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The F-Box Protein Fbp1 Regulates Sexual Reproduction and Virulence in *Cryptococcus neoformans*▽†

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Cryptococcus neoformans **is the leading cause of fungal meningitis in immunocomprised populations. Although extensive studies have been conducted on signal transduction pathways important for fungal sexual reproduction and virulence, how fungal virulence is regulated during infection is still not understood. In this study, we identified the F-box protein Fbp1, which contains a putative F-box domain and 12 leucine-rich repeats (LRR). Although *fbp1* mutants showed normal growth and produced normal major virulence factors, such as melanin and capsule, Fbp1 was found to be essential for fungal virulence, as *fbp1* mutants were avirulent in a murine systemic-infection model. Fbp1 is also important for fungal sexual reproduction. Basidiospore production was blocked in bilateral mating between *fbp1* mutants, even though normal dikaryotic hyphae were observed during mating. *In vitro* assays of stress responses revealed that *fbp1* mutants are hypersensitive to SDS, but not calcofluor white (CFW) or Congo red, indicating that Fbp1 may regulate cell membrane integrity. Fbp1 physically interacts with Skp1 homologues in both *Saccharomyces cerevisiae* and *C. neoformans* via its F-box domain, suggesting it may function as part of an SCF (Skp1, Cullins, F-box proteins) E3 ligase. Overall, our study revealed that the F-box protein Fbp1 is essential for fungal sporulation and virulence in *C. neoformans*, which likely represents a conserved novel virulence control mechanism that involves the SCF E3 ubiquitin ligase-mediated proteolysis pathway.**

Cryptococcus neoformans is a basidiomycetous yeast pathogen that often infects the central nervous system (CNS) to cause meningitis, predominantly in immunocompromised individuals (11). The pathogen has emerged as the leading cause of fungal meningitis, with roughly a million cases that account for ~600,000 deaths annually (50). Besides its medical importance, *C. neoformans* has also been developed as a model organism to study fungal genetics and pathogenesis; it is a fast-growing haploid yeast with a defined sexual cycle, and robust animal models are available in which to study its virulence (11, 32). Since it is an important pathogen with genetic tractability, extensive studies have been conducted in *C. neoformans.* Several virulence factors, including production of a polysaccharide capsule and melanin, as well as the ability to grow at mammalian body temperature (37°C), have been well characterized. Signaling pathways that are important for fungal sexual reproduction and virulence have also been identified and extensively studied (1, 33, 40, 58). These studies revealed that fungal virulence is a complex trait, and virulence-determining mechanisms remain to be explored.

Protein turnover is an important regulatory mechanism of cellular function in eukaryotes. The F-box protein, which contains an F-box domain that was first identified in human cyclin F (5), commonly interacts with Skp1 of the SCF (Skp1, Cullins, F-box proteins) E3 ligase complexes to take part in protein ubiquitination and degradation. F-box proteins are exchangeable subunits that facilitate the capacity of SCF to specifically target a number of structurally and functionally diverse substrates (55). A large number of F-box proteins exist in all eukaryotic model organisms reported so far, and each of them likely binds to multiple substrates for ubiquitination. The fungal F-box proteins have been reported to be important in regulating cellular functions, including the cell cycle, circadian clocks, transcription, development, signal transduction, and nutrient sensing (35).

Saccharomyces cerevisiae has at least 20 proteins containing an F-box domain, and several of them have been well studied, including Grr1 (glucose repression resistant 1). Grr1 is part of the SCF^{GRR1} E3 ligase, which interacts with Skp1 via its F-box domain and with downstream targets via its leucine-rich repeat (LRR) domain. Grr1 was originally identified as a primary element responsive to glucose repression (20) and was also found to control amino acid sensing (8) and cell cycle regulation (9). In the presence of glucose, Grr1 targets Std1 and Mth1 for degradation, which ordinarily repress glucose uptake by binding to the negative transcription regulator Rgt1, causing it to bind to promoters of several hexose transporter genes (*HXT*). Upon the degradation of Std1 and Mth1, freed Rgt1 fails to bind to DNAs, thus releasing repression of the *HXT* genes (51). Grr1 regulates amino acid sensing by degrading two transcription factors, Stp1 and Stp2, which promotes the expression of several amino acid permease genes (8). The involvement of Grr1 in cell cycle regulation was shown by the accumulation of the cyclins Cln1 and Cln2 in a *grr1* mutant strain (41). Grr1 regulates meiosis by interacting with Ime2, a serine/threonine protein kinase important for meiosis (52).

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Schizosaccharomyces pombe is another yeast in which a number of F-box proteins have been studied (35).

Besides *S. cerevisiae* and *S. pombe*, the importance of the F-box proteins in fungal development has been reported in several other ascomyces, such as Grr1 and Cdc4 in *Candida albicans* (2, 10, 42), GrrA in *Aspergillus nidulans* (37), Fwd1 and Scorn2 in *Neurospora crassa* (29, 30, 39), Pth1 in *Magnaporthe grisea* (57), Fbp1 (F-box protein 1) in *Fusarium graminearum* (28), and Frp1 in *Fusarium oxysporum* (18). Interestingly, all three F-box proteins reported in plant fungal pathogens (Pth1, Fbp1, and Frp1) are required for fungal pathogenicity. Despite the importance of F-box proteins in regulating fungal development and virulence, there is no report as yet on their function in fungal phyla other than ascomycete fungi.

In this study, we set out to investigate the function of one F-box protein, Fbp1, in *C. neoformans*. Based on the genome database, there were at least 19 proteins containing the F-box domain in *C. neoformans*. Among them, Fbp1 has both an F-box domain and at least 12 LRRs and shares sequence and structural similarity with Grr1 in *S. cerevisiae*. The expression of Fbp1 is regulated by glucose, suggesting that it may also be involved in glucose sensing. We also found that Fbp1 is essential for sexual reproduction and that *fbp1* mutants no longer produce spores, even though they produce mating-specific dikaryotic filaments. Interestingly, Fbp1 is essential for fungal virulence, as *fbp1* mutants are avirulent, even though mutant strains still produce normal virulence factors, including melanin, capsule, and growth at mammalian body temperature. Thus, the importance of Fbp1 in fungal virulence may represent a novel virulence control mechanism.

MATERIALS AND METHODS

Strains, media, and growth conditions. *C. neoformans* and *S. cerevisiae* strains used in this study are listed in Table 1. Strains were grown at 30°C on yeast extract-peptone-dextrose (YPD) agar medium and synthetic (SD) medium. J774 murine macrophages were grown in 10-cm petri dishes in liquid Dulbecco modified Eagle's (DME) medium with 10% fetal calf serum (FCS) (ATL Biologicals, GA), 10% NCTC-109 (Gibco), and 1% nonessential amino acids (MP Biomedicals, OH). Modified MS medium (Murashige and Skoog medium) was used for mating and sporulation assays and prepared as previously described (62). All other media were prepared as described previously (1, 27).

Detection of *FBP1* gene expression using qRT-PCR. To test the expression of Fbp1 during mating and how Fbp1 is expressed in response to glucose, we measured *FBP1* expression at mRNA levels throughout the mating process and under conditions with or without glucose via quantitative real-time PCR (qRT-PCR). Cultures of the *C. neoformans* var. *grubii* wild-type strain H99 and its near congenic strain KN99**a** were grown overnight on YPD liquid medium with shaking. Cells from an H99 overnight culture were collected and washed with distilled H_2O (dH_2O) and then resuspended in medium with 2% glucose (YPD) or 2% galactose (YPG) and incubated for 2 h. Cells were then collected and washed with dH_2O. The cells collected from YPD were resuspended in YPG, while the cells collected from YPG were resuspended in YPD. Both cultures were incubated for 2 h. Total RNAs were prepared from the cells with each treatment, and cDNA was synthesized as described below.

Mating was performed by mixing H99 and KN99**a**, which were then cocultured on V8 medium (pH 5.0). Mating mixtures were collected from agar surfaces using cell scrapers after 6, 24, 48, 72, or 96 h of incubation. The collected cells were washed with dH_2O, and the pellets were used for total RNA extraction. Total RNAs were extracted using Trizol reagent (Invitrogen) and purified with the Qiagen RNeasy cleanup kit (Qiagen) following the manufacturer's instructions. The purified RNAs were used as templates for PCR amplification with primers of the glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) to determine potential genomic DNA contamination. Purified RNAs were quantified using a Nanodrop spectrometer (Thermo Scientific).

TABLE 1. Strains used in this study

Strain	Genotype	Source/reference
C. neoformans		
H99	*MAT*α	50a
KN99**a**	*MAT***a**	45a
CUX2	*MAT*α *fbp1*::*NEO*	This study
CUX3	*MAT***a** *fbp1*::*NEO*	This study
CUX5	*MAT*α *fbp1*::*NEO FBP1*::*NAT*	This study
CUX6	*MAT***a** *fbp1*::*NEO FBP1*::*NAT*	This study
CUX83	*MAT*α *ura5* (*5-FOA*^{r})	This study
CUX84	*MAT***a** *ura5* (*5-FOA*^{r})	This study
CUX87	*MAT*α *fbp1*::*NEO ura5* (*5-FOA*^{r})	This study
CUX88	*MAT***a** *fbp1*::*NEO ura5* (*5-FOA*^{r})	This study
CUX89	*MAT*α *fbp1*::*NEO FBP1*$^{\Delta FB}$-*NAT*	This Study
CUX90	*MAT***a** *fbp1*::*NEO FBP1*$^{\Delta FB}$-*NAT*	This study
S. cerevisiae		
pJ69-4A	*MAT***a** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4*Δ *gal8*Δ *LYS2*::*GAL1-HIS3 GAL2-ADE2 met2*::*GAL7-lacZ*	34
YSB4742	*MAT*α *his3*Δ*1 leu2*Δ*0 ura3*Δ*0*	ATCC yeast deletion collection
YSB4741	*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*	ATCC yeast deletion collection
YJR090C	*MAT*α *grr1*::*KanMX*	ATCC yeast deletion collection
YJR090C	*MAT***a** *grr1*::*KanMX*	ATCC yeast deletion collection
YUX39	*MAT***a** *grr1*::*KanMX FBP1-LEU2*	This study

First-strand cDNAs were synthesized using a Superscript III cDNA synthesis kit (Invitrogen) following the manufacturer's instructions. Expression of *FBP1* and *GAPDH* was analyzed using SYBR advantage QPCR premix reagents (Stratagene). Primer efficiency was determined by serially diluting the cDNA and monitoring DNA amplification by real-time PCR. Gene expression levels were normalized using the endogenous control gene *GAPDH*, and the relative levels were determined using the comparative threshold cycle (C_T) method (43). Real-time PCRs were performed using an Mx4000 QPCR system (Stratagene) as previous described (61). The specificity of the PCR was further verified by subjecting the amplification products to agarose gel electrophoresis and sequencing them.

Generation of *fbp1*-null mutants and their complemented strains. *fbp1* mutants were generated in both H99 and KN99**a** strain backgrounds by overlap PCR as previously described (14). The 5′ and 3′ regions of each *FBP1* gene were amplified from H99 genomic DNA with primers CX278 and CX279, and CX280 and CX281, respectively (see Table S1 in the supplemental material for primer sequences). The dominant selectable markers (NEO^r) were amplified with the M13 primers (M13F and M13R) from plasmid pJAF1 (21). Each target gene replacement cassette was generated by overlap PCR with primers CX278 and CX281 (see Table S1 in the supplemental material). The purified overlap PCR products were precipitated onto 10-μl gold microcarrier beads (0.6 μm; Bio-Rad), and strain H99 or KN99**a** was biolistically transformed as described previously (15). Stable transformants were selected on YPD medium containing G418 (200 mg/liter). To screen for mutants of the *FBP1* gene, diagnostic PCR was performed by analyzing the 5′ junction of the disrupted mutant alleles with primers CX284 and JH8994 (see Table S1 in the supplemental material). Positive transformants identified by PCR screening were further confirmed by Southern blot analysis.

To generate complemented strains of *fbp1* mutants, a genomic DNA fragment that contains a 1.5-kb upstream promoter region, the *FBP1* open reading frame (ORF), and its 500-bp downstream region was amplified in a PCR using primers CX285 and CX286. This PCR fragment was fused with the NAT^r selective marker gene at its C terminus in an overlap PCR using primers CX285 and M13R. The overlap PCR product was biolistically transformed in both α and **a** mating-type *fbp1* mutant strains. Mating assays were performed to identify transformants that complemented the *fbp1* phenotype.

To generate strains expressing Fbp1 lacking the F-box domain, we deleted the F-box domain sequence by amplifying two fragments from H99 genomic DNA with primers CX225/CX198 and CX199/CX231. Overlap PCR was performed by using the mixture of two fragments as a template with primers CX225 and

CX231. The amplified overlap PCR product contains the *FBP1* gene lacking the F-box domain sequence and was cloned into vector pJAF13, which contains the NAT^r selective marker, to generate the construct pCUX58. pCUX58 was introduced and expressed in the *fbp1* mutant background by biolistic transformation. The expression of Fbp1 lacking an F-box domain was confirmed by Western blot analysis (see Fig. S1 in the supplemental material).

Assays for melanin, capsule production, and mating. Melanin production was assayed by inoculating *C. neoformans* strains into 2 ml YPD liquid medium and incubating them overnight at 30°C. Five microliters of each overnight culture with series dilutions was placed on L-3,4-dihydroxyphenylalanine (L-DOPA) agar medium (13 mM glycine, 15 mM glucose, 29.4 mM KH_2PO_4, 10 mM $MgSO_4 \cdot 7H_2O$, 3 μM thiamine, 5 μM D-biotin, 2% agar, 1 mM L-DOPA, pH 5.5). The agar plates were incubated at 30°C or 37°C for 2 days, and the pigmentation of fungal colonies was assessed.

To examine capsule production, yeast cells were grown in YPD broth overnight at 37°C with constant agitation, washed 3 times with phosphate-buffered saline (PBS), and resuspended at a concentration of 1×10^6 cells/ml in various media: DME medium, PBS, or YPD broth. Cells were added to 6-well plates and incubated with or without the addition of 10% heat-inactivated FCS and in the absence or presence of 10% CO_2 for 24 h. The capsule size was visualized by adding a drop of India ink to the cell suspensions and observing them on an Olympus (Melville, NY) AX70 microscope using a 100× objective. The cells were analyzed using the ImageJ 1.40g software by measuring the distance from the cell wall to the capsule edge (India ink exclusion zone). The average and standard deviation from 15 to 30 cells were calculated for each condition tested.

In a mating assay, *C. neoformans* cells of opposite mating types were mixed and cocultured on V8 or MS agar medium at 25°C in the dark. Mating filaments and basidiospore formation were examined and recorded by photography using the Olympus CX41 light microscope after incubation for 7 to 10 days. Mating results were also examined using scanning electron microscopy (SEM), following the method previously described (60).

DAPI staining and trypan blue staining. DAPI (4′,6-diamidino-2-phenylindole) staining was performed as previously described (3) with minor modification. Cell cultures and mating filaments were fixed with formaldehyde (9.3%) for 10 min. The fixed cells were washed twice with PBS, permeabilized with an equal volume of PBS buffer containing 1% Triton X-100 for 5 min, washed twice again with PBS, and resuspended in PBS. For DAPI staining, equal volumes of cell suspension and DAPI mixture (20 ng/ml DAPI, 1 mg/ml antifade, 40% glycerol) were mixed and observed with a Nikon fluorescence microscope.

Trypan blue staining was performed as previously described (25). Briefly, strains were inoculated in 5 ml YPD liquid medium and grown for 2 days at 30°C. Yeast cells were mixed with an equal volume of 0.4% trypan blue solution and viewed through an Olympus CX41 light microscope. The percentages of cells that were able to take up dye (dark blue) were calculated by counting at least 500 yeast cells for each strain.

Protein-protein interaction assays using the yeast two-hybrid system. Yeast two-hybrid interaction assays were performed as previously described (48). The *FBP1* full-length cDNA and partial cDNA lacking the F-box domain, full-length cDNAs of *SKP1* homologues in strain H99 (*CnSKP1*) and *S. cerevisiae* (*ScSKP1*), and *CRK1* cDNA were cloned into the bait vector pGBKT7 and fused with the BD domain. cDNAs of *FBP1*, *CnSKP1*, and *ScSKP1* were also cloned into the prey vector pGADT7 and fused with the AD domain. All inserted cDNA sequences were confirmed by sequencing. Both bait constructs and prey constructs were cotransformed into the yeast strain PJ69-4A. Transformants growing on SD medium lacking histidine or adenine were considered positive interactions. The expression of the LacZ gene in these transformations was quantified by β-galactosidase enzyme activity assays using chlorophenol red–β-D-galactopyranoside (CPRG) (Calbiochem, San Diego, CA) as a substrate.

Macrophage phagocytosis assays. Phagocytosis assays were performed in 96-well plates, using J774 macrophages at a concentration of 2.5×10^4 cells/well that were allowed to double overnight at 37°C in activating medium (DME medium, 50 U/ml of gamma interferon [IFN-γ], 1 μg/ml of lipopolysaccharide [LPS]). PBS-washed fungal cells were opsonized with 20% mouse complement (Pel-Freez, AK) and added to the macrophages at an effector-to-target ratio of 1:2. Phagocytosis was allowed to occur for 2 h at 37°C in 10% CO_2. The cells were then washed three times with PBS and fixed with methanol at −20°C for 30 min. Giemsa stain was added to the wells at a dilution of 1:20, and the plates were incubated at room temperature for 30 min. The wells were washed once with PBS and analyzed using an inverted microscope. For each well, 3 different fields were counted, for a total of at least 100 macrophages. The percent phagocytosis was determined by dividing the number of macrophages that contained *C. neoformans* by the total number of macrophages counted.

To check macrophage antifungal activity, phagocytosis was allowed to occur for 3 h and 24 h. After each time interval, medium was removed from the well and transferred to an Eppendorf tube containing sterile PBS. Macrophages were lysed by adding sterile dH_2O to each well and incubating the plate for 45 min at room temperature. Fluid was transferred to each respective tube after resuspension. Fungal cells were counted, appropriate dilutions were made, and approximately 200 cells were plated on YPD agar plates. The colonies on each plate were counted after 2 days of incubation at 37°C to determine the actual number of live cells per well.

Virulence studies. Yeast strains were grown at 30°C overnight, and the cultures were washed twice with PBS buffer and resuspended at a final concentration of 2×10^6 CFU/ml. Groups of 10 female A/Jcr mice (NCI, Frederick, MD) were intranasally infected with 10^5 cells of each yeast strain as previously described (13). Over the course of the experiments, animals that appeared moribund or in pain were sacrificed by CO_2 inhalation. Survival data from the murine experiments were statistically analyzed between paired groups using the log-rank test with PRISM version 4.0 (GraphPad Software, San Diego, CA) (P values of <0.001 were considered significant).

Histopathology and fungal burden in infected organs. Infected animals were sacrificed at the endpoint of the experiment according to the University of Medicine and Dentistry of New Jersey (UMDNJ) IACUC-approved animal protocol. For mice infected by the *fbp1* mutant strain, the experiment was terminated 60 days postinfection. To compare the fungal burdens and host inflammatory responses, lungs and brains from mice infected by H99, the *fbp1* mutant, or the complemented strain of the *fbp1* mutant were isolated at 10 or 20 days postinfection, fixed in 10% formalin solution, and sent to the AML laboratory for section preparation (AML Laboratories, Inc. Rosedale, MD). Lungs and brains infected by the *fbp1* mutant were also isolated at the endpoint of the experiment (60 days postinfection). Tissue slides were stained with hematoxylin and eosin (H&E) and examined by light microscopy. Infected lungs, brains, and spleens were also isolated and homogenized using a homogenizer in PBS buffer. Resuspensions were diluted, 100 μl of each dilution was spread on YPD medium with ampicillin and chloramphenicol, and colonies were determined after 3 days of incubation at 30°C.

RESULTS

Identification of the F-box protein Fbp1. To understand the function of F-box proteins in *C. neoformans*, we scanned the genome sequence of *C. neoformans* var. *grubii* strain H99 and identified 20 proteins containing an F-box domain in the H99 genome database (http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/MultiHome.html and data not shown). Among them, six proteins contain WD40 repeats, and only one (CNAG_05280.2) contains LRRs; we named the latter protein Fbp1 (F-box protein 1). Fbp1 contains 928 amino acids with an F-box domain and an LRR domain containing at least 12 LRRs. Fbp1 shows sequence similarity to several F-box proteins reported in fungi, including Grr1 in *S. cerevisiae* and *C. albicans*, GrrA in *A. nidulans*, and Fbp1 in *F. graminearum* (Fig. 1). Among them, *Saccharomyces* Grr1 has been most extensively studied and has been found to play a role in the sensing of glucose, amino acids, and pheromone, as well as cell cycle regulation (35). Fbp1 showed 19% protein sequence identity and 33% sequence similarity with Grr1, suggesting it could also be an important nutrient regulator. Due to the importance of nutrient sensing and its regulation in both the development and pathogenesis of *C. neoformans*, we decided to investigate the function of Fbp1 (4).

Expression of *FBP1* is downregulated during mating. We analyzed *FBP1* expression during different developmental stages using qRT-PCR and Northern blot assays. Mating between H99 and KN99**a** was performed on V8 mating medium, and mating mixtures were collected from plates after incubation for 0, 6, 24, 48, 72, and 96 h. RNAs were purified, and qRT-PCR was performed. Our results showed that the expression of *FBP1* was significantly downregulated during mating,

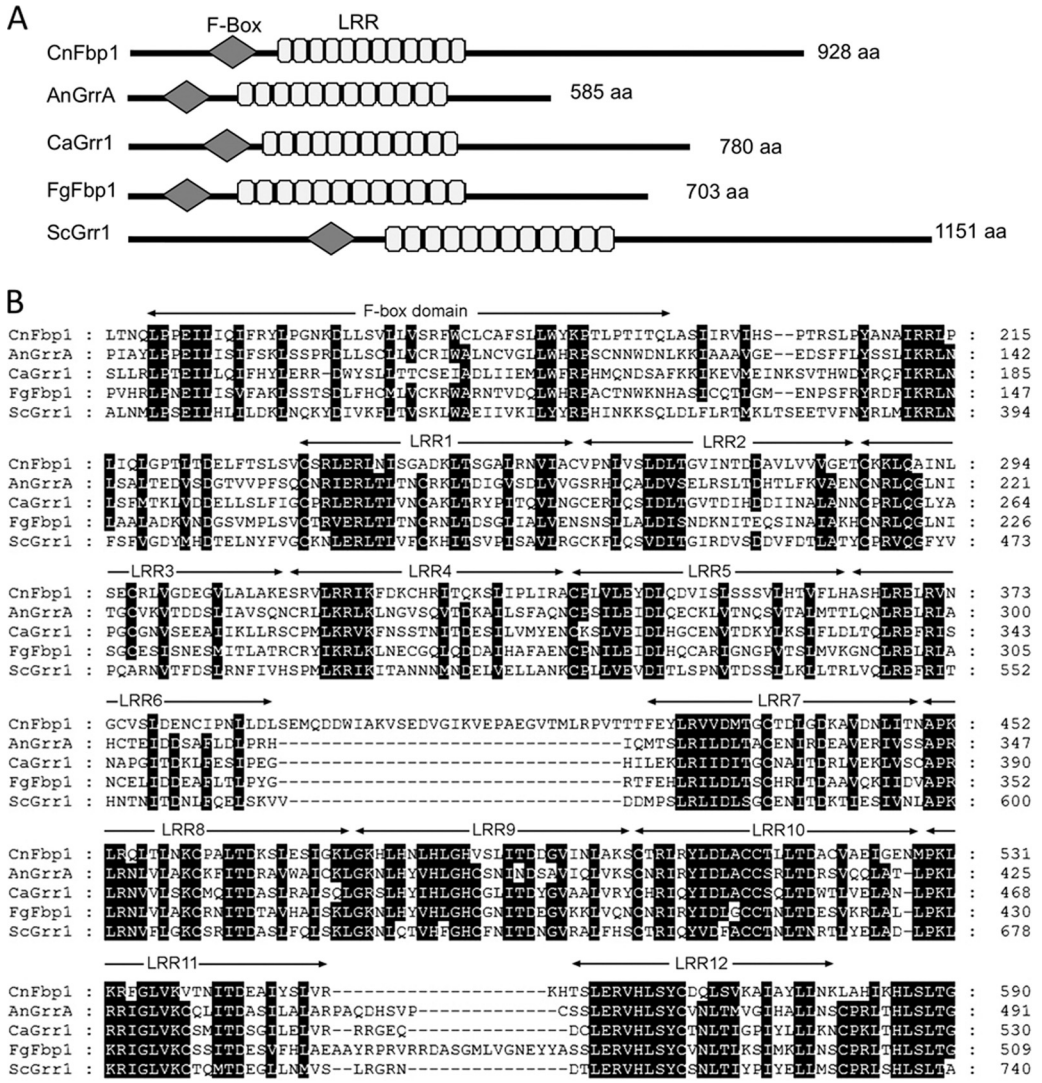

FIG. 1. Alignments of Fbp1 protein sequences and conserved F-box and LRR domains. (A) Schematic illustration of F-box proteins in *C. neoformans* (*CnFbp1*), *A. nidulans* (*AnFbp1*), *F. graminearum* (*FgFbp1*), and *S. cerevisiae* (*ScGrr1*). F-box, F-box domain; aa, amino acids. (B) Comparison of the region spanning F-box and LRR motifs in the *CnFBP1* sequence with those in known F-box proteins. The locations of F-box domains and each LRR are labeled. The amino acid alignments were generated using the CLUSTALX program, and conserved amino acids are indicated by shading.

especially after 72 h, compared to expression at the 0-h time point, an indication that the *FBP1* gene may play a role in later stages of sexual reproduction after cell fusion (Fig. 2A and B).

We also evaluated the effect of the glucose concentration on the expression of the *FBP1* gene. Our qRT-PCR results showed that *FBP1* expression was moderately upregulated in response to the glucose starvation caused by switching cultures from YPD (2% glucose) to YPG (2% galactose) for 2 h (Fig. 2C). On the other hand, the expression of *FBP1* was moderately downregulated in response to the glucose induction caused by switching cultures from YPG to YPD for 2 h (Fig. 2D). These results suggest that the expression of *FBP1* is subject to glucose repression.

Fbp1 expression does not complement *grr1* mutant phenotypes. In *S. cerevisiae*, Grr1 functions as a repressor of glucose repression and is also involved in cell cycle regulation (49, 56). Overnight cultures of *grr1* mutants grown on YPD quickly flocculated without being shaken (not shown), and mutant cells were elongated (Fig. 2E). To examine whether Fbp1 is an ortholog of Grr1, we transformed the pGBKT7 vector expressing Fbp1 or Grr1 into a *grr1* mutant strain. Reintroducing the *GRR1* gene complements the *grr1* mutant on cell morphology (see Fig. S2 in the supplemental material). However, no morphological change was observed when Fbp1 was expressed in a *grr1* mutant strain, which was confirmed by RT-PCR (not shown), indicating Fbp1 could not complement the function of Grr1 in *S. cerevisiae* (Fig. 2E). Because cell morphology was not altered in *C. neoformans fbp1* mutants, Fbp1 may have different functions than *Saccharomyces* Grr1.

Hxt3 is a glucose transporter, and its expression is induced by either low or high glucose concentrations in *S. cerevisiae* in a Grr1-dependent manner. We identified a homologue of

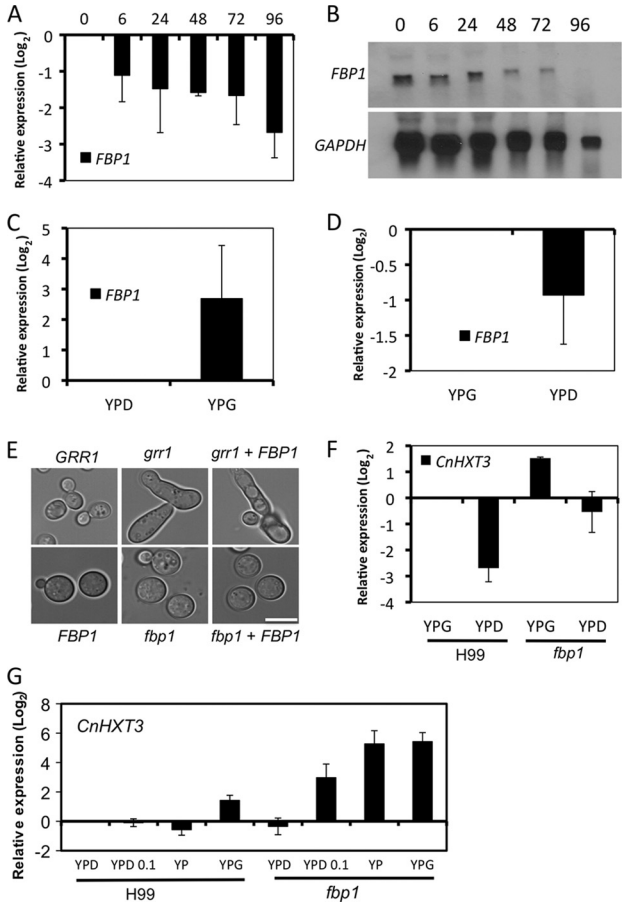

FIG. 2. *FBP1* expression patterns under different culture conditions. (A) Expression of *FBP1* during mating on V8 medium was measured by qRT-PCR. Mating cultures of H99 × KN99**a** were isolated from the plate surface after incubation for 6, 24, 48, 72, and 96 h. RNAs were purified, and cDNA was synthesized for qRT-PCR analyses. The comparative C_T method was used for the relative quantification. Values are expressed as relative expression ($\log_2$; means and standard deviations) of the *FBP1* gene, normalized to the *GAPDH* gene endogenous reference and relative to the 0-h time point (H99 overnight liquid culture on YPD was considered the 0-h time point). The error bars show standard deviations of three repeats. (B) RNAs from mating cultures were also used for detecting the expression of *FBP1* in a Northern blot probed with *FBP1* (top) and *GAPDH* (bottom) that was amplified from H99 cDNA. (C and D) Expression of *FBP1* under glucose depletion (C) or induction (D) conditions was measured by the same qRT-PCR analysis strategy. H99 cells were grown on YPD (2% glucose) liquid medium overnight and were collected and washed with dH_2O before being resuspended on YPD (C) or YPG (YP with 2% galactose) (D) medium and incubated for 2 h. Cells were collected and resuspended on YPG (C) or YPD (D) medium and incubated for another 2 h. The first-strand cDNAs from these samples were used for qRT-PCR with *FBP1* primers and *GAPDH* primers. (E) Expression of *FBP1* in a *Saccharomyces grr1* mutant strain failed to complement Grr1 function. The *S. cerevisiae* control strain BY4742, a *grr1* mutant, and a *grr1* mutant expressing *FBP1*, *C. neoformans* H99, an *fbp1* mutant (CUX2), and an *fbp1* mutant expressing *FBP1* (CUX5) were grown on YPD for 24 h, and yeast cells were visualized under light microscopy. (F and G) Expression of a *Cryptococcus HXT3* homologue was measured by qRT-PCR under glucose induction conditions (F) or glucose depletion conditions (G) by switching cells from YPG to YPD, YP (no glucose), or YP0.1 (0.1% glucose) medium in both wild-type H99 and an *fbp1* mutant background. All medium treatments were the same as those described for panels C and D. An H99 overnight culture grown on YPD was used as the 0-h time point for all qRT-PCR analyses.

HXT3 in *C. neoformans* (*CnHXT3*; CNAG_03372.2). The expression of *CnHXT3* under different glucose conditions was measured by qRT-PCR and compared between wild-type H99 and the *fbp1* strain. When cells grown on medium without glucose (YPG, 2% galactose) were switched to glucose-rich medium (YPD, 2% glucose) for 2 h to generate glucose induction conditions, expression of *CnHXT3* was higher in the *fbp1* mutant background and was downregulated in the H99 background (Fig. 2F). On the other hand, when cells grown on YPD were switched to medium without glucose (YP, no glucose; YP0.1, 0.1% glucose; or YPG, 2% galactose) to generate glucose starvation conditions, *CnHXT3* expression was not significantly altered in the wild-type strain but was upregulated in *fbp1* mutants (Fig. 2G). These results indicated that *CnHXT3* expression was repressed by a high glucose concentration and induced by glucose starvation. Fbp1 negatively regulates *CnHXT3* expression under these conditions.

Fbp1 is important for sexual sporulation. The *fbp1* deletion mutants were generated in both *C. neoformans* H99 and KN99**a** strain backgrounds. The development of dikaryotic hyphae and basidiospores was examined in both *fbp1* unilateral mating (*fbp1* × wild type) and bilateral mating (*fbp1* × *fbp1*). No obvious phenotypic changes were observed in *fbp1* unilateral-mating assays. Interestingly, the bilateral mating between *fbp1* mutants failed to produce basidiospores, even though it produced normal dikaryotic hyphae, indicating that Fbp1 is essential for sporulation (Fig. 3A). As the F-box domain of an F-box protein is important for its function, we investigated the role of the F-box domain in Fbp1 function by generating strains expressing Fbp1 lacking the F-box domain ($Fbp1^{\Delta FB}$), which contains a 6×His tag at the C-terminal end. The expression of $Fbp1^{\Delta FB}$ in *fbp1* mutants was confirmed by Western blot analysis using anti-His antibody (see Fig. S1 in the supplemental material). Our mating results showed that bilateral mating between strains expressing $Fbp1^{\Delta FB}$ ($Fbp1^{\Delta FB}$ × $Fbp1^{\Delta FB}$) could not produce spores, similar to the bilateral mating of *fbp1* mutants, indicating the F-box domain is essential for Fbp1 function in mating.

Fbp1 is involved in meiosis and nuclear division. To understand why *fbp1* mutants fail to produce spores, we investigated the development of fungal nuclei at different stages of sexual reproduction by DAPI staining. A single nucleus in each yeast cell was observed in both the wild-type and the *fbp1* mutant cultures, and two separated nuclei can be observed in each dikaryotic hypha produced from bilateral-mating mixtures after cell fusion (Fig. 3C). A single fused nucleus could be observed in each young basidium of both the wild type and *fbp1* mutants after mating mixtures were incubated for 3 days, indicating that both strains undergo normal nuclear fusion to produce basidia during mating. However, nuclei in the bilateral mating of *fbp1* mutants failed to undergo meiosis after fusion, and only a single nucleus was observed in each mature basidium after14 days of incubation, while all basidia from wild-type mating produced four nuclei. This observation suggested that Fbp1 is important for regulating meiosis during mating, which could explain the defect of sporulation in bilateral mating of *fbp1* mutants.

Because *fbp1* mutants have a normal growth rate and also have normal nuclear division when grown in rich medium, Fbp1 may not be involved in the cell cycle during mitotic

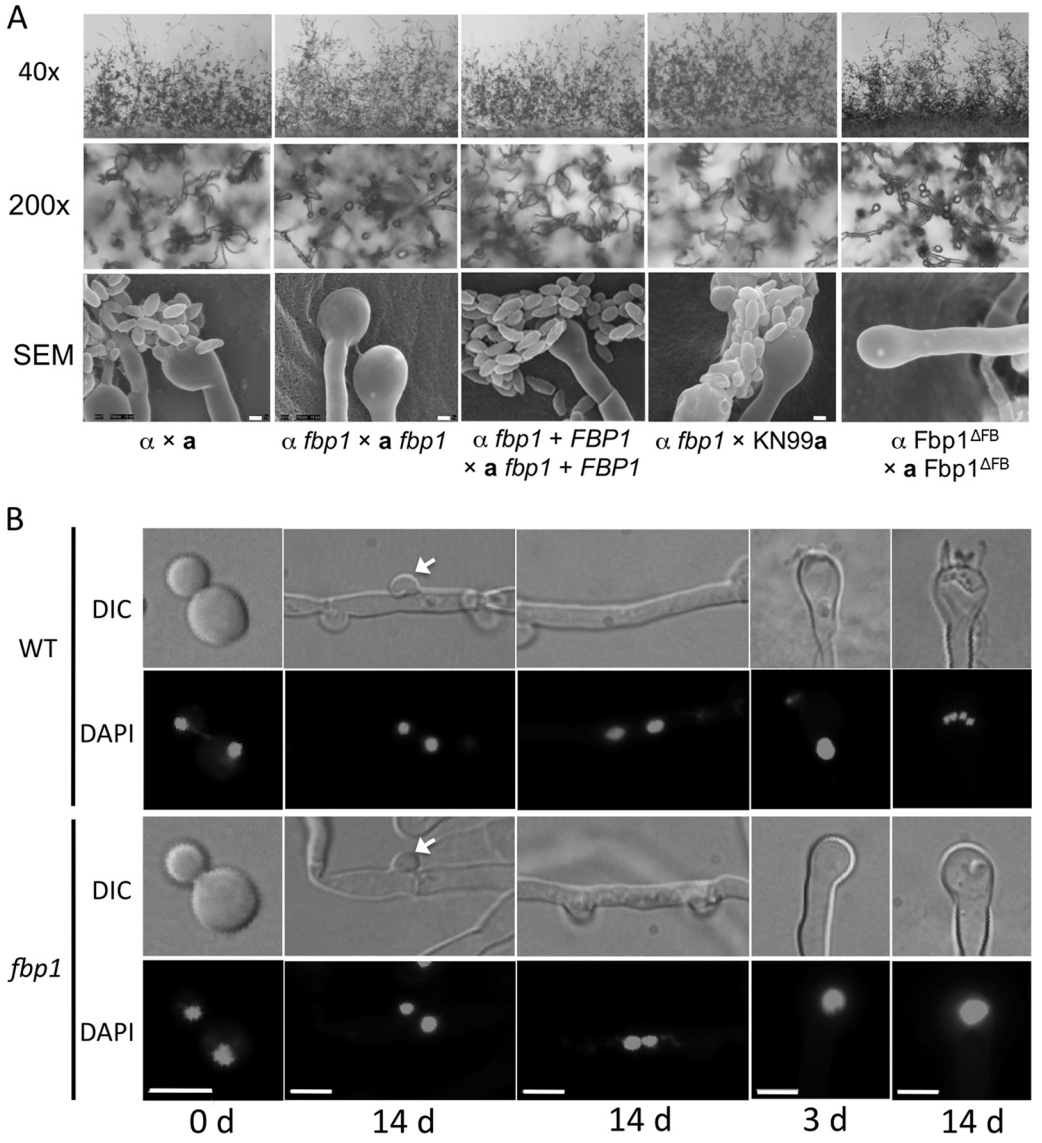

FIG. 3. Mating filament production versus sporulation on the wild-type and mutants. (A) Mating assays for wild-type (H99 × KN99**a**) mating, *fbp1* mutant unilateral (CUX2 × KN99**a**) and bilateral (CUX2 × CUX3) mating, Fbp1$^{\Delta FB}$ strain bilateral (CUX89 × CUX90) mating, and *fbp1* complemented strain (CUX5 × CUX6) mating were performed on MS medium. Mating structures at ×40 magnification (top) were photographed after 2 weeks of incubation in the dark at 25°C. Mating cultures were also fixed and viewed by SEM (bottom). Bar, 1 μm. (B) Nuclear staining for H99 × KN99**a** and α *fbp1* (CUX2) × **a** *fbp1* (CUX3) mating hyphae and basidia. Mating cultures were isolated from mating plates after incubation for 7 or 14 days (d) on MS medium in the dark. Mating culture mixtures were washed with dH_2O twice and fixed with formaldehyde before being coincubated with 2 μg/ml DAPI solution. The staining results were visualized under Nikon fluorescence microscopy following 1 h of staining. Bars, 5 μm.

division; rather, it plays a role only in regulating meiosis. To confirm these observations, we performed fluorescence-activated cell sorter (FACS) analysis for H99 and *fbp1* mutants cultured in YPD liquid medium and minimal medium. No difference was observed between the wild-type strain and the *fbp1* mutant, which confirmed that Fbp1 is dispensable for mitotic cell division in *C. neoformans* (data not shown).

Fbp1 is dispensable for *in vitro* virulence factor production but essential for fungal infection. In *C. neoformans*, several virulence factors that are important for fungal virulence have been well characterized *in vitro*, including production of a polysaccharide capsule and melanin, as well as the ability to grow at mammalian body temperature (37°C). We examined the development of these virulence factors in *fbp1* mutants *in vitro*. *fbp1* mutants produced normal melanin on both Niger seed agar and L-DOPA agar, produced regular capsules on DME medium, and also showed normal growth at 37°C, indicating that Fbp1 is not important for development of these virulence factors *in vitro* (Fig. 4; see Fig. S3 in the supplemental material).

The capsule size of *C. neoformans* can be further induced by the presence of high CO_2 concentrations and serum (27, 63). The capsule enlargement under such inducible conditions is similar to what has been observed *in vivo* during infection,

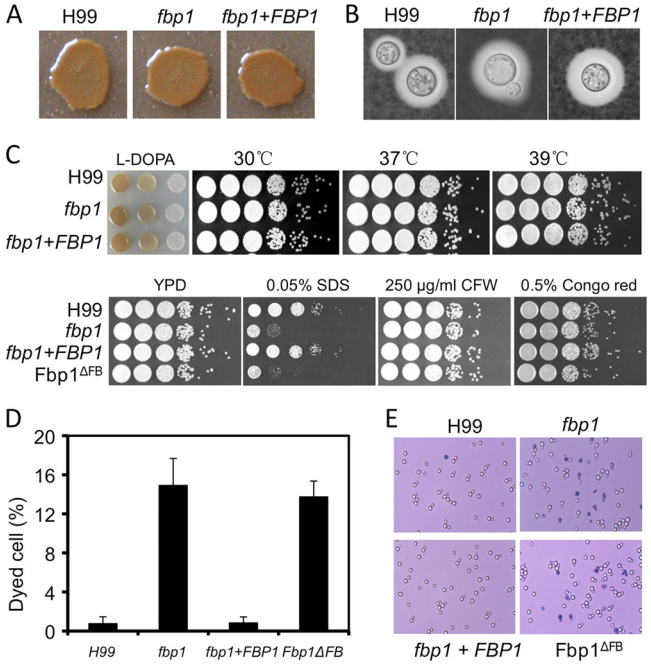

FIG. 4. Fbp1 is dispensable for virulence factor development but required for cell membrane integrity. (A) Melanin production was assayed on Niger seed medium. Melanin levels produced by the strains were observed in photographs after incubation for 3 days at 37°C. (B) Capsule formation was assayed at 37°C on DME medium. Capsule production was visualized by India ink staining after cells were grown on DME medium for 3 days. (C) Cultures were grown overnight in YPD and diluted to an optical density at 600 nm (OD_{600}) of 2.0. Tenfold serial dilutions were made in dH_2O, and 5 μl of each was plated. The plates were grown for 2 days at 30°C for the inhibitor plates and for the indicated temperature for all others. H99 and mutant strains are indicated on the left and the conditions at the top. (D) Trypan blue staining to examine cell membrane integrity. H99 and the *fbp1* mutant (CUX2), its complemented strain (CUX5), and an *fbp1* mutant expressing Fbp1 lacking the F-box domain (CUX89) were inoculated in YPD for 24 h. Yeast cells were mixed with 0.4% trypan blue solution for 30 min before being visualized by microscopy. Over 500 yeast cells for each sample were evaluated for those that were heavily stained. The percentage of heavily stained cells was calculated. The error bars indicate standard deviations. (E) Representative images of trypan blue staining results are shown.

since 10% CO_2 and serum are part of the physiological condition in mammals. The ability of capsule to grow under such conditions has been reported to be important for fungal pathogenicity (63). To test whether Fbp1 plays a role in capsule growth under such inducible conditions, we examined the capsule sizes of *fbp1* mutants after they were treated with 10% CO_2 and 10% heat-inactivated FCS. Yeast cells were grown on different culture media, including rich medium (YPD), PBS buffer, and DME medium, and the cultures were treated with 10% CO_2, 10% FCS, or both. Our results showed that in every medium tested, the capsule sizes of both H99 and *fbp1* mutants were significantly enlarged in the presence of 10% CO_2 or 10% CO_2 plus 10% FCS, consistent with results described in earlier studies (63). However, there was no significant difference between H99 and *fbp1* mutants, indicating Fbp1 is dispensable for capsule growth under these inducible conditions (see Fig. S4 in the supplemental material).

Because fungal virulence is a complex trait, we examined the virulence of *fbp1* mutants in a murine intranasal-inhalation model. Interestingly, the *fbp1* mutants tested are completely avirulent. Consistent with previous studies, mice infected by the H99 wild-type strain were terminated around 18 to 22 days postinoculation due to lethal infection. However, *fbp1*-infected mice stayed healthy and continued to gain body weight even after 60 days postinfection. A complemented strain of the *fbp1* mutant developed lethal infection in mice around 24 days, which confirmed that the avirulent or hypovirulent phenotype in *fbp1* mutants is caused by the deletion of the *FBP1* gene (Fig. 5A).

The fungal burdens of infected mice were evaluated at the endpoint of the infection experiments. Lungs, spleens, and brains from three mice infected by each fungal strain were isolated, and the fungal loads in these organs were measured as yeast CFU per gram fresh organ. Our results showed that when mice infected by the wild-type strain, H99, were sacrificed around 20 days, 10^9, 10^5, and 10^6 CFU were isolated in mouse lung, brain, and spleen, respectively. Mice inoculated with *fbp1* mutant cells were sacrificed 60 days postinoculation, and mouse organs were isolated to examine the fungal burden. No yeast cells were recovered in *fbp1*-infected spleen and brain, and only an average of 10^3 yeast CFU were observed in each gram of lung (Fig. 5B).

Fungal lesion development in lung and brain was also visualized in H&E-stained slides. As shown in Fig. 5C, at either 10 or 20 days postinfection, both the wild-type H99 and the complemented strain caused severe damage in infected lungs, with abundant yeast cells containing big capsules. In contrast, only very limited damage was produced in the *fbp1* mutant-infected lung, with very few yeast cells observed at both time points. There was no obvious difference between brains infected by any of the three strains at 10 days postinfection (not shown). However, at 20 days postinfection, severe organ damage with visible lesion development was observed in brains infected by H99 or the complemented strain, while no detectable damage or lesion was detected in brains infected by the *fbp1* mutant (Fig. 5C). Even at 60 days postinfection, the *fbp1* infection caused only very limited damage in lungs and did not cause any lesion development in brains (see Fig. S5 in the supplemental material). These results demonstrate that Fbp1 is essential for the development of cryptococcosis in the murine model.

Fbp1 is not important in fungal survival in macrophages. *C. neoformans* can proliferate inside macrophages as an intracellular pathogen. To evaluate whether the avirulent phenotype of *fbp1* mutants could be due to increased susceptibility to macrophage killing during fungus-macrophage interactions, we tested the sensitivity of the *fbp1* mutant to phagocytotic killings in the J774 murine macrophage cell line. In our studies, no significant difference was observed in the internalization of yeast cells in macrophages (Fig. 6). The macrophage antifungal activity assay also showed that the number of CFU recovered from the *fbp1* mutant is similar to that from the wild-type strain H99 after coinoculation with activated macrophages in DME medium for either 3 h or 24 h, suggesting that the *fbp1* mutant strain is equally resistant to macrophage killing. Interestingly, *fbp1* mutants appeared to be less viable when incubated in DME tissue culture medium alone or coincubated with inactivated macrophages for 24 h, suggesting that the *fbp1* mutant grows better inside the macrophage, perhaps by obtaining nu-

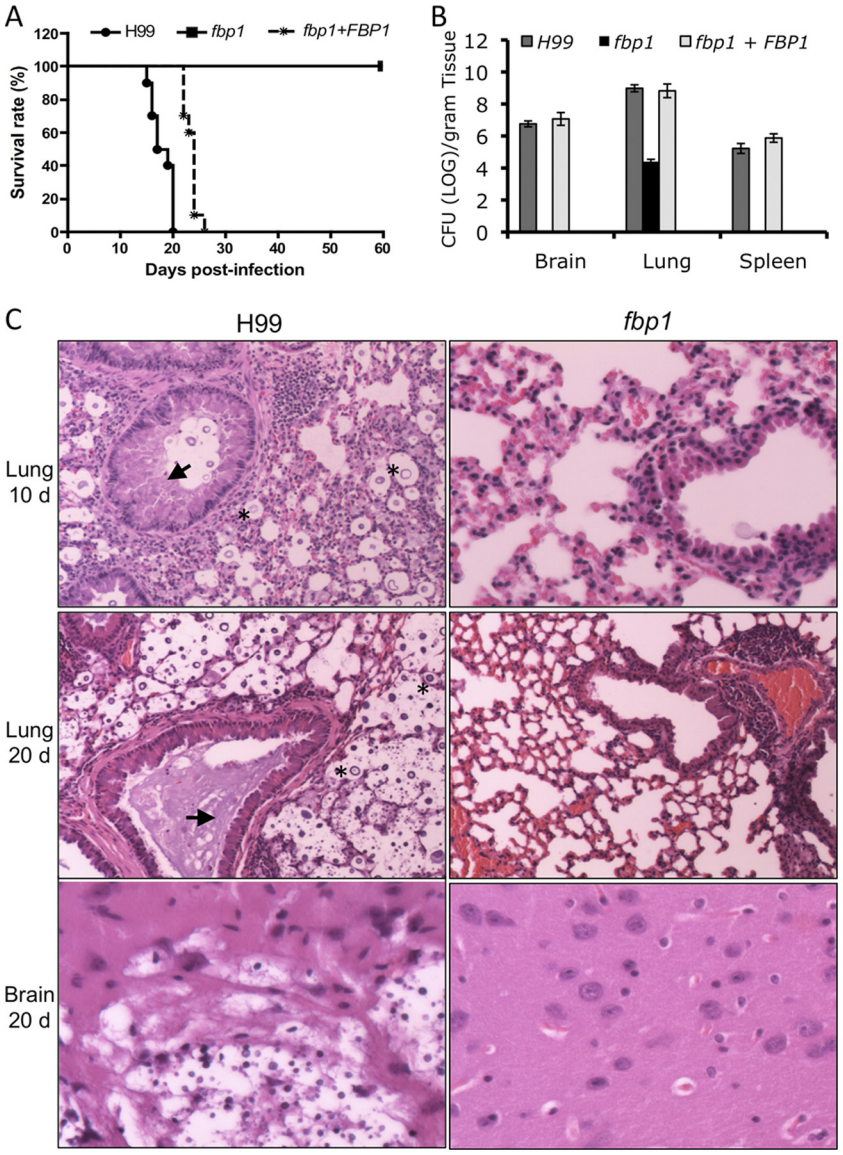

FIG. 5. Fbp1 regulates virulence in a murine inhalation model. (A) Female A/Jcr mice were intranasally inoculated with 10^5 cells of H99, the *fbp1* mutant (CUX2), and the *fbp1* + *FBP1* complemented strain (CUX5). The animals were monitored for clinical signs of cryptococcal infection and sacrificed at predetermined clinical endpoints that predict imminent mortality. The *fbp1* mutant strain is completely avirulent compared with the wild-type strain, H99. (B) Lungs, brains, and spleens from three mice infected with H99 or the *fbp1* mutant were isolated at the end time point of infection. The isolated organs were homogenized and spread on YPD plates containing ampicillin and chloramphenicol after dilution. CFU per gram of fresh organ were also measured in lung homogenates. Each data point and error bar indicates the mean and standard error of the mean for values from three animals. (C) H&E-stained slides were prepared from cross sections of infected lung at 10 days postinfection and infected lung and brain at 20 days postinfection and visualized by light microscopy at ×100 magnification. The cryptococci are indicated by asterisks, and mucus production in the airway is indicated by arrows.

trients from host cells, a phenomenon that has been described before (16).

Fbp1 regulates cell membrane integrity. Because Fbp1 does not play an important role in the development of several well-characterized virulence factors, how Fbp1 controls fungal virulence remains unclear. We evaluated the possibility that Fbp1 could be involved in stress responses and that its absence could lead to virulence attenuation *in vivo* in the hostile host environment. We tested the growth of *fbp1* mutants under osmotic stress (1.5 M NaCl, 1 M or 2.5 M sorbitol) and oxidative stress (2.5 mM H_2O_2), but no growth defect was observed (not shown). We also examined the cell integrity of *fbp1* mutants by applying several chemicals that target cell integrity, such as SDS, CFW, and Congo red. SDS disrupts the plasma membrane and lyses cells with a defective membrane, while CFW specifically binds to chitins and Congo red binds to β-1,4-glucans of the cell wall to disrupt cell wall integrity (19, 59). Our results showed that *fbp1* mutant cells were hypersensitive to 0.05% SDS, but not CFW or Congo red, an indication that *fbp1* mutants have cell membrane defects (Fig. 4). Expressing Fbp1 lacking the F-box domain ($Fbp1^{\Delta FB}$) in an *fbp1* mutant background could not rescue the growth defect on YPD with

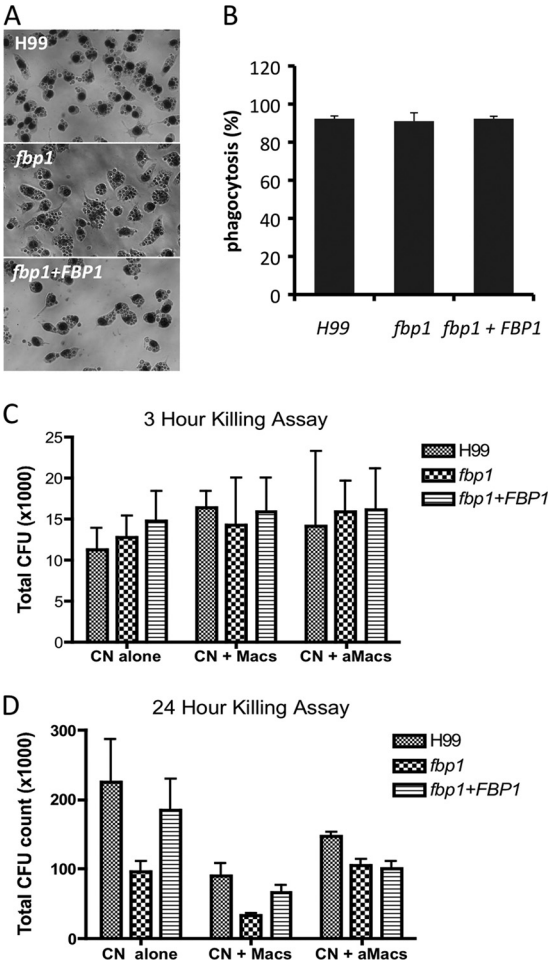

FIG. 6. Fbp1 is not important for *Cryptococcus*-macrophage interactions. (A) Typical field views of phagocytosis for each sample. (B) Phagocytosis assays were performed in 96-well plates using J774 macrophages. PBS-washed *C. neoformans* strain H99, an *fbp1* mutant (CUX2), and an *fbp1* mutant expressing *FBP1* (CUX5) were added to the macrophages and incubated for 2 h at 37°C in 10% CO_2. The percent phagocytosis was determined by dividing the number of macrophages that contained *C. neoformans* by the total number of macrophages counted. The error bars indicate standard deviations. (C and D) Phagocytosis was allowed to occur for 3 h (C) and 24 h (D) to measure both fungicidal and fungistatic activity. After each time interval, macrophages were lysed by adding sterile dH_2O. Cells were counted and plated on YPD agar plates to determine CFU. The assay was performed under three culture conditions. *C. neoformans* strains were incubated on medium without macrophages (CN only), with inactivated macrophages (CN + Macs), or with activated macrophages (CN + aMacs).

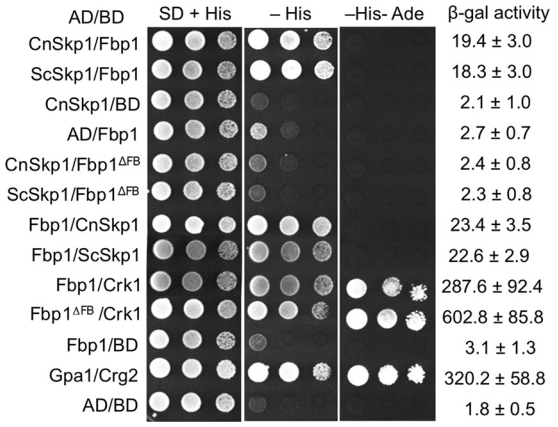

FIG. 7. Fbp1 interacts with both Skp1 homologues in *Cryptococcus* and *Saccharomyces* in a yeast two-hybrid interaction assay. The full-length cDNAs of *FBP1*, *CnSKP1*, and *ScSKP1* were fused with both the AD of vector pGADT7 and the BD of vector pGBKT7. The *FBP1* partial cDNA fragment lacking the F-box domain ($Fbp1^{\Delta FB}$) and *CRK1* cDNA was also fused with the BD. Both fusion constructs were introduced into the yeast host strain PJ69-4A, and colonies grown on SD medium without tryptophan and leucine were tested on media also lacking histidine and lacking both histidine and adenine. The expression of the *LacZ* gene was determined by measuring β-galactosidase enzyme activity. Yeast cells expressing pGADT7 and pGBKT7 empty vectors were used as negative controls, while yeast cells expressing Gpa1-AD and Crg2-BD fusion proteins were used as positive controls. The assay was repeated at least three times with similar outcomes.

SDS, further supporting the idea that the F-box domain is essential for the function of Fbp1.

Because trypan blue only stains cells with membrane defects or dead cells, wild-type H99, *fbp1* mutants, and strains expressing $Fbp1^{\Delta FB}$ were stained with trypan blue after being cultured at 30°C for 48 h in YPD liquid medium with shaking. Our results showed that while approximately 0.7% of H99 cells were heavily stained, over 12% of *fbp1* mutant cells and $Fbp1^{\Delta FB}$ cells were stained under the same conditions. These results further indicate that Fbp1 is important for maintaining cell membrane integrity. A defect of cell membrane integrity could be one reason for the virulence attenuation. It is still unclear how the Fbp1 protein regulates cell membrane integrity and whether damaged membrane integrity is the cause of virulence attenuation.

Fbp1 interacts with Skp1 and Ime2 homologues in a yeast two-hybrid protein-protein interaction assay. F-box proteins involved in SCF E3 ligase function regulate cellular processes mostly through substrate proteins. To examine whether Fbp1 is a subunit of an SCF E3 ubiquitin ligase complex, protein-protein interaction assays were performed between Skp1 in *S. cerevisiae* (ScSkp1) and Fbp1 in a yeast two-hybrid system. We have also identified a homologue of ScSkp1 in *C. neoformans*, CnSkp1 (CNAG_00829.2). Interactions between CnSkp1 and Fbp1 were also performed. Fbp1, ScSkp1, and CnSkp1 were fused with both the activation domain (AD) and the binding domain (BD). Constructs containing Skp1 homologues and Fbp1 were cotransformed into the yeast strain pJ69-4A, and interacting transformants were screened on SD dropout medium (SD-Trp-Leu-His and SD-Trp-Leu-His-Ade). The expression of the *LacZ* gene was determined by detecting the β-galactosidase enzyme activity. Our results showed that both ScSkp1 and CnSkp1 interacted with Fbp1 in a yeast two-hybrid protein-protein interaction system (Fig. 7). Deletion of the F-box domain in Fbp1 eliminated its interaction with either ScSkp1 or CnSkp1, indicating that the F-box domain is essential for these interactions.

Because Fbp1 is essential for sporulation, we searched for homologues of Grr1 substrates in *S. cerevisiae* that are involved in meiosis regulation. In *S. cerevisiae*, the serine/threonine kinase Ime2 is a key regulator of meiosis, and its degradation is

Grr1 dependent. We identified Crk1, a Cdk-related kinase that shares sequence homology with both Ime2 in *S. cerevisiae* and Crk1 in *Ustilago maydis* (22, 23). Crk1 interacts with Fbp1 in our yeast two-hybrid system and could potentially be a substrate of Fbp1 (Fig. 7). In our yeast two-hybrid protein-protein assays, besides the positive control, only colonies expressing both Fbp1 and Crk1 grew on SD-Trp-Leu-His-Ade medium, indicating a strong interaction between the two proteins. This strong interaction was also evident in the high β-galactosidase enzyme activities (Fig. 7).

DISCUSSION

SCF E3 ubiquitin ligase complexes are a key part of the ubiquitin-proteasome system (UPS) and play an essential role in a wide range of cellular processes in eukaryotes. Their importance in higher eukaryotes has been recognized following the recent discovery that F-box proteins are receptors of several important plant hormones (45) and human diseases caused by mutations in the SCF E3 ligase pathway (54). The therapeutic potential of the UPS pathway for human diseases has been actively evaluated and has resulted in the development of drugs, such as bortezomib, a proteasomal inhibitor that has been used to treat human neoplasias (44).

Fungi, particularly unicellular yeasts, have been proven to be valuable models for understanding fundamental developmental processes in eukaryotic cells, such as cell cycle control and nutrient sensing. Our study of Fbp1, a Grr1-like F-box protein in *C. neoformans*, is the first functional study of F-box proteins in basidiomycetes. Significantly, we have shown that Fbp1 is essential for fungal sporulation and virulence in *C. neoformans*. Since several homologues of Grr1 in other plant-pathogenic fungi have also been reported to play a role in fungal infectivity, the involvement of F-box proteins in virulence is likely conserved in fungal pathogens.

Cryptococcus Fbp1 shares sequence and structural similarity with Grr1 in *S. cerevisiae* and *C. albicans*. Both Grr1 proteins in the two yeasts are important for cell morphology, and *grr1* mutants produced pseudohyphae (10, 20). However, Fbp1 does not regulate cell morphology in *C. neoformans*, as *fbp1* mutants produced normal yeast cells when grown in liquid medium. The expression data of both *FBP1* and *CnHXT3* under glucose induction or depletion conditions also support the conclusion that Fbp1 and *Saccharomyces* Grr1 regulate glucose sensing differently. The downregulation of *CnHXT3* expression under glucose induction is different from what has been reported in *S. cerevisiae*. It is possible that *CnHXT3* is not a true ortholog of *ScHXT3*, even though they share the best sequence identity based on genome sequence analysis. In *S. cerevsiaie*, Grr1 positively regulates glucose sensing by targeting Mth1 and Std1 to dissociate the binding of Rgt1 with promoters of hexose transporters. Interestingly, no obvious sequence homologue can be identified for either Mth1, Std1, or Rgt1 in *C. neoformans* genomes. Such an outcome could be another indication that glucose-sensing and regulation systems are different in the two organisms.

Signaling pathways important for regulating classical virulence factors (polysaccharide capsule, melanin, and growth at body temperature) of *C. neoformans* have been identified (32, 33, 36, 58). Several pathways are well understood, such as the Gpa1-cyclic AMP (cAMP) pathway (17, 40, 60), the Hog1 mitogen-activated protein (MAP) kinase pathway (3), the protein kinase C (PKC) pathway (12, 24, 31), and the calcium-calcineurin pathway (38). It is interesting to observe that Fbp1 is essential for fungal virulence yet is not involved in regulation of any of these well-studied virulence factors, an indication that additional virulence-controlling mechanisms are involved. Fungal virulence is a complex trait, and additional mechanisms that control fungal virulence independent of these common virulence factors have been reported, such as the production of urease (13, 47) and phospholipase B (46) and sensing inositol (61). Whether the function of Fbp1 is related to any of these additional factors remains to be explored.

Although Fbp1 is not involved in the development of virulence factors, it is important for maintaining cell membrane integrity. Many enzymes important for either cell wall construction or cell wall maintenance have been found to be critical for maintaining cell integrity, such as α-1,3-glucan synthase (Ags1) (53); the chitinase gene family and chitin/polysaccharide deacetylases, which control chitosan biosynthesis (6); and the KRE family, which is involved in β-1,6-glucan synthesis (26). However, most of these genes are important for cell wall integrity, and strains carrying mutations in the genes are sensitive to not only SDS, but also calcofluor white and/or Congo red, as well as body temperature. The effect of Fbp1 on cell integrity is more specific to cell membranes, but not the cell wall, since *fbp1* mutants are sensitive only to SDS. It is possible that Fbp1 targets certain membrane proteins that are important for membrane integrity for degradation. It would be very interesting to identify such Fbp1 substrates.

Although *fbp1* mutants exhibited a clear defect in membrane integrity, our assays of *Cryptococcus*-macrophage interactions indicated that *fbp1* mutants had a response to phagocytosis similar to that of the wild type, H99. Thus, the cause of virulence attenuation of *fbp1* mutants in mice remains unclear. Whether the membrane integrity defect is the main cause of the avirulent phenotype *in vivo* remains to be explored. Examining the fungal load of *fbp1*-infected mouse organs revealed that no yeast cell was recovered in brain and spleen infected by *fbp1* mutants and only small amounts of *fbp1* cells were recovered in infected lung. Thus, it is possible that mutant cells cannot exit the macrophage after phagocytosis and thus fail to move from cell to cell to disseminate. Such a hypothesis could explain why only a limited number of mutant cells were recovered in the infected lung, because the initial inoculum could be trapped in the macrophage and not killed, and they might still replicate but could not exit to infect other macrophage cells. In addition, although the macrophage action represents an important host defense mechanism against *Cryptococcus* infection, results obtained from *in vitro* culture interaction tests cannot exclude the possibility that additional host factors in the hostile *in vivo* environment may prevent *fbp1* mutants from causing infection and thus exhibit the hypovirulent phenotype in the murine model.

It would be very interesting to identify Fbp1-interacting proteins that are responsive for virulence or sporulation. Over a dozen substrates have been identified for Grr1 in *S. cerevisiae* that are important for cell cycle regulation, glucose sensing, or amino acid sensing (7, 35). Crk1 is a Ser/Thr kinase and a homologue of the meiosis-specific regulator Ime2, and it inter-

acts with Fbp1 in our assays. Further functional study of Crk1 may promote our understanding of Fbp1-dependent sporulation in *C. neoformans.* Overall, our study demonstrated a potentially novel virulence control mechanism in *C. neoformans* that is independent of the classical virulence factors. Because the involvement of F-box proteins in virulence has also been reported in several plant-pathogenic fungi, the Fbp1-mediated virulence regulation mechanism is likely conserved in fungi.

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