Ubiquitin Degradation of the AICAR Transformylase/IMP Cy-Clohydrolase Ade16 Regulates the Sexual Reproduction of Cryptococcus neoformans

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Article

Ubiquitin Degradation of the AICAR Transformylase/IMP Cyclohydrolase Ade16 Regulates the Sexual Reproduction of *Cryptococcus neoformans*

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Abstract: F-box protein is a key protein of the SCF E3 ubiquitin ligase complex, responsible for substrate recognition and degradation through specific interactions. Previous studies have shown that F-box proteins play crucial roles in *Cryptococcus* sexual reproduction. However, the molecular mechanism by which F-box proteins regulate sexual reproduction in *C. neoformans* is unclear. In the study, we discovered the AICAR transformylase/IMP cyclohydrolase Ade16 as a substrate of Fbp1. Through protein interaction, protein stability, and *in vivo* ubiquitination experiments, we demonstrated that Ade16 is a substrate for Fbp1. To examine the role of *ADE16* in *C. neoformans*, we constructed the i*ADE16* strains and *ADE16*^{OE} strains to analyze the function of Ade16. Our results revealed that the i*ADE16* strains had a smaller capsule and showed growth defects under NaCl, while the *ADE16*^{OE} strains were sensitive to SDS but not to Congo red, which is consistent with the stress phenotype of the *fbp1*Δ strains, indicating that the intracellular protein expression level after *ADE16* overexpression was similar to that after *FBP1* deletion. Interestingly, although i*ADE16* strains can produce basidiospores normally, *ADE16*^{OE} strains can produce mating mycelia but not basidiospores after mating, which is consistent with the *fbp1*Δmutant strains, suggesting that Fbp1 is likely to regulate the sexual reproduction of *C. neoformans* through the modulation of Ade16. Fungal nuclei development assay showed that the nuclei of the *ADE16*^{OE} strains failed to fuse in the bilateral mating, indicating that Ade16 plays a crucial role in the regulation of meiosis during mating. In summary, our findings have revealed a new determinant factor involved in fungal development related to the post-translational regulation of AICAR transformylase/IMP cyclohydrolase.

Keywords: *Cryptococcus neoformans*; sexual reproduction; F-box protein Fbp1; AICAR transformylase/IMP cyclohydrolase; Ade16

1. Introduction

Cryptococcus neoformans is a yeast-like pathogenic fungus that is widely found in natural environments such as soil, plant surface, and bird feces [1]. The basidiospores or dried yeast cells of *C. neoformans* can be inhaled into the host's lungs through the respiratory tract and can be eliminated or in a state of latent infection in the immunocompetent hosts [2]. When the host's immunity is impaired, or the hosts are immunocompromised individuals, *C. neoformans* can proliferate in the lungs of the host and cause cryptococcal pneumonia, penetrating the blood-brain barrier (BBB) to cause deadly cryptococcal meningitis [3]. Statistically, 15% of the deaths related to acquired immune deficiency syndrome (AIDS) are caused by cryptococcal meningitis, and about 220,000 cases of cryptococcal meningitis are caused yearly [4].

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As a basidiomycete, *C. neoformans* has two mating types, α and **a**, which can reproduce sexually or asexually [5]. Typically, *C. neoformans* grows in the form of budding yeast and can switch from yeast growth to filamentous growth by mating and monokaryotic fruiting [6,7]. The transition of morphotypes from yeast to hyphae marks the beginning of the sexual development of *C. neoformans* [8]. During the mating of *C. neoformans*, the yeast cells of different matting types fused and formed dikaryotic mating hyphae, and eventually, a basidium, the specialized sporulation structure, was produced at the tip of the hyphae. Then after completing meiosis inside the basidium, four chains of basidiospores were generated at the top of the basidium [7,8]. Similar environmental factors also affect the monokaryotic fruiting of *C. neoformans* but happen between the haploid yeast cells of the same mating type, such as α cells [9,10]. Compared with mating, the hyphal cells produced by monokaryotic fruiting are mononucleate diploid cells with unfused clamp connections [9].

The sexual reproduction of *C. neoformans* was regulated by many environmental factors and genetic circuits. Environmental factors such as light, darkness, ambient temperature, nutrition deficiency, pheromone, and metal ions can regulate cell-cell fusion and mating hypha growth during the sexual reproduction of *C. neoformans* [6,10], and the mechanisms of these environmental factors affecting the sexual cycle are usually rooted in gene regulatory circuits. The genetic circuits, such as the Cpk1 MAPK pathway and the pheromone response pathway, also regulate the sexual reproduction of *C. neoformans*, as mutation of components in these pathways blocks the sexual reproduction of *C. neoformans* [6,11–14]. Besides these pheromone response pathways, other factors, such as zinc finger proteins, have also been identified as common regulators for sexual reproduction in *C. neoformans* [13,15,16]. Our previous studies showed that SCF E3 ligases play a vital role in the sexual reproduction of *C. neoformans*, as disruption of the key proteins of SCF E3 ligases such as Fbp1 and Cdc4 block the basidiospore production in *C. neoformans* [17,18]. However, the molecular mechanism by which the F-box protein regulates *C. neoformans* sexual reproduction remains unclear. Here, we identified an AICAR transformylase/IMP cyclohydrolase Ade16 and proved that Ade16 is a downstream target of Fbp1. Overexpression of the *ADE16* gene blocks the basidiospores formation by affecting the nuclei fusion of the meiosis process during mating. Our findings uncovered a new determinant of fungal development involving the post-translational regulation of an AICAR transformylase/IMP cyclohydrolase.

2. Materials and Methods

2.1. Strains and Growth Conditions

The wild-type strains of *C. neoformans*, H99 and KN99**a**, and their derived strains were preserved routinely in our laboratory and cultured on a YPD agar or liquid medium at 30°C. *C. neoformans* strains expressing the genes controlled by the *CTR4* promoter were cultured on a YPD medium supplemented with 200 μM bathoproproinediulonic acid (BCS) or 25 μM CuSO4 with 1 mM ascorbic acid [19]. The yeast strain used in the yeast two-hybrid assay was Y187, whose transformants were cultured on a synthetic defined medium without leucine, tryptophan, histidine, or adenine. The *C. neoformans* and yeast strains used in the study are shown in Table S1. All other media used in the study were prepared as described previously [17].

2.2. Quantitative Real-Time PCR

To detect the *ADE16* gene expression under different conditions, the quantitative real-time PCR (qRT-PCR) was used to measure the expression *ADE16* gene at mRNA levels as previously described [20]. Briefly, the yeast cells or mating mixtures of each cryptococcal strain were collected and cleaned with distilled water (dH_2O), and total RNA was extracted and purified using an Omega total RNA kit (Omega Bio-tek, USA). The purified RNAs was quantified using a Nanodrop spectrometer (DeNovix, USA), and the first-strand cDNA synthesis was synthesized with a Hifair® II 1st Strand cDNA Synthesis Kit (Yeasen, Shanghai, China), as described by the manufacturer. The analysis of the *ADE16* gene expression was performed using FastStart Essential DNA Green Master (Roche), and the gene expression level was normalized using the endogenous control gene *GAPDH*. The relative gene

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expression levels were measured using the previously described comparative threshold cycle (CT) method [21]. The qRT-PCRs were carried out using a LightCycler®96 QPCR system (Roche) as described previously [22].

2.3. Generation of Fluorescence Strains

To detect the *ADE16* gene expression in *C. neoformans* at different developmental stages, we amplified a 1996-bp *ADE16* gene promoter sequence using the genomic DNA of H99 as a template with primers TL1166/1167 (see Table 2 for primer sequences) and inserted it into the pTBL5 [16] plasmid to construct the plasmid pTBL196. Then the pTBL196 plasmid was linearized by *Sal*I, concentrated, and biolistically introduced into the wild-type strains, H99 and KN99**a**, respectively, as previously described [23]. Stable transformants were further screened by growing on YPD plates with nourseothricin sulfate (100 mg/L) and fluorescence examination under an Olympus inverted confocal microscope (Olympus, FV1200). Finally, two mating types of the P_{ADE16}-mCherry fluorescence fusion expression strains (α and **a**) were obtained and named TBL310 and TBL378, respectively.

To determine the Ade16 sub-cellular localization, we amplified the *ADE16* gene coding sequence from the H99 genomic DNA using primers TL1164/TL1165 and inserted it into the pCN19 vector to construct the GFP and *ADE16* fusion gene expression vector pTBL186. The *Xba*I-linearized pTBL186 was biolistically introduced into the H99 or KN99**a** strains, respectively. Stable transformants were further confirmed on YPD plates with nourseothricin sulfate (100 mg/L) and named TBL308 and TBL309, respectively. The fluorescence of the transformants was examined by confocal microscopy (Olympus, FV1200).

2.4. Yeast Two-Hybrid Assays

We first carried out a yeast two-hybrid interaction assay to detect the interaction between Ade16 and Fbp1 proteins as described previously [17,24]. The full-length cDNAs of *ADE16* and *FBP1* genes were amplified with primers TL879/880 and 588/589 and cloned into the bait vector pGBKT7 to fuse with the BD domain (pTBL106 and pTBL100), respectively. Meanwhile, the cDNAs of *ADE16* and *FBP1* were also amplified with primers TL883/884 and TL572/573 and inserted into the pGADT7 prey vector (pTBL145 and pTBL142), respectively, fusing with the AD domain. The inserted cDNAs were verified by sequencing to ensure the proteins were translated correctly. Both prey and bait constructs were co-transformed into the yeast strain Y187. After transformation, the yeast cells were transferred to an SD-Leu-Trp plate and incubated for 2-3 days at 30°C. The transformants growing on SD-Leu-Trp-His or SD-Leu-Trp-His-Ade plates were considered positive interactions. The transformants carrying pGADT7-T7/pGBKT7-LAM and pGADT7-T7/pGBKT7-53 served as negative and positive controls, respectively (Clontech, Dalian, China).

2.5. Generation of Tagged Protein Strains

To obtain *C. neoformans* strains expressing the Ade16-HA protein, we first amplified the *ADE16* cDNA with primers TL893/894. We then cloned it into the *Bam*HI sites of the vector pCTR4-2 [19] to generate the Ade16:HA fusion plasmid pTBL149 using the In-Fusion HD cloning kit (Clontech, Dalian, China). The *C. neoformans* strain (TBL81) expressing the Fbp1-Flag fusion proteins was generated by our previous studies [25]. To detect the interaction between Ade16 and Fbp1 in *C. neoformans*, we biolistically introduced the *EcoR*I-linearized pTBL149 into TBL81, the Fbp1-Flag strain, and the wild-type H99, respectively, generating the *C. neoformans* strains TBL248 and TBL264, which express Fbp1:Flag/Ade16:HA proteins and Ade16:HA proteins, respectively.

Total proteins were extracted and examined by Western blotting with anti-HA or anti-Flag antibodies to ensure the correct tagged strains were obtained. Protein pull-down was performed using SureBeads™ anti-HA or anti-Flag Magnetic Beads (Bio-RAD) as described previously [20] to collect the tagged proteins, and immunoblotting was used to detect the Ade16-HA or Fbp1-Flag proteins with anti-HA or anti-Flag antibodies, respectively.

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To detect the Ade16 stability in H99 and *fbp1*Δ mutant strains backgrounds, we introduced the *Eco*RI-linearized pTBL149 into the *fbp1*Δ mutant ballistically to construct the Ade16:HA strains TBL264 and TBL265. Then the yeast cells of TBL264 and TBL265 strains were grown in liquid YPD to mid-logarithmic phase, transferred to YPD containing 25 μM CuSO4 and 1 mM ascorbic acid, and incubated for an additional 1, 2, 4, and 6 h. Yeast cells were harvested, and protein extracts were prepared as described above. The signals of Ade16:HA was detected by Western blotting using a monoclonal anti-HA antibody (Sigma).

2.6. Ubiquitination assay of Ade16 in vivo

Besides the stability assay of the Ade16 in the backgrounds of the wild-type H99 and *fbp1*Δ mutant, we also investigated the ubiquitination of Ade16 in the above strain backgrounds. Strains H99, H99::P_{CTR4}-*ADE16:HA*(TBL264), and *fbp1*Δ::P_{CTR4}-*ADE16:HA* (TBL265) were inoculated into 50 mL YPD broth and grown overnight at 180 rpm at 30 °C. The yeast cells of the above three strains were harvested, washed, and lyophilized, and 1 ml of non-denaturing lysate and 1 microliter of deubiquitinase inhibitor b-AP15 were added for protein extraction. Then the supernatant of the yeast lysates was purified by EZviewTM Red ANTI-HA M2 Affinity Gel (Sigma). Meanwhile, the same amount of the H99 cells were added with 1 ml of Breakage Buffer for protein extraction. The protein samples obtained above were separated by SDS-PAGE gel and detected by Western blotting using Ubiquitin and HA antibodies, respectively.

2.7. Generation of ADE16 Interferences and Overexpression Strains

To generate the *ADE16* promoter replacement strain, we use a split marker strategy to replace the *ADE16* promoter with a copper-repressible *CTR4* promoter. In the first round of PCR, as shown in Figure 3A, the 5′ fragment, the *NEO* marker, and the P_{CTR4}-*ADE16* fusion fragment were amplified with primers TL1036/TL1037, TL17/TL18, and TL1034/TL1038 using the H99 genomic DNA, pJAF1, and pTBL149 as templates, respectively. In the next round of PCR, the fusion fragment of the 5′ fragment and *NEO* marker was amplified with primers TL1036/TL20 using the mixture of the 5′ fragment and *NEO* marker as templates. Similarly, using the mixture of the *NEO* marker and P_{CTR4}-*ADE16* fragment as templates, the fusion fragment of the *NEO* marker and the P_{CTR4}-*ADE16* fragment was amplified with primers TL19/TL1038. The above two fusion fragments were mixed, concentrated, and biolistically introduced into the H99 strain to construct the P_{CTR4}-*ADE16* strains TBL270. The TBL270 strain was further confirmed by PCR with primers TL373/TL59 and Southern blotting. To verify whether *ADE16* is an essential gene for *C. neoformans*, we dropped the H99 and TBL270 strain onto YPD, YPD+200 μM BCS, and YPD+25 μM CuSO4+1 mM ascorbic acid plate, respectively, after series dilution. The growth of each cryptococcal strain was observed after 2-3 days of incubation at 30 °C.

To generate the *ADE16 interferences* strains, we first constructed a plasmid P_{iADE16} for RNA interference of the *ADE16* gene in three steps. Step I, a 200-bp intron of the *ADE16* gene, was amplified using primers TL1334/1335 and inserted into the *Bam*HI/*Pac*I sites of pTBL5 to generate the plasmid pTBL226. Step II, a 480-bp *ADE16* 5′-3 fragment, the *ADE16* RNA interference sequence, was amplified using primers TL1336/1337 and inserted into the *Bam*HI site of pTBL226 to generate pTBL227. Step III, a reversed 480-bp *ADE16* RNA interference sequence was amplified using primers TL1467/1468 and inserted into the *Pac*I/*Spe*I sites of pTBL227 to generate pTBL237. After verification by sequencing, the SalI-linearized pTBL237 was biolistically introduced into the wild-type strains (H99 and KN99**a**). After PCR verification with primers TL1336/1340, the expression level of the *ADE16* gene was quantitated by quantitative real-time PCR in each transformant, and two mating types of *ADE16* interference strains were named TBL414 and TBL415, respectively.

To obtain the *ADE16* gene overexpression strain in *C. neoformans*, we first amplified the *ADE16* gene using primers TL1092/1093 and inserted it into the *Bam*HI site of the pTBL153 plasmid to generate pTBL174. Then the *Apa*I-linearized pTBL175 was biolistically introduced into the wild-type H99 and KN99**a** strains. Stable transformants were further confirmed on a YPD medium containing 100 mg/L nourseothricin sulfate. The *ADE16* gene overexpression (TBL288 and TBL302) was verified

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by qRT-PCR. To monitor the nuclei positioning in the *ADE16* overexpression strains during mating, pTBL59, a *NOP1-mCherry-NEO* cassette, was biolistically transformed into the *ADE16* overexpression strain of both α and **a** mating types to generate TBL445 and TBL446.

2.8. Analysis of Melanin and Capsule

To examine the role of Ade16 in *C. neoformans* melanin production, we grew the wild-type H99 strain and the Ade16-related strains in a YPD liquid medium overnight at 30 °C. One hundred microliters of each culture was dropped on the Niger seed medium to evaluate the melanin production by incubating the agar plates for 2-3 days at 30°C or 37°C. The pigmentation of each cryptococcal strain was evaluated and photographed.

To assess the role of Ade16 in *C. neoformans* capsule production, overnight cultures of each cryptococcal strain were washed with 1×PBS buffer three times and incubated overnight in diluted Sabouraud medium (DSM) or MM with mannitol at 37°C [26,27]. The quantification of the capsule size was performed as previously described [17].

2.9. Mating assay

To investigate the role of Ade16 in *C. neoformans* mating, equal amounts of the α and **a** mating type yeast cells were mixed and grown on MS or V8 agar plates in the dark at 25 °C. After 14 days of incubation, the formation of mating hyphae and basidiospores was visualized and recorded using an Olympus CX41 light microscope.

3. Results

3.1. Identification of the ATIC enzyme Ade16

Our previous study revealed that the F-box protein Fbp1, a critical protein of the E3 ligases, plays a crucial role in *C. neoformans* sexual reproduction, as in bilateral mating of *fbp1*Δ mutants, the basidiospore production was blocked despite the observation of normal dikaryotic hyphae during mating. To determine the downstream targets of Fbp1 in *C. neoformans*, we conducted an iTRAQ analysis coupled with LC-MS/MS in a previous study to investigate the enriched proteins in *fbp1*Δ mutants [20]. The partially enriched proteins are shown in Table 1 and have been selected as potential target candidates for further study.

Table 1. The partial enriched proteins identified in the *fbp1*Δ mutant of *C. neoformans*.

Accession	Description	Average *fbp1*Δ/H99	No. of PEST Domain
CNAG_05514	Uncharacterized protein	1.899038	0
CNAG_01974	Ribosomal protein	1.631707	2
CNAG_06195	Uncharacterized protein	1.595274	0
CNAG_00700	ATIC Ade16	1.544354	0
CNAG_05498	Uncharacterized protein	1.541769	1
CNAG_02860	Endo-1,3(4)-beta-glucanase	1.522101	2
CNAG_01019	Superoxide dismutase	1.470508	0
CNAG_02344	Uracil phosphoribosyltransferase	1.431079	1
CNAG_02738	Uncharacterized protein	1.400934	0
CNAG_01109	Uncharacterized protein	1.384226	1

One of the candidate genes, CNAG_00700, was highly enriched in *fbp1*Δ mutants and its encoded protein is annotated as AICAR transformylase/IMP cyclohydrolase. Annotation information from the FungiDB database [28] showed that the CNAG_00700 gene is 2396-bp long and contains five exons

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that encode a protein with 605 amino acids (Figure 1A). The sequence analysis revealed that the protein encoded by CNAG_00700 contains two domains, one is the MGS (methylglyoxal synthase) domain, and the other is the AICARFT_IMPCHas (AICARFT/IMPCHase bienzyme) domain (Figure 1B). Sequence blast analysis showed that the CNAG_00700-encoded protein has 69% and 65% sequence identities with the ATIC enzymes Ade17 and Ade16 in *Saccharomyces cerevisiae*, respectively (Figure 1B and Table 2). Sequence blast analysis also showed that Ade16 shows high sequence similarity to its homologies in ascomycetes and basidiomycetes (Table 2). Recently, Wizrah et al. identified the CNAG_00700 gene in *C. neoformans* and named it *ADE16* [29]. Therefore, we will follow their name and name CNAG_00700 *ADE16*.

Table 2. Homologs of *C. neoformans* Ade16 in other species.

Organism	Protein	Query cover	Expect	Identities	Positives
Saccharomyces cerevisiae	ATIC Ade17	91%	0	69%	78%
Saccharomyces cerevisiae	ATIC Ade16	92%	0	65%	77%
Schizosaccharomyces pombe	ATIC	91%	0	69%	78%
Ustilago maydis	ATIC Ade17	100%	0	67%	78%
Candida albicans	ATIC Ade17	98%	0	67%	78%
Candida auris	bifunctional purine biosynthesis protein Ade17	98%	0	67%	78%
Mus musculus	bifunctional purine biosynthesis protein ATIC	92%	0	62%	75%
Homo sapiens	bifunctional purine biosynthesis protein ATIC	91%	0	62%	75%

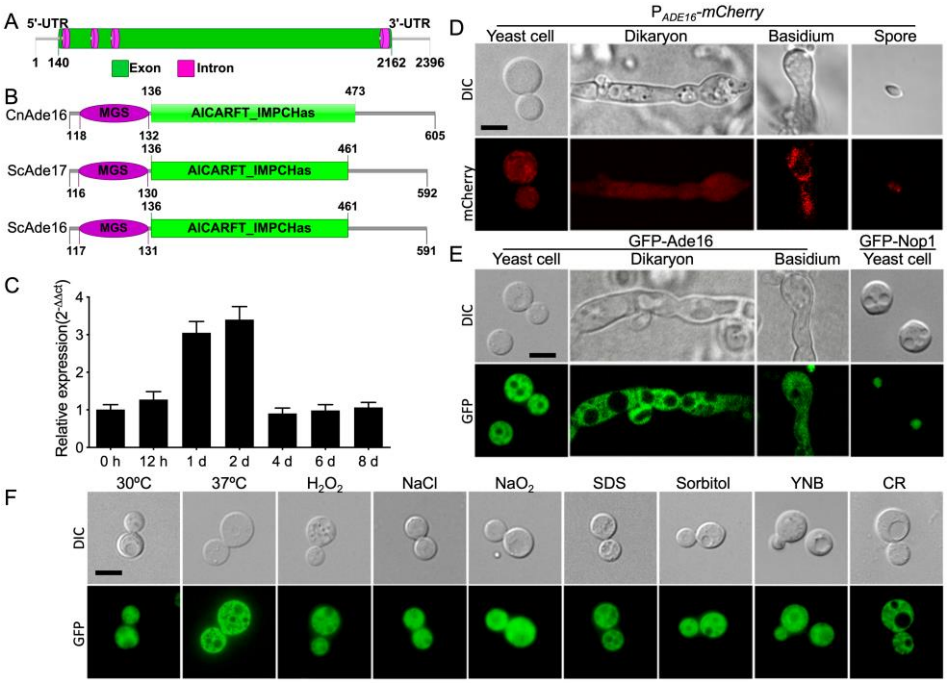

Figure 1. Identification of *C. neoformans* Ade16. Schematic illustration of the *ADE16* gene (**A**) and the Ade16 protein (**B**) in *C. neoformans*. MGS: methylglyoxal synthase domain; AICARFT_IMPCHas: AICARFT/IMPCHase bienzyme domain. (**C**) The *ADE16* expression during bilateral mating on the V8 medium was qualified by qRT-PCR. The error bars represent the standard deviations of three

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independent repeats. Expression of mCherry (**D**) under the control of *ADE16* promoter and subcellular localization of the GFP-Ade16 fusion protein (**E**) in *Cryptoccus* different development stages. Representative fluorescence and bright-field images of the yeast cells, dikaryons, basidia, and basidiospores are shown. The nuclear localization of GFP-Nop1 was used as a positive control. Bar, 5 μm. (**F**) Subcellular localization of the GFP-Ade16 fusion protein in yeast cells of *C. neoformans* under different stressors. DIC, differential interference contrast; Bar, 5 μm.

To investigate the function of *C. neoformans* Ade16 during mating, we first detected the *ADE16* gene expression H99 and KN99**a** strains using qRT-PCR. Our results revealed that the expression of *ADE16* was significantly upregulated during the mating process, reached the highest value at the 2-d time point, and then decreased and remained comparable to the expression at the 0-h timepoint, suggesting that Ade16 may play a role in sexual reproduction of *C. neoformans* (Figure 1C).

To more intuitively observe the *ADE16* expression in *C. neoformans* at different developmental stages, we generated fluorescence strains (TBL310 and TBL378, see Table S2) expressing mCherry under the control of the native promoter of the *ADE16* gene in both mating types. The mCherry strains (TBL310 and TBL378) were crossed on MS medium, and the fluorescence at different developmental stages of mCherry was visualized by a confocal microscope (Olympus, FV1200). Our results revealed that the mCherry was expressed in yeast cells, dikaryons, basidia, and basidiospores (Figure 1D), suggesting that Ade16 may play a key role in the sexual reproduction of *C. neoformans*.

To detect the Ade16 localization in *C. neoformans*, the *Xba*I-linearized *GFP-ADE16* plasmid was biolistically introduced into the wild-type H99 and KN99**a** strains, and stable transformants were named TBL308 and TBL309, respectively, after examination with a fluorescence microscope. In contrast to the nuclear localization of GFP-Nop1(TBL84), the GFP-Ade16 fusion protein is localized in the cytoplasm of *C. neoformans* yeast cells and dikaryotic hyphae except for the vacuoles (Figure 1D). Meanwhile, the subcellular localization of GFP-Ade16 in yeast cells was also examined under different stress conditions, and the results showed no difference between the GFP-Zfp1 localizations under the above-tested stress conditions (Figure 1E).

3.2. Ade16 Interacts With Fbp1

Our previous proteomics data suggested that Ade16 is enriched in *fbp1*Δ mutants and might be a target for Fbp1-mediated ubiquitination (Table 1). we first conducted a yeast two-hybrid protein-protein interaction assay to explore whether there is an interaction between Ade16 and Fbp1. Expression vectors containing Ade16 and Fbp1 by fusion with the binding domain (BD) or the activation domain (AD) were co-transformed into the Y187 yeast strain, and transformants growing on SD-Leu-Trp-His or SD-Leu-Trp-His-Ade media were considered positive interactions. Our results showed that Ade16 interacts with Fbp1 in a yeast two-hybrid protein-protein interaction system (Figure 2A).

To further investigate the Ade16-Fbp1 interaction, we generated an Ade16:HA fusion expression vector (pTBL149) by cloning the *ADE16* gene into the pCTR4-2 plasmid, which the expression of *ADE16* was regulated by the copper repressible CTR4 promoter [19]. The resulting construct, pTBL149, linearized by *Eco*RI, was biolistically transformed into H99, the *fbp1*Δ mutants, and a previously constructed Fbp1:Flag overexpression strain[25], respectively. The expression of Ade16:HA fusion proteins was confirmed by Western blot (Figure 2B). The total proteins from strains expressing Ade17:HA and Fbp1:Flag were purified with anti-HA Magnetic Beads (Bio-RAD), and Western blotted with anti-HA and anti-Flag antibodies. Both HA and Flag signals were detected from the co-immunoprecipitation (Co-IP) products, indicating that Ade17 interacts with Fbp1 in *C. neoformans* (Figure 2C).

3.3. The Ade16 Stability Depends on Fbp1 Function

To test our hypothesis that Ade16 is an Fbp1 downstream target, we investigated whether the Ade16 protein stability depends on the function of the SCF(Fbp1) E3 ligase. The Ade16:HA protein was expressed in the wild-type H99 strain (TBL264) and the *fbp1*Δ mutants (TBL265), and the Ade16

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protein stability was examined in the backgrounds of the above two strains. The Ade16:HA expressing strains were first cultured in a YPD medium containing 200 μM BCS to induce the *CTR4* promoter. Then the cultures were washed with PBS and transferred to a YPD medium containing 1 mM ascorbic acid and 25 μM CuSO4 to block the transcription of *ADE16:HA*. The yeast cells were harvested after incubation for another 0, 1, 2, 4, and 6 h, and the abundance of the Ade16:HA protein was evaluated by Western blot. Our results of the protein stability assay revealed that the Ade16:HA protein was hydrolyzed in the wild-type H99 background in a time-dependent manner during the examined period (0 to 6 h) but was relatively stable in the *fbp1*Δ mutants, suggesting that the Ade16:HA stability is dependent on the function of Fbp1 (Figures 2D).

Next, to detect the Ade16-HA polyubiquitination, we extracted and purified the Ade16-HA proteins from the H99 or *fbp1*Δ mutants using anti-HA antibodies and examined them by Western blotting using the ubiquitin antibody. As shown in Figure 2E, the polyubiquitination of Ade16-HA in *fbp1*Δ mutants decreased compared to that of the H99 strain, suggesting that the normal polyubiquitination of Ade16 depends on the Fbp1 function in *C. neoformans*.

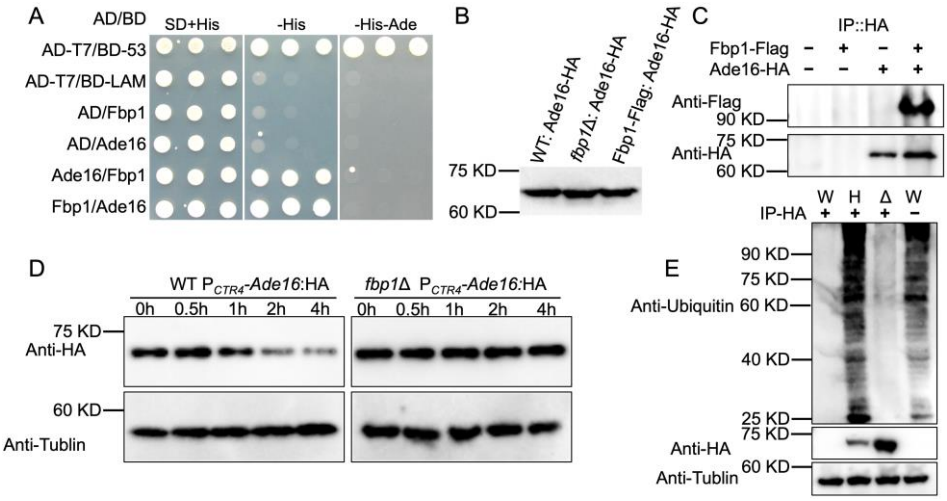

Figure 2. Ade16 interacts with Fbp1 and is a downstream target of Fbp1. (**A**) Ade16 interacts with Fbp1 in a yeast two-hybrid assay. The *ADE16* and *FBP1* full-length cDNAs were fused with the activation domain (AD) of the pGADT7 vector or the binding domain (BD) of the pGBKT7 vector. The two fusion vectors were transformed into the Y187 yeast host strain, and yeast colonies that grew on SD-Leu-Trp medium were further tested on SD-Leu-Trp-His and SD-Leu-Trp-His-Ade media. Yeast cells expressing AD-T7 and BD-53 fusion proteins were used as the positive controls, while that expressing AD-T7 and BD-LAM were used as the negative controls. (**B**) The expression of the Ade16-HA fusion protein in the wild-type H99, *fbp1*Δ mutants, or the Fbp1-Flag strains (TBL264, TBL265, and TBL248) was confirmed by Western blotting with anti-HA antibodies. (**C**) Ade16 interacts with Fbp1 in a Co-IP assay. Proteins from *C. neoformans* strains expressing Fbp1-Flag (TBL81), Ade16-HA (TBL270), or both Ade16-HA and Fbp1-Flag (TBL248) were extracted and purified. The potential interaction between Ade16 and Fbp1 was analyzed by Co-IP using anti-Flag (top panel) or anti-HA antibodies (bottom panel) and examined by Western blotting. (**D**) The Ade16 stability depends on the Fbp1 function. The *PCTR4-ADE16:HA* construct (pTBL149) was expressed in H99 (TBL264) or *fbp1*Δ mutants (TBL265). Yeast cells of TBL264 and TBL265 growing to the mid-logarithmic phase in YPD were collected at the indicated times after CuSO4 addition to stopping *ADE16* transcription, and the Ade16 abundance was detected by Western blotting with HA antibody. Tubulin was used as a loading control. (**E**) Detection of the Ade16 polyubiquitination. Proteins from the yeast cells of the wild-type H99 (W), the wild-type H99 expressing (H), or the *fbp1*Δ mutants (Δ) expressing Ade16-HA were extracted and purified with anti-HA antibodies. The polyubiquitination of Ade16-HA was examined by Western blotting using an anti-ubiquitin antibody. Western blotting with HA antibody also confirmed the Ade16-HA expression in the wild-type H99 or *fbp1*Δ mutants. The expression of the tubulin gene was used as a loading control.

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3.4. ADE16 is Essential for the Growth of C. neoformans

To investigate the role of Ade16 in *C. neoformans*, we tried to knock out the *ADE16* gene using a split-marker strategy as previously described [16]. However, we failed to obtain the *ADE16* gene knockout mutants after repeated attempts, and we speculated that the *ADE16* might be an essential gene and could not be disrupted in *C. neoformans*. Therefore, we decided to substitute the native promoter of the *ADE16* gene with the copper-ion-suppressing *CTR4* promoter to construct a P_{CTR4}-*ADE16* strain (TBL270) to verify whether the *ADE16* gene is an essential gene for *C. neoformans*. The strategy of the P_{CTR4}-*ADE16* strain construction is shown in Figure 3A.

Overnight cultures of the wild-type H99 strain and P_{CTR4}-*ADE16* strain (TBL270) were dropped on YPD, YPD with 200 μM BCS, and YPD with 25 μM CuSO4 and 1 mM ascorbic acid, and incubated at 30°C for 2-3 days after series dilution. Our results showed that the growth of the P_{CTR4}-*ADE16* strain was comparable to that of the wild-type strain when grown on YPD or YPD with 200 μM BCS. However, when grown on YPD with 25 μM CuSO4 and 1 mM ascorbic acid, the P_{CTR4}-*ADE16* strain almost lost its growth ability, while the growth capacity of the wild-type strain was not affected (Figure 3B), indicating that *ADE16* is essential for the growth of *C. neoformans* on YPD.

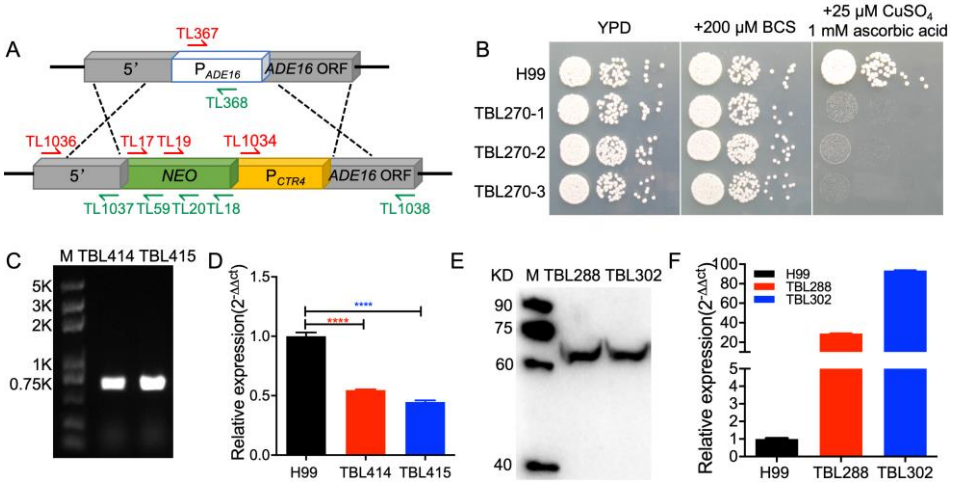

Figure 3. Generation of the ADE16 interference strains and overexpression strains in C. neoformans. (**A**) The strategy of the ADE16 native promoter substitution. 5', ~1kb DNA fragment upstream of ADE16 gene promoter; P_{ADE16}, ADE16 native promoter; ADE16 ORF, ADE16 open reading frame; NEO, NEO marker gene; P_{CTR4}, the copper-ion-suppressing CTR4 promoter. (**B**) The growth of the P_{CTR4}-ADE16 strains on YPD with 200 μM BCS and YPD with 25 μM CuSO4 and 1 mM ascorbic acid. Yeast cells grown overnight of the P_{CTR4}-ADE16 strains were diluted to an OD_{600} value of 2.0, followed by ten-fold dilutions. 5-μL of each diluent was plated on YPD agar plates with different stressors and incubated at 30°C for 2 days. The C. neoformans strains are indicated on the left, and the incubation conditions are on the top. (**C**) PCR verification of the iADE16 interference strains (TBL414 and TBL415) with primers TL1336/1340. (**D**) Determination of the interference efficiency of the ADE16 gene by qRT-PCR. The ADE16 gene expression levels in the H99 strain grown on YPD were set as 1, and the GAPDH gene expression was used as an internal control. The data shown are the mean ± standard deviation from three repeats. ****, $P < 0.0001$. (**E**) The expression of the Ade16-HA fusion protein in $ADE16^{OE}$ overexpression strains (TBL288 and TBL302) was confirmed by Western blotting. (**F**) Overexpression of ADE16-HA TBL288 and TBL302 was also measured by relative qRT-PCR analysis. ****, $P < 0.0001$.

3.5. Ade16 Regulates Capsule Formation in C. neoformans

To investigate the role of *ADE16* in *C. neoformans*, we constructed an *ADE16* RNA interference vector (pTBL237, see materials and methods section for information) and introduced it into the *C. neoformans* wild-type strains to obtain the i*ADE16* interference strains (TBL414 and TBL415) in both

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α and a mating type. The construction of the *ADE16* interference strains and the *ADE16* gene interference efficiency was verified by PCR and qRT-PCR, respectively (Figure 2C,D). Meanwhile, the *ADE16*^{OE} overexpression strains (TBL288 and TBL302) were also constructed by introducing the *ADE16* ^{OE} overexpression vector pTBL237 into the H99 and KN99**a** strains. The overexpression of *ADE16* in both mating types was confirmed by Western blot and qRT-PCR (Figure 3E,F).

To test the role of Ade16 in *C. neoformans* virulence, we first examined the virulence factor production of i*ADE16 and ADE16*^{OE} strains *in vitro*. Interestingly, RNA interference of the *ADE16* gene led to a significant decrease in capsule size, while overexpression of it led to a significant increase in capsule production in *C. neoformans* (Figure 4A,B). The average relative size of the capsule produced by the i*ADE16* strain or the *ADE16*^{OE} strain was reduced by 35% or increased by 111%, respectively, compared with the H99 strain or *fbp1*Δ mutants (Figure 4B). This decrease or increase in capsule production is statistically significant ($P < 0.0001$) based on a Student's t-test, indicating that Ade16 plays an important role in *C. neoformans* capsule production. We also examined the melanin formation of each *C. neoformans* strain and found that neither *ADE16* interference nor overexpression affected melanin formation in *C. neoformans*.

Meanwhile, we also investigated the growth of the i*ADE16* strain and the *ADE16*^{OE} strain under different stress conditions. The results showed that the i*ADE16* strain was sensitive to 1.5M NaCl but not sensitive to cell integrity-targeting chemicals, such as SDS and Congo red, suggesting that Ade16 may play a role in maintaining the osmotic pressure in *C. neoformans* cells (Figure 4C).

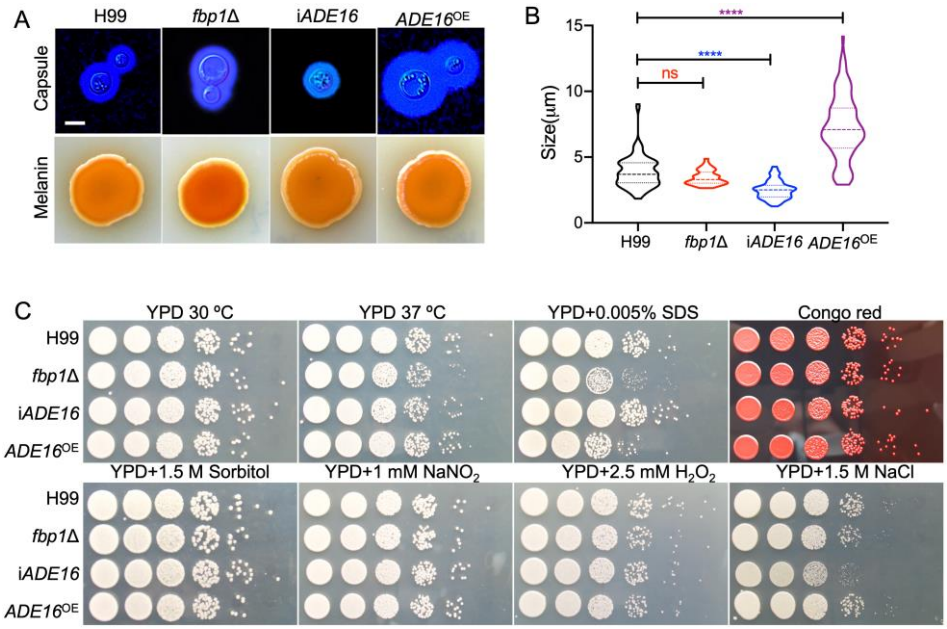

Figure 4. Ade16 is involved in the formation of Cryptococcus capsules. (**A**) Capsule formation and melanin production of Ade16-related strains. Melanin production by the C. neoformans strains was induced on Niger seed plates and photographed after incubation at 37°C for two days. Capsule formation was examined in a diluted SAB medium. Each C. neoformans strain was grown in a diluted SAB medium for 24 hours at 37°C, and the capsule formation was examined by India ink staining. Bars=5 μm. (**B**) Statistical analysis of the capsule formation in each C. neoformans strain in diluted SAB medium. The capsule sizes of more than 100 yeast cells were measured, and the data shown were the mean and standard deviation of the three replicates. ns: not significant; ****, $P < 0.0001$. (**C**) The growth of the C. neoformans strains under different stress conditions. Yeast cells grown overnight were diluted to an OD_{600} value of 2.0, followed by ten-fold dilutions. 5-μL of each diluent was plated on YPD agar plates with different stressors and incubated at 30°C for 2 days. The C. neoformans strains are indicated on the left, and the incubation conditions are on the top.

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3.6. Ade16 Regulates sexual reproduction of C. neoformans

Our previous study showed that knockout of the *FBP1* gene blocked the basidiospore production in bilateral mating between *fbp1*Δ mutants. Since Ade16 is the downstream substrate of Fbp1, we speculated that Fbp1 might affect *Cryptococcus* sexual reproduction by regulating Ade16. To investigate the role of Ade16 in fungal mating, we constructed the i*ADE16* interference strains and *ADE16*^{OE} overexpression strains in both H99 and KN99**a** strain backgrounds. The development of dikaryotic hyphae and basidiospores was examined in bilateral mating in i*ADE16* interference strains or *ADE16*^{OE} overexpression strains. As with the wild-type strains, the i*ADE16* interference strains generated normal mating hyphae and basidiospores (Figure 5). However, the bilateral mating between *ADE16*^{OE} overexpression strains generated normal mating hyphae but failed to produce spores, consisting with the phenotype of the *fbp1*Δ mutants (Figure 6), suggesting Fbp1 may regulate the sexual reproduction by regulating the Ade16.

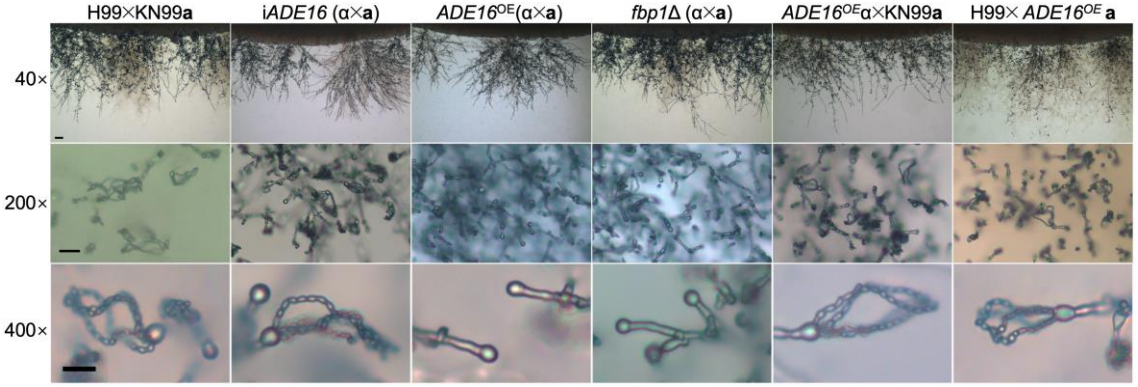

Figure 5. Ade16 is involved in the sexual reproduction of C. neoformans. The wild types, iADE16 interference strains, and ADE16^{OE} strains were crossed on MS medium for bilateral or unilateral mating assays. Mating hyphae and basidiospores at ×40 magnification (top panel, bar = 100 μm), ×200 magnification (middle panel, bar = 50 μm), and ×400 magnification (bottom panel, bar = 10 μm) were imaged after 2 weeks of incubation at 25°C in the dark.

To explore the mechanism of the failure of *ADE16*^{OE} overexpression strains to produce basidiospores, we constructed a nuclear-located Nop1-mCherry fusion expression vector. We introduced it into both α and **a** mating types of the wild type and *ADE16*^{OE} overexpression strains to monitor the fungal nuclei positioning at different stages of mating in *C. neoformans*. The wild types (TBL101 and TBL102) or the *ADE16*^{OE} overexpression strains (TBL445 and TBL446) expressing the Nop1-mCherry were crossed, respectively, and their nuclear positioning was monitored during the mating. As shown in Figure 7A, a single nucleus was visualized in the yeast cells of both the wild type and the *ADE16*^{OE} overexpression strain, while two separated nuclei were found in each dikaryon produced after cell fusion. During mating, the wild-type strains had a single fused nucleus in the young basidium and four nuclei in each mature basidium, consistent with our previous findings [16]. However, the nuclei of the *ADE16*^{OE} overexpression strains failed to fuse in the bilateral mating, and only two separated nuclei were observed in both the young and the mature basidia (Figure 6A,B). These findings suggested that Ade16 regulates meiosis during mating, which may help explain why the *ADE16*^{OE} overexpression strains could not produce basidiospores when crossed with a partner in bilateral mating. However, when grown in rich media, the growth rate and nuclear division of the *ADE16*^{OE} overexpression strains were normal, suggesting that Ade16 is not involved in the cell cycle during mitosis.

Taken together, we identified a downstream substrate of Fbp1, the AICAR transformylase/IMP cyclohydrolase Ade16, and found that Fbp1 regulates the sexual reproduction of *C. neoformans* by regulating ubiquitination degradation of its downstream substrate, Ade16.

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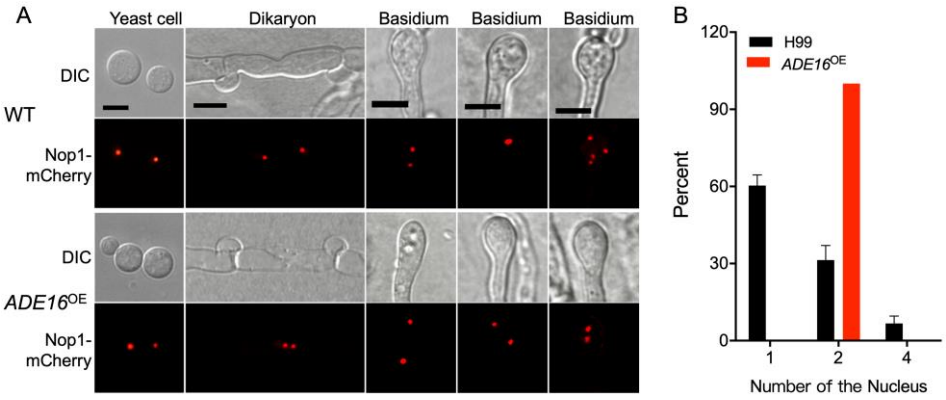

Figure 6. Fungal nuclei development in ADE16^{OE} overexpression strains. (**A**) Yeast cells, dikaryons, and basidia of the wild types or ADE16^{OE} overexpression strains were isolated and observed by a confocal microscope after incubation on MS medium for 1 or 2 weeks in the dark at 25 °C. DIC, differential interference contrast; WT, wild-type strains H99 and KN99**a**. Bars=5 μm. (**B**) Statistical results of nuclei in basidium of wild types or ADE16^{OE} overexpression strains.

4. Discussion

The F-box proteins are the critical component of the SCF E3 ligases and play a vital role in fungal virulence and development. Our previous studies have shown that the F-box protein Fbp1 is required for the sexual reproduction of the human fungal pathogen *C. neoformans*, as the basidiospore formation is blocked in bilateral mating of the *fbp1*Δ mutants [17]. However, the mechanism by which Fbp1 regulates the sexual reproduction of *C. neoformans* remains unclear. To identify the potential downstream targets of Fbp1, we carried out an iTRAQ analysis coupled with LC-MS/MS in a previous study to investigate the enriched target proteins in *fbp1*Δ mutants [20]. In this study, we identified the AICAR transformylase/IMP cyclohydrolase Ade16 and demonstrated that Ade16 interacts directly with Fbp1, and the degradation and ubiquitination of Ade16 depend on the function of Fbp1. The gene expression analysis revealed that the *ADE16* gene was expressed at all developmental stages, and the Ade16 protein was localized in the cytoplasm of *C. neoformans* cells. RNA interference of the *ADE16* gene led to a significant decrease in capsule size, while overexpression led to a significant increase in capsule size in *C. neoformans*. Overexpression of *ADE16* resulted in the production of normal mating hyphae but a failure of basidiospore formation, which is consistent with the phenotype of the *fbp1*Δ mutants (Figure 6), suggesting Fbp1 may regulate the sexual reproduction by regulating the Ade16. Our findings suggest that the SCF(Fbp1) E3 ligase-mediated UPS pathway regulates *C. neoformans* sexual reproduction by regulating the ATIC Ade16.

5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (AICARFT/IMPCHase, ATIC) is a bienzyme catalyzing the last two steps of the purine *de novo* biosynthesis pathway [30]. In *S. cerevisiae*, the ATIC isozyme is encoded by two genes, *ADE16* on chromosome XII and *ADE17* on chromosome XIII, respectively [31]. The amino acid sequences of the two genes are 84% identical to each other, which is 60%-63% identical to the chicken and human bifunctional AICAR transformylase/IMP cyclohydrolase [31]. Sequence blast analysis revealed that the *C. neoformans* CNAG_00700 has 69% and 65% sequence identities with the Ade17p and Ade16p in *S. cerevisiae*, respectively (Figure 1B and Table 2). However, the CNAG_00700 has been identified and named *ADE16* by Wizrah et al.; therefore, we will follow their name and name CNAG_00700 *ADE16*.

We first analyzed the *ADE16* gene expression and found that the *ADE16* was expressed at all development stages of *C. neoformans*, and the Ade16 protein was localized in the cytoplasm of *C. neoformans* cells, consisting with the Ade17p localization in *S. cerevisiae* [32]. Since Ade16 was highly enriched in the *fbp1*Δ mutants and could be a downstream target of Fbp1, we then performed the protein interaction, stability, and ubiquitination assays and found that Ade16 interacts with Fbp1 and its stability and ubiquitination is dependent on the function of Fbp1. These findings suggest that the

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SCF(Fbp1) E3 ligase-mediated UPS pathway might regulate the purine *de novo* biosynthesis pathway in *C. neoformans* by regulating the ATIC Ade16. So far, to our knowledge, there have been no reports on the regulation of Ade16 protein degradation, so our findings may reveal a new regulatory pathway for Ade16 protein degradation.

Given the importance of the Ade16 protein in the purine *de novo* biosynthesis, we attempted to knock out the *ADE16* gene in *C. neoformans*. However, we tried several rounds of transformation, screened hundreds of transformants, and still failed to get the *ADE16* gene knockout mutants. Later, we adopted the strategy of replacing the native promoter of the *ADE16* gene with a copper-repressible *CTR4* promoter, examined the growth of the transformants on the medium containing $CuSO_4$, and found that the P_{CTR4}-*ADE*16 strains showed great growth defect (Figure 2B). However, in *S. cerevisiae*, the null mutants of *ADE17* or the paralog *ADE16* are viable [31]. Disruption of both *S. cerevisiae ADE17* and *ADE16* led to adenine auxotrophy, while the expression of either gene alone was sufficient to support growth without adenine [31], indicating that the two genes were redundant in function. Why do we try to knock out the *ADE16* gene in *C. neoformans* but fail to get the *ADE16* knockout transformants? Could only one *ADE16* be present in *C. neoformans*, unlike *S. cerevisiae*, which has an Ade16 paralog, Ade17? To answer this question, we used the *C. neoformans* Ade16 sequence as a query and did a BLASTp search against the genome database of *C. neoformans* in FungiDB [28]. The results showed that the protein with the highest identity to Ade16 was CNAG_07373, a large subunit of the Carbamoyl-phosphate synthase with a sequence identity of 25% with Ade16, indicating that there is no paralog of Ade16 in *C. neoformans* genome. Thus, disruption of the *ADE16* gene resulted in adenine auxotrophy in *C. neoformans,* and the *ade16*Δ mutants could not grow on the YPD medium without additional adenine and histidine, which led to the mistaken belief that the *ADE16* gene was essential and could not be knocked out. This is confirmed by the growth of *ade16*Δ mutants obtained by Wizrah et al. [29] on YPD, consistent with our strain P_{CTR4}-ADE16 strains on YPD containing $CuSO_4$.

The polysaccharide capsule is a virulence factor that plays a key role in the interaction between *C. neoformans* cells and the immune system, protecting *C. neoformans* cells from being phagocytosis by immune cells [33].In this study, we found that the *ADE16* interference would decrease the capsule size of *C. neoformans*, while the *ADE17* gene overexpression would lead to the enlargement of the capsule, indicating that the formation of *C. neoformans* capsule is related to the expression level of *ADE17*. In the Wizrah et al. study, the *ade16*Δ strain also displayed reduced capsule size at 30 °C and 37 °C [29]. The results from both groups suggested that Ade16 plays an important role in the capsule formation of *C. neoformans*.

Sexual reproduction allows genetic material from two parents to recombine, resulting in recombinant offspring with the potential for variable adaptation, and allows natural selection to more effectively remove harmful mutations that accumulate in the parental genomes [34]. In *C. neoformans*, sexual reproduction links to virulence, antifungal drug resistance, and rapid adaptive evolution [35,36]. Many environmental factors and genetic circuits regulate sexual reproduction in *C. neoformans* [10]. Our previous study showed that the SCF(Fbp1) E3 ligase-mediated UPS regulates sexual reproduction in *C. neoformans* [17]. However, the molecular mechanism by which Fbp1 regulates sexual reproduction in *C. neoformans* remains unclear. In this study, we identified the AICAR transformylase/IMP cyclohydrolase (ATIC) Ade16 as a substrate of Fbp1 and demonstrated that Ade16 is involved in the sexual reproduction of *C. neoformans*. As a bienzyme catalyzing the last two steps of the purine *de novo* biosynthesis pathway, Ade16 is involved in the sexual reproductive process of *C. neoformans*, which is truly amazing. Until now, why and how Ade16 is involved in the sexual reproductive process of *C. neoformans* is unknown. There are few studies on Ade16 in fungi, mainly in *S. cerevisiae* [31,32,37,38]. In *C. neoformans*, the Ade16 was identified by Wizrah et al. and proved to be essential for *de novo* purine biosynthesis [29]. The *ADE16* gene was only mentioned in research on a new high-throughput screening procedure for the detection of sporulation defects in 624 non-lethal homozygous deletion mutants created by the European Joint research project EUROFAN [39]. So far, the mechanism of Ade16's involvement in the regulation of fungal sexual reproduction remains unclear, which is, of course, what we will tackle in the future of this study.

In conclusion, our study discovered a novel substrate of Fbp1 in *C. neoformans* and unveiled a novel sexual reproduction regulatory pathway involving the SCF(Fbp1) E3 ligase-mediated UPS and its regulation of the AICAR transformylase/IMP cyclohydrolase in *C. neoformans*. Given the importance of the AICAR transformylase/IMP cyclohydrolase, it would be interesting to investigate how the AICAR transformylase/IMP cyclohydrolase regulates sexual reproduction in *C. neoformans*.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author Contributions: Conceptualization, L.-T.H and T.-B.L.; methodology, L.-T.H and Y.-J.W.; software, L.-T.H and Y.-J.W.; validation, L.-T.H., Y.-J.W. and S.-C.X.; formal analysis, L.-T.H and Y.-J.W.; investigation, L.-T.H.; resources, L.-T.H and Y.-J.W.; data curation, L.-T.H and Y.-J.W.; writing—original draft preparation, T.-B.L.; writing—review and editing, T.-B.L.; visualization, L.-T.H and Y.-J.W.; supervision, T.-B.L.; project administration, T.-B.L.; funding acquisition, T.-B.L. All authors have read and agreed to the published version of the manuscript.

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