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The *Candida albicans* Ddr48 protein is essential for filamentation, stress response, and confers partial antifungal drug resistance

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C Statistical Analysis
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Summary

Background: *Candida albicans* is a dimorphic pathogenic fungus that causes mucosal and systemic infections. *C. albicans* pathogenicity is attributed to its ability to exist in different morphologic states and to respond to stress by up regulating several key genes. *DDR48* is a stress-associated gene involved in DNA repair and in response to antifungal drug exposure.

Material/Methods: One allele of *DDR48* was knocked out by homologous recombination that inserted a marker cassette in its position. Furthermore, reintroducing *DDR48* on a plasmid created a revertant strain. Strains were grown on filamentation inducing and noninducing media, subjected to an oxidative stress challenge, injected into mice to assess virulence, and assayed for antifungal susceptibility by the E-test method.

Results: *DDR48* was found to be haploid insufficient and possibly essential, since only a heterozygote, but not a homozygous, null mutant was generated. The mutant was filamentation defective on all hyphal media tested including serum and corn meal agar. Discrepancies in drug resistance profiles also were present: Compared with the parental strain, *DDR48/ddr48* heterozygote strain was susceptible in a dose-dependent manner to itraconazole and fluconazole and susceptible to ketoconazole. The mutant also appeared to be hypersensitive to a potentially lethal hydrogen peroxide challenge. However, no reduction in virulence of the mutant was observed.

Conclusions: The present findings provide evidence that *DDR48* is essential for filamentation, stress response, and possibly viability of *C. albicans,* making it a prime target for antifungal drug design.

key words: ***Candida albicans*** **•** ***DDR48*** **• filamentation • virulence • drug susceptibility • stress response**

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BACKGROUND

Candida albicans is a harmless colonizer of mucosal surfaces [1]. However, when the balance between *C. albicans* and the host is disturbed, this pathogenic yeast can cause superficial, mucosal, and systemic infections [2]. Systemic and disseminated candidosis is seen primarily among HIV-infected patients and vulnerable intensive care patients, especially those undergoing immunosuppressive therapy [3]. In the United States, the direct cost of treating *C. albicans* bloodstream infections is between $1 and $2 billion annually [4], and despite aggressive therapies, mortality approaches 50% [5].

An interesting feature of *C. albicans* is its ability to grow in a variety of morphologic forms. Dimorphism, which refers to the ability of *C. albicans* to switch between yeast and filamentous hyphae, is often considered to be necessary for virulence. Hyphae develop from an unbudded yeast cell blastospore. It has parallel sides along its length, and no constriction at the neck of the mother cell. The hyphal form can be induced from the unbudded yeast in fetal bovine serum, hyphal-inducing serum, or hyphal-inducing medium at a growth temperature of approximately 37°C or at neutral to slightly basic pH [3]. The hyphal form helps evade the host's defense mechanism and penetrate tissue, whereas the yeast form can disseminate more thoroughly [6]. Prolonged incubation of *C. albicans* under unfavorable conditions of nutrient, and dryness such as corn meal tween 80 agar, forms extensive agar-invading hyphal filaments capped by chlamydospores; thick-walled terminal spores that form on the ends of hyphae [7,8]. Chlamydospores probably function as a storage form or dormant growth under nonfavorable conditions. Moreover, chlamydospores might have a functional role in *C. albicans* infection, although this role has yet to be elucidated [9]. Chlamydospores have been found in the lung of an AIDS patient [10].

In response to various environmental signals such as starvation, serum, slightly alkaline pH, and hypoxia, *C. albicans* utilizes converging pathways to regulate its yeast to hyphae transition. The 2 main signal transduction cascades include a Cph1-mediated MAP kinase pathway and an Efg1-mediated cAMP pathway. A deletion of both *cph1* and *efg1* blocks filamentation under most conditions and causes avirulence [11]. On the other hand, many transcriptional repressors control virulence and filamentation in *C. albicans* [12], since filament-specific genes become active via inactivation of transcriptional repressors, such as Tup1, Nrg1, and Rfg1. Tup1, a global transcriptional repressor, is recruited to genes via interaction with Nrg1 and Rfg1, promoter sequence specific DNA binding proteins that might recruit histone deacetylases to the promoter, remodel the chromatin on yeast promoters, and thereby inhibit assembly of the transcriptional apparatus [12,13–17].

The specific mechanism by which *C. albicans* transforms itself into an opportunistic pathogen is not well understood. Although disruption of *C. albicans* genes encoding secreted aspartyl proteinases, glycosylphosphatidylinositol (GPI)-anchored superoxide dismutase, cell wall adhesins, and cell surface hydrophobicity genes, have been shown to attenuate mortality in animals with disseminated candidiasis, no individual genes encode a dominant determinate of candidal virulence [18]. Rather, pathogenesis depends upon the ability of *C. albicans* to adapt to changes in the local host environment by coordinating the expression of multiple genes [19].

Ddr48 is a 212 amino acid immunogenic stress-associated protein on the cell wall, isolated in cell surface extracts of biofilms cultures where it has been shown to be overexpressed [20]. Survival in the hostile environment of the blood and escape from blood vessels into tissues are essential steps for *C. albicans* to cause systemic infections [21].

Exposure of *C. albicans* cells to macrophages leads to 8.0-fold up-regulation of *DDR48,* suggesting that *DDR48* is predominantly induced in response to macrophages. Thus, *C. albicans* engulfed by macrophages can filament, lyse the phage, counterattack neutrophils, and cause systemic infections [22]. In response, oxidative stress *C. albicans* detoxifies nitric oxides by flavohemoglobin, *YHB1*, which reacts with oxygen to form ferric flavohemoglobin and nitrate [23]. Using *C. albicans* genomic DNA microarrays, Hromatka and associates [23] showed that exposure of *C. albicans* cells to nitric oxides for 10 minutes led to 1.9-fold up-regulation of *DDR48,* suggesting that Ddr48 is involved in combating the effects of nitric oxide. Moreover, *DDR48* was persistently induced 57.1 times in the *yhb1/yhb1* null mutant strain, providing evidence that *DDR48* is involved in DNA repair [23]. Under amino acid starving conditions, expression of *DDR48* depends on the amino acid biosynthesis transcriptional activator Gcn4. Transcription profiling revealed that the expression of *DDR48* was enhanced 2.1 times in the presence of Gcn4 compared with a *gcn4* strain [24].

Regarding transcriptional activators, Cph1 has been shown to activate *DDR48* under hyphal inducing media [11], while in a *tup1/tup1* and *nrg1/nrg1* null mutant strain *DDR48* was overexpressed 15 times [25]. These results are interesting bearing in mind that Tup1 and Nrg1 are repressors of many filamentation and virulence required genes, suggesting a possible role in filamentation and virulence for Ddr48.

Using microarray analyses, Nantel and associates [26] have shown that when cells are cultured in fetal bovine serum (FBS) at 37°C, hyphae formation was correlated with 2.9-, 3.01-, and 6.5-fold increased expression of *DDR48,* the putative cell wall protein *RBT1*, and the hyphal cell wall protein *HWP1*, respectively. Interestingly, Rbt1 is required for virulence [27], while Hwp1 is not [28]. Singh and associates [22] showed that expression of *DDR48* was increased in YPD plus 10% FBS and Spider's medium, a hyphal inducing medium.

Furthermore, *DDR48* contains multiple repeats of the amino acid sequence NNNDSYGS [22] that might be involved in drug resistance, since differences in drug resistance of various *C. albicans* isolates have been attributed to mutations in regions surrounding this amino acid sequence [29]. Recently by examining genome-wide expression profiles of *C. albicans* in response to amphotericin B, Liu and associates [30] observed a 3.4-fold up-regulation of *DDR48.* On the other hand, upon exposure to fluconazole and benomyl *DDR48,* led to a 6.6-fold increase in up-regulation [31]. In response to caspofungin, *C. albicans* up-regulates transcription factors Cas5 and Ada2, which are activators of many

cell wall protein genes and are required for resistance to numerous cell wall perturbing treatments. Microarray analysis reveals that on exposure to caspofungin, *ada2/ada2* and *cas5/cas5* null mutants repress expression of *DDR48* 4.27 and 1.8, respectively [32]. Hence, it is possible that *DDR48* plays a role in conferring caspofungin resistance.

By doing a BLASTP search of the *Candida albicans* database using the Ddr48 ORF, the gene most similar to *DDR48* was *ASR1*, a putative heat shock protein. It is worth noting that Tup1 and Nrg1 also regulate the expression of *ASR1* [33]. Interestingly *IHD1*, *HYR1*, and *ALS7* showed up in this search. All 3 genes are expressed under hyphal conditions [26] and *ALS7*, a member of the *ALS* family of cell surface glycoproteins, is thought to be involved in virulence and adhesion [34,35]. This again suggests a possible role for *DDR48*.

To date, no study has reported the creation of a *ddr48* null mutant strain. Therefore, the present study attempts to knock out *DDR48* and characterize any essential role it plays in filamentation, under hyphal inducing and noninducing synthetic liquid media, growth in serum, filamentation on solid media, oxidative stress response, and virulence through a mouse model of disseminated infection. Furthermore, since *DDR48* expression is up-regulated in response to antifungal drugs, this study attempts to asses 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.

Material and Methods

Yeast strain, media preparation, and cell growth

Candida albicans strain BWP17 (*ura3: : 1imm434/ura3: : 1imm434 his1: : hisG/ his1: : hisG arg4: : hisG/arg4: : hisG*) was used in this study [36]. Cells were maintained and grown without selection on rich PDA medium [37]. SC synthetic medium lacking specific nutrients was used for selective growth [37]. For filamentation assays on solid media, cultures were grown to a density of 5×10^6 cells/mL, washed, and resuspended in PBS buffer at the same density. 2-µL cultures were spotted on M199 with Earle's salts, L-glutamine, and 150 mM HEPES, at pH 4 or buffered to pH 7.5 (Sigma-Aldrich Corp. St. Louis, MO, USA), Lee's media buffered to pH 6.8 [38], 50% Fetal Bovine Serum (FBS, BioWhittaker Incorporated, Walkersville, MD, USA) (2% dextrose, 2% agar, 50% FBS), and PDA (Himedia Laboratories, Marg, Mumbai, India) plus 25% FBS (BioWhittaker). 100% FBS liquid was used for filamentation assays on liquid media. Hyphal formation on corn meal agar supplemented with 1% Tween 80 was prepared according to the manufacturer's instructions (HiMedia). Media was inoculated with a cut streak and covered with a glass cover slip as described previously [39], incubated at 28°C and monitored daily. For agar spotting and liquid assays, cultures were incubated at 37°C and monitored daily for 7 days, or at 2-hour interval over a period of 12 hours for the presence of germ tubes, respectively. All media were supplemented with 0.005% uridine and arginine. Photographs were generated with a Canon IXUS 55 digital camera (Canon U.S.A, Inc, Lake Success, NY, USA) coupled to an Olympus CH 30 light microscope for cellular morphology (Olympus Imaging America Inc, Center Valley, PA, USA), and to a Leica Stereozoom 4 dissecting microscope (Leica Camera AG, Solms, Germany) for colony morphology.

Table 1. Primers generated in this study.

Primer	Sequence
1. *DDR48-HIS1* F	5'ATGGTTTTCGGTTTCGGTAAAGACGAC ACAAAGACAAGAACGACAGAAGAGAT CAGAAGTTAGTAGTAACAATTTG 3'
2. *DDR48-HIS1* R	5'ATGGTTTTCGGTTTCGGTAAAGACGACG ACAAAGACAAGAACGACAGAAGAGAT CAGAAGTTAGTAGTAACAATTTG 3'
3. *DDR48* Integration Verification	5'CTGTGGATTATTCTCTTTGAA 3'
4. *HIS1* Internal	5'CGGTCTGGTAAATGATTGACTAAATCC TCC 3'
5. *Act1* F	5'GTTGCTGCTTTAATTATCGATAACGGT TCTGG 3'
6. *Act1* R	5'GGTGAACAATGGATGGACCAGATTCG TC 3'
7. *DDR48* Internal F	5'AGATAACGATTCTTACGGATC 3'
8. *DDR48* Internal R	5'CAGCCATATGAGTCGGTCTTG3'
9. *pABSK2-DDR48* F	5'GCGCTGCAGATGATAATTGTGGA ATGTATGTCTAATTG3'
10. *pABSK2-DDR48* R	5'GCGCTCGAGGCTGTAAATGAATGGCT TCAA3'

F denotes a forward primer while R denotes a reverse primer.

Plasmid construction

All plasmids were maintained in the *Escherichia coli* strain DH5α as described previously [39]. Plasmid constructions and enzymatic manipulations were done with standard procedures [40] and conditions recommended by the enzyme vendors. The sequences are numbered with the A of the ATG translational initiation codon as +1, and sequences 3' are numbered consecutively in positive integers and sequenced 5' in negative integers.

pABSK2-*DDR48* was constructed by amplifying the *C. albicans DDR48* gene from –430 to +990 using primers that placed the *Xho*I and *Pst*I sites into the 5' and 3' ends of the fragment. The fragment was ligated into the homologous sites of the *URA3*-containing *C. albicans* transforming plasmid pABSK2. (See Table 1 for primer sequences.)

Strain construction

The *DDR48/ddr48: : HIS1* strain was generated by transforming cells with PCR fragments containing the complete *HIS1* allele sequence flanked by 50bp of the *DDR48* allele. This fragment was generated using 1 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'. Verification of integration was confirmed by PCR analysis of the heterozygous colonies. Verification involved the use of 3 sets of primers. The first set involved *DDR48* internal reverse primer that hybridized to sequences internal to the heterozygous allele at nucleotide position +270 to +291, and *DDR48* internal forward primer that hybridized to sequences internal to the heterozygous allele at nucleotide position +51 to +72, to verify the presence of 1 *DDR48* allele. For the second set of primers, a *DDR48* forward primer hybridized outside the integration site at –270 to –248 and a *HIS1* reverse primer hybridized to the end of the *HIS1* sequence at nucleotide position +1 to +30 such that a PCR product would be generated only from the *ddr48: : HIS1* allele. Finally, actin primers were used as a control for the quality of the DNA preparations [16]. See Table 1 for a sequence of primers used.

DNA extraction

DNA was extracted from all strains prior to PCR analyses as described previously [41].

Yeast transformation

Strain BWP17 was transformed with purified PCR fragments or with plasmids pABSK2, or pABSK2-*DDR48* as indicated using a modification of the Alkali Cation Yeast Transformation kit (Q Biogene, MP Biomedicals, Santa Ana, CA, USA) as described previously [16].

Oxidative stress assay

Oxidative stress was performed as described previously [42]. Briefly strains were grown in rich PDB media until the exponential phase (OD_{600} 0.8–1.2), adjusted to a cellular density of around 3×10^6 cells/mL, and treated for 120' with H_2O_2 at a final concentration of 50 mM. Cellular viability was monitored at 30' intervals for 2 hours. Untreated BWP17 +pABSK2, and *DDr48/ddr48: : HIS1* +pABSK2 strains were used for controls.

Antifungal susceptibility assays

The antifungal susceptibility test was performed on RPMI 1640 medium (with L-glutamine and 165 mM MOPS, BioWhittaker) using the Epsilometer test (E-test) method. E-test strips of fluconazole (FL), voriconazole (VO), ketoconazole (KE), itraconazole (IT), amphotericin B (AP), and caspofungin (CS) were supplied by AB Biodisk (Solna, Sweden). Inoculum preparation, inoculation, and strip application were performed according to the manufacturer's instructions. Minimal inhibitory concentration (MIC) readings were done after 48-hour incubation at 35°C. As recommended by the manufacturer, amphotericin B MICs were read as the lowest concentration on the E-test strip at which there was 100% inhibition, that is, at the point where the border of the elliptical inhibition zone intersected the strip, whereas caspofungin and azole MICs were read as the lowest concentration at which there was 80% growth inhibition or where there was a significant decline in growth. The MIC interpretive breakpoint criteria for itraconazole and fluconazole were as follows: for itraconazole: susceptible (S), ⩽0.125 μg/mL; susceptible dose dependent (S-DD), 0.25–0.5 μg/mL; resistant (R), ⩾1 μg/mL; for fluconazole: (S), ⩽8 μg/mL; (S-DD), 16 to 32 μg/mL, and (R), ⩾64 μg/mL [43,44]. And for voriconazole: (S), ⩽1 μg/mL; (S-DD), 2 μg/mL; (R), ⩾4 μg/mL [45]. Even though the interpretive breakpoints have not been well established for ketoconazole, amphotericin B, and caspofungin, the following criteria were used in this study: (R), ⩾1 μg/mL for ketoconazole [46]; (R), ⩾0.38 μg/mL for amphotericin B [47,48]; and (S), ⩽1 μg/mL for caspofungin [49,50]. The control limit for the Clinical Laboratory Standard Institute reference strain *Candida parapsilosis* ATCC 22019 is: CS: 0.25 to 2 μg/mL; AP: 0.25 to 1 μg/mL; VO: 0.016 to 0.064μg/mL; KE: 0.032 to 0.125 μg/mL; IT: 0.064 to 0.25 μg/mL; and FL: 1 to 8 μg/mL [51].

Murine model of disseminated candidiasis

Nine 4- to 6-week-old female BALB/c mice (Lebanese American University stock) weighing 20 g to 30 g were injected via the lateral tail vein with 200 μL of 1.5×10^8 cells of either the heterozygote, revertant, or wild-type strains suspended in PBS solution, pH 7.5. Mice were given food and water ad libitum and monitored 3 times daily for survival over a period of 20 days.

DDR48 homologues

Homologue genes to *DDR48* were identified in the *Candida albicans* database. ORF 19.4082 corresponding to *DDR48* was obtained from the *C. albicans* website at *http://genolist.pasteur.fr/CandidaDB*. A BLASTP comparison of the ORF 19.4082 of *DDR48* with the *C. albicans* genome database (*www.candidagenome.org*) was performed.

Statistical analyses

Data were analyzed with the chi-square test using data collected with a Sigmastat 3.5 computer software (Systat Software, Inc, San Jose, CA, USA) to assess the significance of any discrepancy.

RESULTS

Verification of integration

The *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. A homologous recombination displaced the wild-type *DDR48* allele creating a heterozygote *DDR48/ddr48: HIS1* strain (Figure 1). The integration was verified by amplifying with 1 primer that hybridized upstream of the integration site and a *HIS1* internal primer that hybridized within the *HIS1* locus (Figure 2A, lane 2). 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 fragment of the *DDR48* ORF (Figure 2B, lane 2).

Growth on solid media

On corn meal agar, the control BWP17 strain formed an abundant number of hyphae within 4 days of incubation,

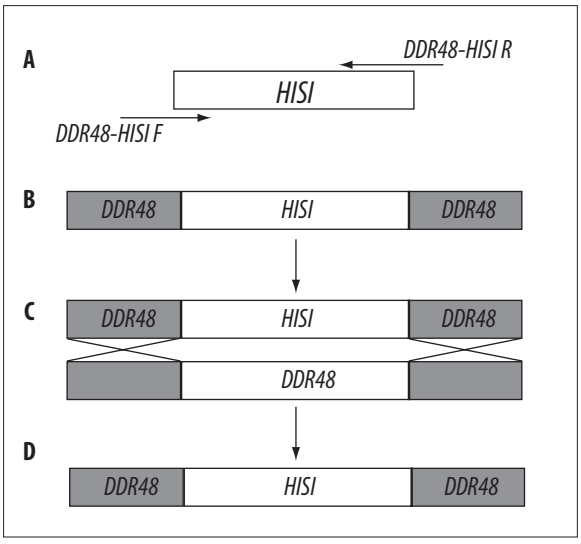

Figure 1. *DDR48* deletion strategy. (**A**) Primers with flanking *DDR48* sequence were used to amplify the *HIS1* gene, generating the integration cassette (**B**). Upon transformation, homologous recombination at the 5′ and 3′ flanking regions should occur (**C**) generating a functional His1gene and a *ddr48* mutant allele (**D**).

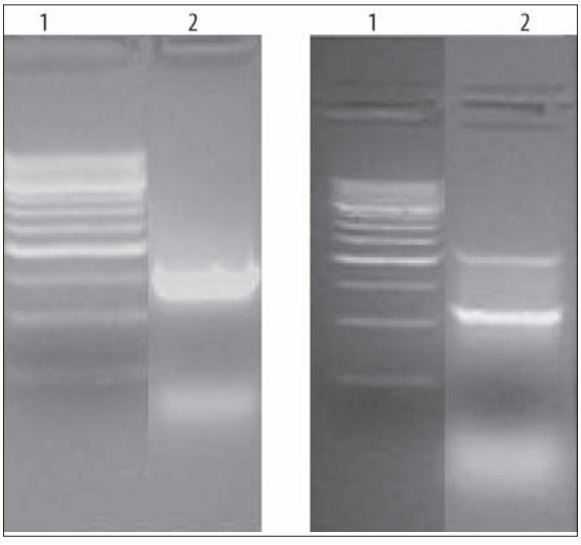

Figure 2. Verification of integration and heterozygosity. (**A**): lane 1: 250bp molecular weight marker. Lane 2: *ddr48: : HIS1* integration fragment. (**B**): lane 1: 250bp molecular weight marker, lane2: upper band, actin control; lower band, *DDR48* internal fragment, confirming the presence of one *DDR48* allele.

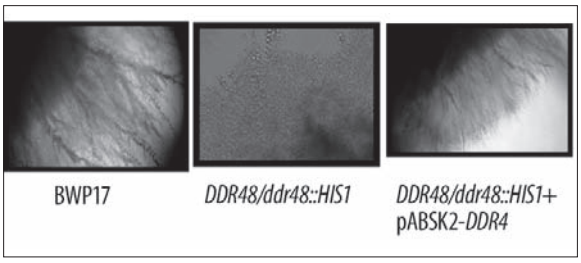

Figure 3. Phenotypes of BWP17, *DDR48/dd48*, and the revertant *DDR48/ddr48: : HIS1*+ pABSK2-*DDR48* on corn meal tween 80 agar after four days of incubation. Note the extensive hyphal formation of the wildtype, the almost complete abolishment of hyphal formation in the heterozygote and the restoration of filamentation in the revertant, confirming that the observed phenotype is due to the deletion of one *DDR48* allele, and not to a secondary mutation.

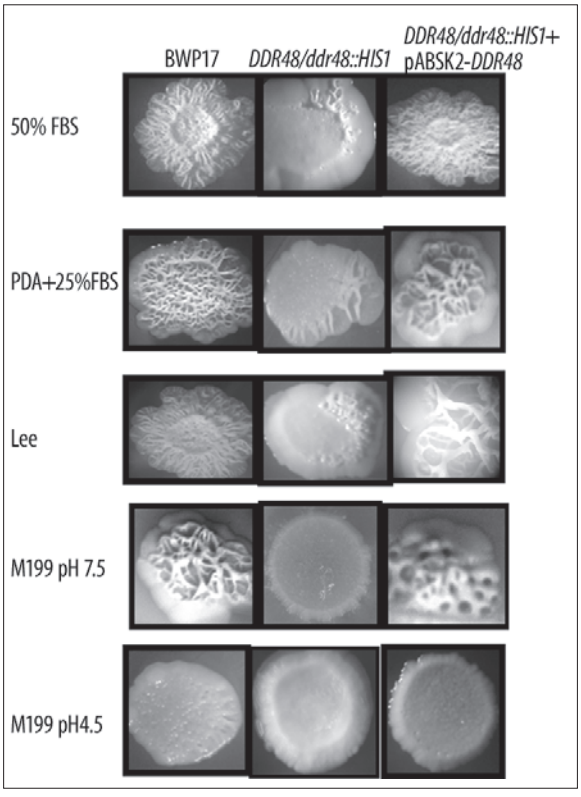

Figure 4. Growth on solid media. Control, mutant, and revertant strains were spotted on various hyphal inducing and non-inducing solid media at 37°C. Note the difference in colony morphology between the wildtype and mutant strains reflecting the lack of hyphal production in the mutant strain, which was completely restored following retransformation of *DDR48*.

whereas the *DDR48/ddr48* heterozygote strain produced mainly round-shaped yeast blastopores with markedly less hyphal elongations. This mutant phenotype was rescued upon retransforming *DDR48* (Figure 3). However, by day 8, the *DDR48/ddr48* mutant strain formed an extensive number of long hyphal filaments comparable to BWP17 (data not shown).

When spotted on a variety of filamentation-inducing media such as agar plus 50% fetal bovine serum (FBS), potato dextrose agar (PDA) plus 25% FBS, Lee's medium, and medium M199 pH 7.5, at 37°C and monitored for a week, BWP17 colonies exhibited the wrinkled aspect associated with extensive hyphal filament morphology, while the heterozygote mutant exhibited creamy smooth colonies with some peripheral hyphal sectoring produced peripheral filamentation after 7 days. Retransforming *DDR48* again rescued the mutant phenotype. On the other hand, no differ-

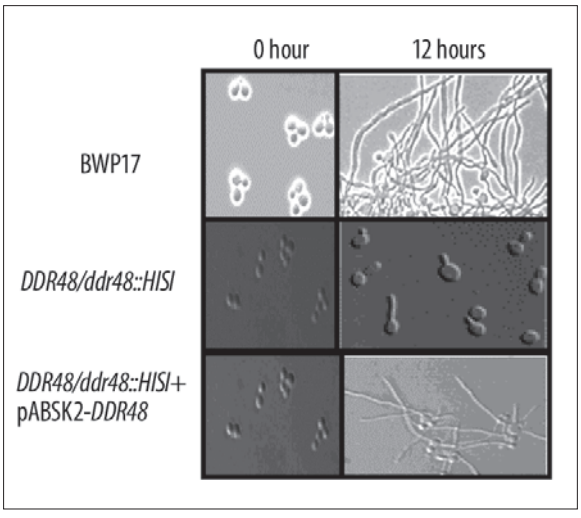

Figure 5. Germ tube and hyphal formation in 100% Liquid FBS at 37°C. Note the lack of hyphal formation by the mutant and the restoration of hyphal morphology by the revertant, confirming that the observed mutant phenotype is not due to secondary mutations. The experiment was repeated three times for each strain.

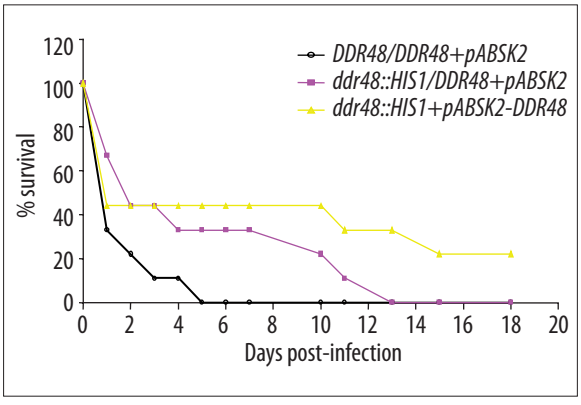

Figure 6. Ddr48 is not essential for virulence. Nine BALB/c mice were injected with 1.5×10^8 cells of the parent (BWP17 + pABSK2), mutant (*DDR48/ddr48: : HIS1*+ pABSK2) and revertant (*DDR48/ddr48: : HIS1*+ pABSK2-*DDR48*) strains. Note that both the wild type and heterozygote strains were able to kill all intravenously injected mice.

Table 2. Antifungal drug susceptibility assay.

Antifungal agent	BWP17	*DDR48/ddr48: : HIS1*
Fluconazole	64 µg/ml	32 µg/ml
Ketoconazole	1 µg/ml	0.25 µg/ml
Itraconazole	1 µg/ml	0.023 µg/ml
Voriconazole	0.016 µg/ml	0.08 µg/ml
Amphotericin B	0.5 µg/ml	0.5 µg/ml
Caspofungin	0.5 µg/ml	0.38 µg/ml

All strains were tested on RPMI1640 media. Note the increased sensitivity of the mutant to most antifungal drugs assayed. The essay was repeated twice to confirm results.

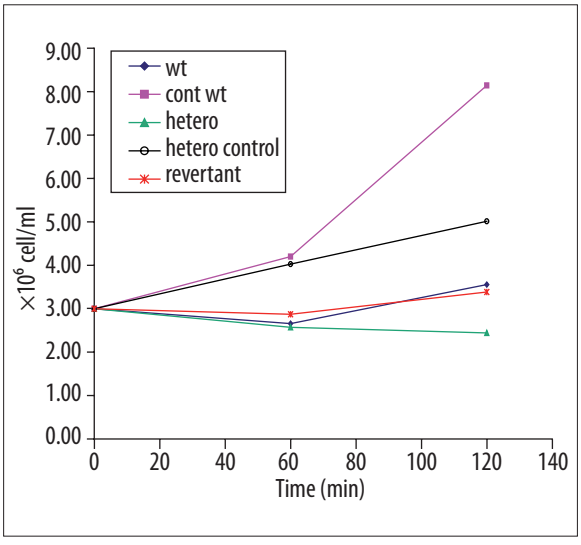

Figure 7. Oxidative stress response test. Cells were challenged with 50mM Hydrogen peroxide and monitored for two hours at 30' intervals. Note the increased sensitivity of the mutant compared to the wild type and revertant strains. **Wt**: BWP17 +pABSK2, **cont wt**: BWP17 + pABSK2 unchallenged, **hetero**: *DDr48/ddr48: : HIS1*+ pABSK2, **hetero control**: *DDr48/ddr48: : HIS1*+ pABSK2 unchallenged, **revertant**: *DDr48/ddr48: : HIS1*+ pABSK2-*DDR48*.

ence was observed on medium M199 at pH 4.5, a nonhyphal inducing medium (Figure 4).

Filamentation in liquid media

Both parent revertant and mutant strains, were incubated in FBS at 37°C to induce filamentation. Under these conditions, and as can be seen in Figure 5, both BWP17 and the revertant strain formed uniform and rapid induction of germ tubes that formed an extensive mycelial network by 12 hours. In contrast, very few germ tube formations were observed in the *DDR48/ddr48* strain after 12 hours; there was no extensive hyphal network. Furthermore, these few mutant cells that did exhibit aberrant short projections failed to extend into true hyphae even after 72 hours of growth in serum (data not shown).

Murine model of disseminated candidiasis

Using a mouse systemic infection model, it was observed that all mice infected with the wild-type or heterozygote strain died after 5 and 13 days, respectively (Figure 6).

Antifungal drug susceptibility

The antifungal susceptibility E-test was done to determine sensitivity of the heterozygous and parent strain to azoles, amphotericin B, and caspofungin on RPMI 1640 medium. After 48 hours of incubation, the MIC values were determined for the parental strain BWP17, the *DDR48/ddr48* mutant, (Table 2) and the reference strain *C. parapsilosis* ATCC 22019 at the lowest drug concentration at which there was either a complete inhibition of growth, or an

80% inhibition, depending on the manufacturer's suggestion for each drug.

Oxidative stress test

To assess a possible role for Ddr48 in combating oxidative stress, strains were challenged with 50 mM H_20_2. As can be seen from Figure 7, the heterozygote mutant strain showed a constant decrease in viability whereas both the heterozygote and revertant manifested an initial decrease in cell count 1-hour after challenge, but resumed normal cell growth afterwards.

DISCUSSION

Owing to the diploid nature of *Candida albicans*, molecular analysis of the *DDR48* function requires knocking out both alleles. In this study, 50 bp of PCR primer homology were sufficient to create the *DDR48/ddr48: : HIS1* strain. In fact, 2 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 the *C. albicans* strain BWP17. However, increasing the homology region to 100 bps increases the rate of correctly targeted mutants to 20% to 80% [52]. Primers with 100 bps *DDR48* homology were designed and utilized to amplify a *URA3* deletion cassette, but upon transformation and screening of 15 His^+/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, 1 allele can be deleted but the second cannot. 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 [23,53].

This study provides clear evidence that *DDR48* has a critical role in filamentation and virulence. An unexpected feature was that the *DDR48/ddr48* heterozygous strain exhibited a phenotype usually associated with homozygous mutant strains suggesting that *DDR48* is haploid insufficient. Haploinsufficiency is a condition in which a heterozygote copy of the gene is unable to maintain a wild-type phenotype [54]. Uhl and associates [55] proposed that haploinsufficiency occurs in *C. albicans* as a consequence of the evolution and subsequent slight differentiation of 2 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 ensues [55]. 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 a haploinsufficiency phenotype as reduced levels of infection in a rabbit's cornea and avirulence in a mouse model. *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 [27]. Finally, both the *CSH3* heterozygote and homozygote mutant strains, required for wild-type amino-acid responsive hyphal growth, exhibited attenuated virulence defects in a mouse model compared with the wild type [56].

Since Ddr48 has been labeled as a stress response protein, we wanted to determine whether the heterozygote mutant generated had any increased susceptibility to a potentially lethal H_2O_2 oxidative stress challenge by comparing its response to that of the wild-type and revertant strains. Interestingly, the viability of the mutant decreased constantly after a 2-hour challenge while both wild-type and revertant restored normal cell growth after an hour, possibly through up-regulating *DDR48* mRNA transcription. Such a result implies a role for Ddr48 in protection against stress.

When grown on PDB plus 25% FBS and 50% FBS medium the heterozygote strain was filament-deficient compared with the parent BWP17 strain. Furthermore, 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, nonfilamentation inducing pH. Altered filamentous growth is correlated with a defect in pH sensing.

In the presence of fetal bovine serum, no germ tube formation was detected in the heterozygote mutant strain; whereas the revertant strain rescued the mutant phenotype by exhibiting near wild-type levels of hyphal formation confirming that the observed mutant phenotype is not due to any secondary mutations. Inhibition of germ tube formation was consistent with the weakly induced filamentation phenotype observed on the other synthetic hyphal inducing media. On corn meal agar, the *DDR48/ddr48* strain formed hyphae more slowly than the parental strain, but ultimately, the extent of hyphal formation was similar in each. However, DDr48 does not seem to play a role in virulence, as all mice injected with the heterozygote strain were killed within 2 weeks after the injection Interestingly, the revertant strain was slightly less virulent than the parent strain. This could be explained by the fact that *DDR48* was reintroduced on a plasmid, which might be lost in nonselective media such as blood, the loss of the plasmid would render the strain a uridine auxotrophic strain and thus less virulent [57]. The lack of a reduction in virulence is interesting, as Thewes and associates [58] recently 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. Such a finding implies that the haploinsufficiency phenotype observed *in vitro*, through a reduction in filament formation and stress response, is not mirrored *in vivo*, with one copy of *DDR48* sufficient for wild-type virulence. Thus, even though Ddr48 appears to be an essential component of the cell wall, a wall that is essential for pathogenicity, adhesion, and virulence [20] through its constituents, including mannoproteins, chitin, and glucan that are capable of depressing the immune response of the host and regulating the actions of natural killer cells, phagocytic cells, and cell mediated immunity [59], it is not necessary for virulence.

The *Saccharomyces cerevisiae DDR48* gene is the closest phylogenetic homologue of *DDR48*. The *S. cerevisiae* gene is not only similar to *DDR48* in sequence, but it is similar to it in function too. *S. cerevisiae DDR48* displayed increased transcription response to heat shock stress and to treatments that produced DNA lesions, although a *ddr48* null mutant showed a slightly greater survival curve than the wild-type strain and was more resistant to exposure to methyl methanesulfonate and UV radiation resulting in a 10-fold reduction of spontaneous mutation rates [60]. *Saccharomyces cerevisiae* is not a pathogen, however, and thus does not traditionally exhibit any virulence characteristic or even true hyphal formation, making any comparison between the phenotypes of an *S. cerevisiae ddr48* deletion and our mutant unfeasible. Nevertheless, *S. cerevisiae* in its diploid state, and under certain growth conditions, can produce filaments in the form of pseudohyphae [61]. Giaever and associates [62] tested for the formation of pseudohyphae on YPD serum in both a *DDR48/ddr48* heterozygote strain and a *ddr48* deletion strain. Both strains were not defective in pseudohyphal growth and even exhibited normal pseudohyphal cell morphology suggesting that the *ddr48* mutation did not affect cell division, or cell growth. Thus, even though both *DDR48* genes converged on stress response, the *C. albicans DDR48* gene has sufficiently diverged to acquire additional roles, notably in filamentation and virulence.

Based on the previously mentioned antifungal susceptibility breakpoints, our *DDR48/ddr48* mutant strain generated in this study was susceptible in a dose-dependent manner to fluconazole and itraconazole compared with the parent strain, on RPMI 1640 medium. This is not surprising because previous data showed that exposure to fluconazole and itraconazole led to increased expression of *DDR48* RNA [63]. Although interpretive breakpoints have not been well established for ketoconazole, the *DDR48/ddr48* strain appeared to be sensitive to ketoconazole with respect to the parent strain. However, statistical significance was only reached with itraconazole ($P<0.01$). Interestingly, no difference in sensitivity was noticed with the other drugs tested, as both the heterozygous and the parent strain were resistant to amphotericin B but sensitive to voriconazole and caspofungin, even though up-regulation of *DDR48* in the presence of amphotericin B, and down-regulation of *DDR48* in cells carrying a deletion of key caspofungin response genes has been previously reported [30,32].

Conclusions

The present findings provide compelling evidence that *DDR48* is essential for the filamentation and possibly viability of *C. albicans,* making it a prime target for antifungal drug design. Recently, *DDR48* and other cell wall proteins have been used in vaccine formulations against candidiasis in animal models. A Ddr48 vaccine evokes a protective immune response because it becomes readily accessible to the cells of the host immune system [20]. In addition, this study provides more evidence of the complex relation between morphogenesis and filamentation in *C. albicans* pathogenesis because abolishment of filamentation did not abolish virulence. However, to confirm that *DDR48* is indeed an essential gene, more experimental work should be done such as placing the gene under the control of an inducible promoter and screening for viability under noninducing conditions, or by applying the heterozygote trisomy test designed by Enloe and associates [64] whereby a strain would retain an additional copy of an essential gene from translocation or trisomy after disruption of both alleles.

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