficiently diverged to acquire additional roles, notably in filamentation and virulence.

4.5 Future work

The *DDR48/ddr48* phenotype observed in this study requires additional experimental confirmation. Reintroduction of the *DDR48* into the heterozygous mutant either by integration into the genome or by subcloning on a plasmid should reverse its virulence and filamentation defect demonstrating that the heterozygous mutant, and not some secondary mutation, is directly responsible for the observed defects. Furthermore, it would be interesting to determine whether overexpression of *DDR48*, under the control of a strong constitutive promoter such as actin (*ACT1*), will display the opposite phenotype observed with the heterozygous mutants, such as filamentation on rich non filamentous inducive media and even an increased virulence *in vivo* compared to the wild type.

To confirm that *DDR48* is an essential gene, the *DDR48/ddr48* heterozygote strain should be placed under the control of the inducible *MET3* promoter. *MET3* encodes sulphate adenylyltransferase and is repressed by both methionine and cysteine (Care et al., 1999). Furthermore, the heterozygote triosmy test designed by Enloe et al. (2000) is an alternative method to identify essential genes.

4.6 Conclusion

In conclusion, the present findings demonstrate that *DDR48* is essential for the virulence and possibly viability of *C.albicans*. On hyphal inducing medium and serum, the heterozygote strain exhibited defective filamentation formation. This observation was consistent with the avirulent phenotype in a mouse model of disseminated candidasis. Thus, this study provides more evidence of the importance of morphogenesis and filamentation in *C.albicans* pathogenesis.

Chapter V

References

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Chapter VI

APPENDIX I

Table 4: MIC Interpretive breakpoints of *C.albicans* and *C.parapsilosis*

Antifungal agents	MIC Interpretive Breakpoints <i>C.albicans</i>	Control limits <i>C.parapsilosis</i>
Fluconazole	S: $\leq 8 \mu\text{g/ml}$ S-DD: 16 to 32 $\mu\text{g/ml}$ R: $\geq 64 \mu\text{g/ml}$	1-8 $\mu\text{g/ml}$
Ketoconazole	R: $\geq 1 \mu\text{g/ml}$	0.032-0.125 $\mu\text{g/ml}$
Itraconazole	S : $\leq 0.125 \mu\text{g/ml}$ S-DD: 0.25-0.5 $\mu\text{g/ml}$ R: $\geq 1 \mu\text{g/ml}$	0.064-0.25 $\mu\text{g/ml}$
Voriconazole	S: $\leq 1 \mu\text{g/ml}$ S-DD: 2 $\mu\text{g/ml}$ R: $\geq 4 \mu\text{g/ml}$	0.016-0.064 $\mu\text{g/ml}$
Amphotericin B	R: $\geq 0.38 \mu\text{g/ml}$	0.25-1 $\mu\text{g/ml}$
Caspofungin	S: $\leq 1 \mu\text{g/ml}$	0.25-2 $\mu\text{g/ml}$

MIC interpretive breakpoints of *C.albicans* were adapted from the following papers: Fluconazoles & Itraconazole: (Rex et al., 1997). Voriconazole (Pfaller et al., 2006). Ketoconazole (Therese et al., 2006). Amphotericin B (Clancy and Nguyen, 1999 ; Park et al., 2006). Caspofungin (Fung et al., 2002, Letscher-Bru and Herbrecht , 2003). Control limits of the *C.parapsilosis* were adapted from Rex et al. (1997).

APPENDIX II

Media and Solutions preparation

2.1 Media preparation

2.1.1 YNB+ARG+URA medium

0.05% uridine and 0.05% arginine were prepared as described by the manufacture's suggestion (Fluka, Switerzland) with the addition of 0.34% yeast nitrogen base (YNB) (Fluka, Switerzland) and 1% of ammonium sulfate (Himedia, India). 4% bactoagar was prepared as described by the manufacture's suggestion (Himedia, India) with the addition of 4% glucose (Surchem, England). Then, the solutions, YNB+ARG+URA and agar+glucose, were mixed together and poured into petri dish plates.

2.1.2 Corn Meal Tween 80 agar plates

1.7 % Corn meal agar plates were prepared as described by the manufacture's suggestion (HiMedia, India) with the addition of 1% Tween 80 (Surechem, England).

2.1.3 Lee's medium

Lee's media was prepared as described by Singh et al. (2005). Media was supplemented with 25mg/ml uridine and 25 mg/ml arginine as needed.

2.1.4 Medium 199

2.1.4.1 Medium 199 at pH4

3% Medium 199 was prepared as described by the manufacture's suggestions (Sigma-Aldrich, USA) with the addition of 4% bactoagar and adjustment of the pH to 4. Media was supplemented with 25mg/ml uridine and 25 mg/ml arginine as needed.

2.1.4.2 Medium 199 pH7.5

3% Medium 199 was prepared as described by the manufacture's suggestion (Sigma-Aldrich,USA) with the addition of 0.25M of MOPS (Amersco, Ohio, USA) and adjustment of pH to 7.5. Media was supplemented with 25mg/ml uridine and 25 mg/ml arginine as needed.

2.1.5 50% FBS medium

50% FBS medium was prepared as follows: 4% bactoagar was prepared as described by the manufacture's suggestion (Himedia,India) with the addition of 50% FBS (Biowhittaker,Belgium). Media was supplemented with 25mg/ml uridine and 25 mg/ml arginine as needed.

2.1.6 PDA plus 25% FBS medium

PDA plus 25% FBS plates was prepared as follows: 4% PDA was prepared as described by the manufacture's suggestion (Himedia, India) with the addition of 25% FBS. Media was supplemented with 25mg/ml uridine and 25 mg/ml arginine as needed.

2.1.7 Sabourad Dextrose agar (SAB) medium

6.5% SAB plates were prepared as described by the manufacture's suggestion (Oxoid, U.K). Media was supplemented with 25mg/ml uridine and 25 mg/ml arginine as needed.

2.1.8 RPMI 1640 medium

RPMI 1640 medium was prepared as follows: 0.6% glucose was prepared as described by the manufacture's suggestion with the addition of 9% bactoagar and 500ml of filter sterilized RPMI 1640 serum (Biowhittaker, USA). Media was supplemented with 25mg/ml uridine and 25 mg/ml arginine as needed.

2.2 Solutions preparation

2.2.1 DNA extraction solutions

DNA extraction solutions were prepared as described by Yazbeck et al. (2007).