RESEARCH ARTICLE

Essential Gene Discovery in the Basidiomycete *Cryptococcus neoformans* for Antifungal Drug Target Prioritization

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ABSTRACT **Fungal diseases represent a major burden to health care globally. As with other pathogenic microbes, there is a limited number of agents suitable for use in treating fungal diseases, and resistance to these agents can develop rapidly. *Cryptococcus neoformans* is a basidiomycete fungus that causes cryptococcosis worldwide in both immunocompromised and healthy individuals. As a basidiomycete, it diverged from other common pathogenic or model ascomycete fungi more than 500 million years ago. Here, we report *C. neoformans* genes that are essential for viability as identified through forward and reverse genetic approaches, using an engineered diploid strain and genetic segregation after meiosis. The forward genetic approach generated random insertional mutants in the diploid strain, the induction of meiosis and sporulation, and selection for haploid cells with counterselection of the insertion event. More than 2,500 mutants were analyzed, and transfer DNA (T-DNA) insertions in several genes required for viability were identified. The genes include those encoding the thioredoxin reductase (Trr1), a ribosome assembly factor (Rsa4), an mRNA-capping component (Cet1), and others. For targeted gene replacement, the *C. neoformans* homologs of 35 genes required for viability in ascomycete fungi were disrupted, meiosis and sporulation were induced, and haploid progeny were evaluated for their ability to grow on selective media. Twenty-one (60%) were found to be required for viability in *C. neoformans*. These genes are involved in mitochondrial translation, ergosterol biosynthesis, and RNA-related functions. The heterozygous diploid mutants were evaluated for haploinsufficiency on a number of perturbing agents and drugs, revealing phenotypes due to the loss of one copy of an essential gene in *C. neoformans*. This study expands the knowledge of the essential genes in fungi using a basidiomycete as a model organism. Genes that have no mammalian homologs and are essential in both *Cryptococcus* and ascomycete human pathogens would be ideal for the development of antifungal drugs with broad-spectrum activity.**

IMPORTANCE **Fungal infections are very common in humans but may be neglected due to misdiagnosis and inattention. *Cryptococcus neoformans* is a yeast that infects mainly immunocompromised people, causing high mortality rates in developing countries. The fungus infects the lungs, crosses the blood-brain barrier, and invades the cerebrospinal fluid, causing fatal meningitis. *C. neoformans* infections are treated with amphotericin B, flucytosine, and azoles, all developed decades ago. However, problems with antifungal agents highlight the urgent need for more-effective drugs to treat *C. neoformans* and other invasive fungal infections. These issues include the negative side effects of amphotericin B, the spontaneous resistance of *C. neoformans* to azoles, and the inefficacy of the echinocandin antifungals. In this study, we report the identification of *C. neoformans* essential genes as targets for the development of novel antifungals. Because of the level of evolutionary divergence between *C. neoformans* and the ascomycetes, a subset of these genes is likely essential in all fungi. Genes identified in this study represent an excellent starting point for the future development of new antifungals by pharmaceutical companies.**

Received 16 November 2014 **Accepted** 4 March 2015 **Published** 31 March 2015

Citation Ianiri G, Idnurm A. 2015. Essential gene discovery in the basidiomycete *Cryptococcus neoformans* for antifungal drug target prioritization. mBio 6(2):e02334-14. doi:10.1128/mBio.02334-14.

Invited Editor James W. Kronstad, University of British Columbia **Editor** Bernhard Hube, Friedrich Schiller University Jena

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The majority of the world's population will suffer with fungal infections at some stage during their lifetime. For example, dandruff, ringworm, athlete's foot, toenail disfiguration, thrush and other yeast infections, necrotic diseases in diabetics, and fatal pulmonary or central nervous system disease can all be caused by fungi. Invasive fungal infections are responsible for about 1.5 million deaths every year, more than tuberculosis or malaria. However, fungal infections are not commonly recognized, and epidemiological data for fungal infections are poor due to misdiagnosis, negligence, or cause of death not being accurately reported. For example, the only public agency that conducts a mycological surveillance program is the U.S. Centers for Disease Control and Prevention (CDC). Consequently, the lack of accurate incidence data, especially from developing countries, may underestimate the real burden of invasive infections (1). More than 90% of fatal fungal infections are caused by species belonging to the genera *Cryptococcus*, *Candida*, *Aspergillus*, and *Pneumocystis*, with *Cryptococcus*

neoformans, *Candida albicans*, *Aspergillus fumigatus*, and *Pneumocystis jirovecii* being the most common species.

C. neoformans is primarily a pathogen of immunocompromised patients. The fungus has a worldwide distribution and is found ubiquitously in association with bird guano, soil, and certain tree species (2, 3). There are two varieties of *C. neoformans*, each with different properties. Strains of *C. neoformans* var. *grubii* (or serotype A) are most commonly isolated in clinical settings. The advent of antiviral therapies for AIDS patients reduced the incidence of cryptococcosis to ~8,000 hospitalizations and ~300 deaths a year in the United States (4, 5), although there are still an estimated 624,000 deaths globally each year (6). In sub-Saharan African countries where HIV remains untreated, *C. neoformans* may account for up to half a million deaths annually. The CDC estimated that *C. neoformans* kills more people than tuberculosis does in sub-Saharan Africa (6), highlighting the importance of this fungal pathogen. A close relative of *C. neoformans*, *Cryptococcus gattii*, is rarer in incidence but causes disease more often in non-AIDS patients. North American outbreaks caused by *C. gattii* were identified in 1999 on Vancouver Island and are ongoing there and in the northwestern United States (7–9).

Antifungal drug regimens based on azoles, flucytosine, and amphotericin B can be effective in treating both species of *Cryptococcus*. HIV-infected individuals who experience a fungal infection are subsequently prescribed lifelong prophylactic azole drugs to reduce chances of contracting cryptococcosis or other fungal diseases. This leads to concerns about the development of drug resistance, which is particularly relevant for *C. neoformans* due to its inherent "heteroresistance" to azoles, triggered by spontaneous high-frequency duplication of chromosomes (10, 11). The newest class of antifungal agents, and the only class developed in more than 20 years, is the echinocandins. However, the echinocandins are ineffective against *C. neoformans* despite the target protein being present in the fungus (12). Thus, the identification and development of new drug targets for treating *Cryptococcus* constitute areas requiring more research.

The aim of this study is to identify essential genes of *C. neoformans*. "Essential" genes cannot be deleted or mutated without causing inviability to the organism, although this definition depends on the growth conditions. Such genes are candidate drug targets because ideally the complete prevention of growth by their targeted inhibition is preferable to partial inhibition (such as targeting nonessential genes required for virulence). The essential nature of fungal genes can be demonstrated by a number of experimental methods. While most fungi are haploid, many others have a stable diploid stage or grow as filamentous hyphae with multiple nuclei. A common approach is a three-step process: first, to mutate one copy of the gene at the stages in which the other copy remains functional and enables the fungus to grow; then, to force a reduction to a single-chromosome state through meiosis or a parasexual process; and finally, to demonstrate that a haploid mutant is inviable (reviewed in reference 13). At present, 21 essential genes are known in *C. neoformans* (see Table S1 in the supplemental material).

Essential genes have been identified from mutant screens in other fungi such as the pathogens *A. fumigatus* (14–16) and *C. albicans* (17) and include a complete list from two strains of the model fungi *Saccharomyces cerevisiae* (18, 19) and *Schizosaccharomyces pombe* (20). These four species are ascomycetes. *C. neoformans* is within the basidiomycete phylum, a lineage that diverged from the ascomycetes at least 500 million years ago (21). Thus, essential genes identified in a basidiomycete that have essential orthologs in ascomycetes are expected to be essential in the majority of fungi and represent broad-spectrum targets for drug development.

In this study, we applied forward and reverse genetics to identify genes required for the viability of *C. neoformans*. The approach was to transform an engineered diploid strain of *C. neoformans* and assess, by classical Mendelian analysis, the viability of haploid progeny isolated from the transformants. For the reverse genetics, a bioinformatic analysis of fungal and human gene similarity identified target genes specifically suitable for future antifungal development. Finally, a series of growth assays were performed to determine drug- and stress-induced haploinsufficiency of the heterozygous mutant strains and the phenotypes of viable haploids where applicable. The results of this study provide much-needed information about essential genes in *C. neoformans* and pave the way for development of new agents effective in treating fungal infections.

(This work was presented at the 12th European Conference on Fungal Genetics, 23 to 27 March 2014, Seville, Spain [22].)

RESULTS

Development of a forward genetic screen to identify essential genes in *C. neoformans*. The underlying approach of the screen is to generate insertional mutants of a diploid strain of *C. neoformans*, sporulate these strains, and evaluate the inability of haploid progeny to grow on selective medium due to the loss of the dominant nourseothricin acetyltransferase (*NAT*) marker used. The selection relies on two systems. *NAT* is introduced by transformation. Resistance to 5-fluoroorotic acid (5-FOA) is due to mutation of the *ura5* gene in the fungus that blocks the incorporation of this toxic molecule into nucleic acids in haploid *ura5* mutants but not in heterozygote *URA5*/*ura5* cells. Thus, 5-FOA selects for the haploid cells achieved by sporulation (Fig. 1).

The *NAT* marker was delivered in the diploid strain by *Agrobacterium*-mediated transformation (AMT) or biolistic transformation. The latter was performed as an alternative insertional mutagenesis approach attempting to circumvent problems met with AMT. Insertional mutants were selected on nourseothricin, sporulation was induced on Murashige-Skoog (MS) medium, and the spores were plated onto yeast extract-peptone-dextrose (YPD) medium at pH 4, supplemented with nourseothricin, 5-fluoroorotic acid (5-FOA), and adenine. This low-pH medium enables both 5-FOA and nourseothricin to be selective and for the screen to be performed under rich growth conditions, rather than the nutrient-limiting yeast nitrogen base (YNB) in which 5-FOA is usually employed.

A total of 2,665 insertional mutants of *C. neoformans* were isolated and tested, and 127 (~4.5%) were selected for further phenotypic and molecular characterization as showing no growth or strongly reduced growth after transfer of sporulating cultures to YPD plus NAT plus 5-FOA. Southern blot analysis revealed that 94 strains had one transfer DNA (T-DNA) insertion, 26 had two, 6 had three, and one strain had four. Figure 2A shows a representative Southern blot wherein each hybridization band represents a T-DNA insertion in the genome of 19 transformants selected in the primary screen.

In order to identify the genes affected by the *NAT* insertion, strains were subjected to inverse or splinkerette PCRs and the

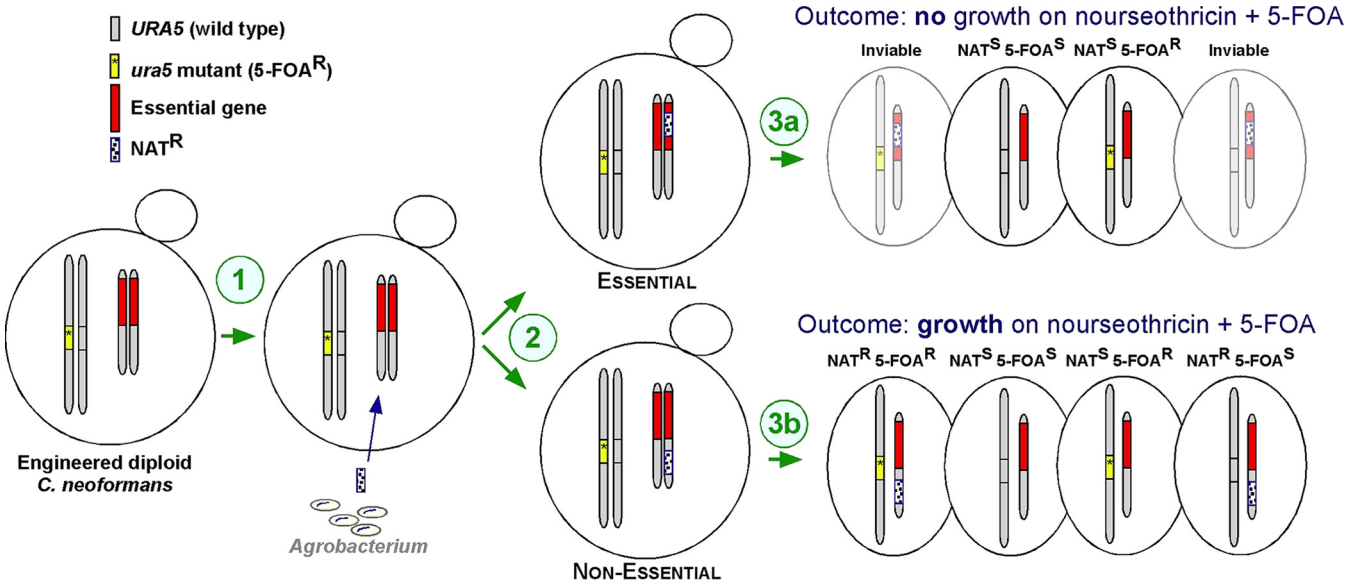

FIG 1 Overview of the genetic screen procedure (1). The diploid *C. neoformans* strain is transformed with *Agrobacterium* T-DNA molecules that confer nourseothricin resistance (NAT^R) (2). The *NAT* gene can integrate into essential genes or nonessential regions (3a and b). The strains are induced to undergo meiosis, and the basidiospores are plated onto medium with nourseothricin and 5-fluoroorotic acid. The primary screen results are confirmed by Mendelian genetic analysis on basidiospores by micromanipulation.

sequences obtained from those amplification products were compared using BLASTn on the updated genome sequence of *C. neoformans* strain H99 (23), which is congenic with the diploid strain. Fifty-six strains (~44%) were characterized. The T-DNA was found within the open reading frame (ORF) of a gene in 20 strains. In five cases, the T-DNA was found in 5′ and 3′ untranslated regions (UTRs). In the remaining characterized strains, the T-DNA was found to be inserted in intergenic regions. BLASTp comparison of the predicted protein sequences of *C. neoformans* was performed against the GenBank, *Saccharomyces*, and *Schizosaccharomyces* genome databases to assign gene names and predict protein functions.

Strains bearing single *NAT* insertions, or in some cases strains with two *NAT* insertions and predicted chromosomal rearrangements, were retested on MS medium to induce meiosis and the production of the haploid basidiospores. A low germination rate and the presence of all eight combinations from the three markers *URA5*, *ADE2*, and *MAT* but the absence of nourseothricin resistant (NAT^R) progeny indicate an essential function. Twenty-seven strains were unable to produce chains of basidiospores, and PCR analysis revealed that five of them had only one *MAT* gene (Fig. 2B), likely preventing the formation of sexual structures. Sexual reproduction was successfully restored in these 5 strains after crossing them with the opposite mating type, and two of these mutants (2aGI646 and 2aGIPlate17H3) did not yield viable NAT^R progeny (details are discussed in the text below).

In total, 15 heterozygous mutants that were unable to produce viable NAT^R progeny were isolated through forward genetics (Table 1), with 11 having a single T-DNA insertion and the remaining strains having two insertions.

For strain 1d2, only the left border of the T-DNA was identified, as inserted in the first exon of the gene *RRP40* encoding the exosome complex component Rrp40. In *S. cerevisiae*, *RRP40* is an essential gene involved in 3′-to-5′ RNA processing and degradation in the nucleus and the cytoplasm (24).

Mutant 1d53 had a T-DNA insertion in the last exon of the gene *TRR1*, which encodes a thioredoxin disulfide reductase (Fig. 3A.1). This gene was already reported as essential in *C. neo-*

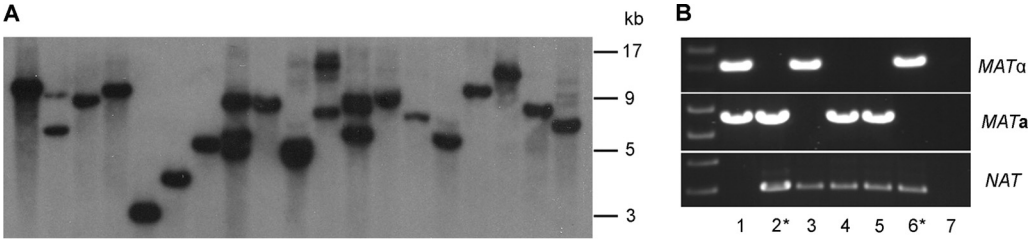

FIG 2 Molecular analysis of insertional mutants. (A) Representative Southern blot analysis of 19 transformants selected in the primary screen. Genomic DNAs were digested with ClaI, which does not cut inside the T-DNA region, and hybridized with the ORF of the *NAT* gene. Each hybridization band corresponds to one T-DNA insertion. (B) PCR analysis to score the presence of the *MATα*, *MAT***a**, and *NAT* genes in five transformants (lanes 2 to 6) that were not able to produce sexual structures. Lane 1 is the diploid strain AI187 of *C. neoformans*, and lane 7 represents the negative control. Asterisks in lanes 2 and 6 indicate insertional mutants 2aGI646 and 2aGIPlate17H3, respectively; both of them did not yield NAT^R haploid progeny following backcrosses with KN99*α* (Fig. 3D) and KN99**a**, respectively.

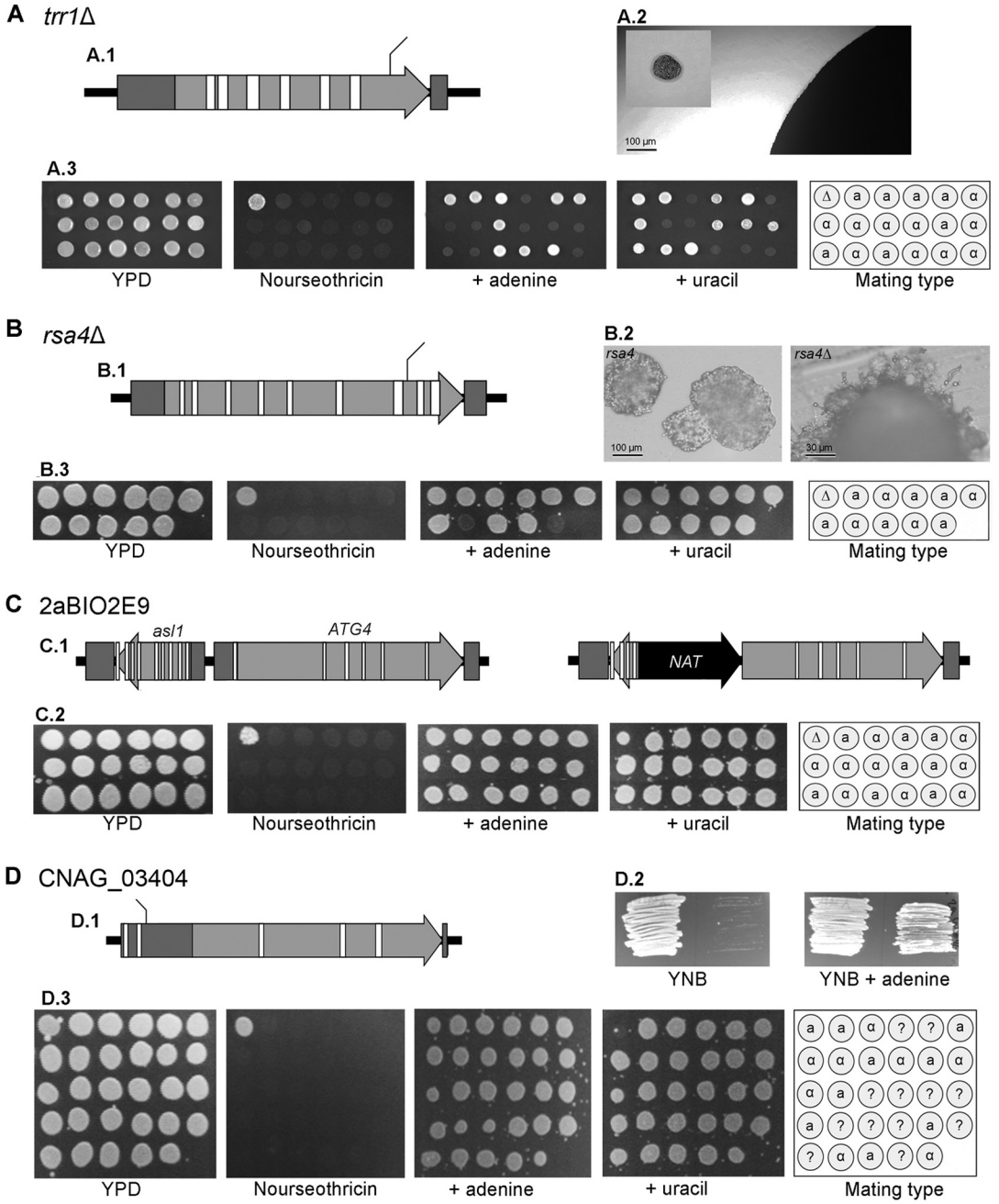

FIG 3 Representative examples of mutants with insertions in essential genes identified by forward genetic screens. In all cases, the diagrams of the structure of the genes indicate exons in light gray, introns in white, and UTRs in dark gray. The first colony in the top left corner of all the panels represents the original heterozygous mutant from which the progeny analyzed comes. (A) (A.1) In mutant 1d53, the T-DNA inserted in the final exon of *TRR1* and the allele replacing the complete ORF was generated through specific homologous recombination. (A.2) The inset small colony is *trr1*, while the large colony edge is from the wild-type strain. (A.3) Seventeen basidiospore progeny isolated for the heterozygous *TRR1/trr1*Δ strain were unable to grow on nourseothricin medium, indicating the deletion allele as inviable. (B) (B.1) In strain 2aGIPlate18E5, the T-DNA inserted in the eighth exon of *RSA4* and a *RSA4/rsa4*Δ targeted mutant was generated. (B.2) The *RSA/rsa4* insertional mutant was unable to form chains of haploid basidiospores, whereas the *RSA4/rsa4*Δ deletion mutant underwent meiosis and produced basidiospores. (B.3) Segregation analysis revealed that *RSA4* is an essential gene, as shown by the loss of the *NAT*-dominant marker in the progeny. (C) In the insertional mutant 2aBIO2E9 generated through biolistic transformation, the *NAT* insertion caused a 2,139-bp deletion involving the 5′ regions of two adjacent genes, *asl1* and *ATG4.* (C.1) On the left are represented the wild-type structures of the two genes, while on the right are shown the genes lacking the 5′ regions replaced by *NAT* (black arrow). Genetic segregation analysis of progeny isolated from 2aBIO2E9 shows that this mutant is likely aneuploid as no *ura5* and *ade2* progeny were obtained. (C.2) Also, the absence of growth on nourseothricin indicates that the mutation does not allow for the isolation of viable homozygous progeny. (D) (D.1) Transformant 2aGI646 had a T-DNA insertion in the 5′ UTR of CNAG_03404 encoding a protein of unknown function. (D.2) The mutant was also unable to grow on YNB without adenine and was thus aneuploid for the *ADE2*-containing chromosome and unable to produce chains of basidiospores because of the missing *MAT*α allele (this is shown in Fig. 2B, lane 2). (D.3) After crosses with KN99α, dissected haploid progeny showed regular segregation only for the *NAT* and the *MAT* markers. The absence of growth on nourseothricin indicates that transformant 2aGI646 produces NAT^R progeny that are inviable.

TABLE 1 *C. neoformans* heterozygous insertional mutants generating inviable progeny identified through forward genetics

C. neoformans heterozygous insertional mutants	H99 Broad annotation[a]	Gene name[a,b]	T-DNA position[a,c]	Description[a,d]	No. of germinated basidiospores/total no. dissected	NAT^R	Essential in *S. cerevisiae* or *S. pombe*
One *NAT* insertion							
id2	CNAG_01141	*RRP40*	ORF	Exosome complex component RRP40	24/46	0	Yes
id53	CNAG_05847	*TRR1*	ORF	Thioredoxin-disulfide reductase	17/27	0	Yes
*trr1*Δ targeted mutant	CNAG_05847	*TRR1*	NA[g]	Thioredoxin-disulfide reductase	9/36	0	Yes
2a445	CNAG_01800	NA	5-bp 5′ UTR	Related to NADH:ubiquinone oxidoreductase	21/36	0	NA
2aGIPlate14C8	CNAG_03368	*STU2*	ORF	Microtubule-associated protein	7/34	0	Yes
2aGIPlate18E5	CNAG_ 04117	*RSA4*	ORF	Ribosome biogenesis	NA	NA	Yes
*rsa4*Δ targeted mutant	CNAG_ 04117	*RSA4*	NA	Ribosome biogenesis	10/42	0	Yes
2aBIO2E9	CNAG_02661-CNAG_02662	*asl1-ATG4*	ORF-ORF	*O*-Glucosyl hydrolase–cysteine protease	17/24	0	No-no
2a63	CNAG_02033/CNAG_03113	*ARC19/PIB2*	chr6:chr8[h]	Arp2/3 complex subunit/proteinase B inhibitor	27/90	0	Yes/no
2a129	CNAG_00194/CNAG_01080	NA/*CYB2*	chr1:chr5	Hypothetical protein/cytochrome *b*	13/73	0	No/no
2a46	CNAG_07485/NA	*YTP1*/NA	chr2:chr8	Membrane protein/NA	17/35	0	No/NA
2aBIO1F11	NA	NA	NA	NA	22/36[e]	0	NA
2aGIPlate17H3[f]	NA	NA	NA	NA	10/30	0	NA
Two *NAT* insertions							
2aPlate2D9	CNAG_06548-CNAG_06549	*EMC1-CET1*	ORF-ORF	Endoplasmic reticulum protein–mRNA-capping enzyme subunit beta	10/25	0	No-yes
2aPlate4C11	CNAG_03105	NA	ORF	Hypothetical protein	14/36	0	NA
2aGI646[f]	CNAG_03404	NA	5′ UTR	Hypothetical protein	27/33	0	NA
2a13	CNAG_07485/CNAG_03212	*YTP1/HCM1*	chr2:chr8	Membrane protein/Forkhead transcription factor	4/16	0	No/no

[a] The hyphen or dash in columns 2, 3, 4, and 5 indicates two adjacent genes; the slash (/) indicates genes originally located in different chromosomes (chromosomal rearrangements).
[b] The *S. pombe* orthologs are indicated in italic lowercase.
[c] The distance from the closest 5′ or 3′ UTR is indicated in base pairs.
[d] Gene description is based on the *C. neoformans* H99 Broad database. Where it was not available, the description of the *S. cerevisiae* or *S. pombe* ortholog was used.
[e] Phenotype assessed after backcross of a NAT^R progeny with the opposite mating type of KN99.
[f] For mutants 2aGI646 and 2aGIPlate17H3, PCR revealed the presence of only one *MAT* allele. In these aneuploid mutants, sexual reproduction was restored by crossing with the opposite mating type of the congenic KN99 pair, and for both of them, haploid basidiospores were unable to grow on selective medium containing nourseothricin.
[g] NA, not available.
[h] chr, chromosome.

formans based on using a conditional promoter strategy (25). Segregation analysis of haploid basidiospores that germinated from strain 1d53 (62%) revealed that they were all nourseothricin sensitive (NAT^S), confirming that *TRR1* is an essential gene in *C. neoformans*. However, 9 microcolonies too small for phenotypic analysis were also obtained from dissected haploid basidiospores (Fig. 3A.2). A targeted deletion mutant of *TRR1* was generated in the diploid using the gene replacement procedure described below. All of the progenies obtained after sporulation were NAT^S (Fig. 3A.3), but a lower percentage of germination was obtained (25%) and no microcolonies were observed, suggesting that the T-DNA insertional allele of *trr1* maintains residual function.

For strain 2a445, only the left border of the T-DNA was identified 5 bp before the 5′ UTR of the gene CNAG_01800. The closest *S. cerevisiae* gene is *KRS1*, which encodes a cytoplasmic lysyl-tRNA synthetase (26). However, the expected value after BLASTp is very low at 0.63, and bidirectional best-hit analysis revealed CNAG_04179 as the closest *C. neoformans* ortholog of *S. cerevisiae KRS1*. Interestingly, the most informative hit from BLASTp analysis of CNAG_01800 on GenBank is a protein designated "related to NADH:ubiquinone oxidoreductase 14 kDa," with no further available information. Complex I NADH:ubiquinone oxidoreductase is the first enzyme of the respiratory chain that catalyzes the transfer of electrons from NADH to coenzyme Q10, and in eu-

karyotes, it is located in the inner mitochondrial membrane. Hence, CNAG_01800 encodes an essential protein of unknown function that may be involved in the mitochondrial electron transport chain.

For strain 2aGI14C8, the T-DNA inserted in the 10th exon of *STU2*, a gene encoding a microtubule-associated protein. *C. neoformans* Stu2 contains two N-terminal CLASP domains that are conserved in microtubule plus-end-tracking proteins to regulate the stability of dynamic microtubules. Stu2 also contains a heat repeat domain that is a solenoid protein domain found in a number of cytoplasmic proteins that forms a rod-like helical structure involved in intracellular transport.

In strain 2aGIPlate18E5, the T-DNA inserted in the eighth exon of the *S. cerevisiae* homolog *RSA4* (Fig. 3B.1). This gene contains WD40 domains that are implicated in a variety of functions ranging from signal transduction and transcription regulation to cell cycle control, autophagy, and apoptosis. In *S. cerevisiae*, *RSA4* is involved in ribosome biogenesis and it is required for maturation and efficient intranuclear transport of pre-60S ribosomal subunits. However, strain 2aGIPlate18E5 was not able to produce haploid basidiospores on MS medium. To test whether the lack of sexual reproduction was due to *RSA4*, the gene was replaced by targeted mutagenesis in the diploid strain. Differing from 2aGIPlate18E5, the heterozygous *RSA4/rsa4*Δ strain was able to form sexual structures on MS medium. Figure 3B.2 shows a lack of reproductive structures for the insertional heterozygous mutant 2aGIPlate18E5 (*RSA4/rsa4*) and basidiospore chains for the targeted heterozygous mutant *RSA4/rsa4*Δ. This indicates that *RSA4* is not involved in sexual reproduction, and the T-DNA insertional mutant has another phenotype unassociated with *RSA4*. Importantly, the haploid progenies derived from *RSA4/rsa4*Δ were all NAT^S, indicating that *RSA4* is required for viability in *C. neoformans* (Fig. 3B.3).

In strain 2aBIO2E9, generated with biolistic transformation, the *NAT* insertion produced a 2,139-bp deletion that involved the 5′ regions of two adjacent genes, the *S. pombe* ortholog *asl1*, not present in *S. cerevisiae*, and *ATG4* (Fig. 3C.1). *S. pombe* als1 is a nonessential *O*-glucosyl hydrolase involved in cell wall organization and biogenesis and carbohydrate metabolic processes. *ATG4* encodes a conserved cysteine protease required for autophagy. Atg4 cleaves the carboxy-terminal arginine residue of Atg8 in preparation for conjugation with phosphatidylethanolamine and also cleaves the modified, membrane-bound form of Atg8p to reverse conjugation (27). Atg4 also interacts with Tub1 and Tub2 for the attachment of autophagosomes to microtubules (28). In *S. cerevisiae*, *ATG4* is not essential, but null mutants have reduced viability, and homozygous *atg4* diploid cells show drastically reduced sporulation (28). Segregation analysis of the haploid basidiospores isolated from 2aBIO2E9 showed that all of them were unable to grow on medium containing nourseothricin, and based on the function of the two affected genes, it is likely that this is due to the deletion of *ATG4* (Fig. 3C.2). If so, *C. neoformans ATG4* has more extensive functions in the cell than does the gene of *S. cerevisiae*.

T-DNA caused chromosomal rearrangements in three other insertional mutants that showed "inviable" phenotypes (2a63, 2a129, and 2a46), since inverse PCR yielded genes that originally were located on different chromosomes. For strain 2a63, the genes involved in the rearrangement were the essential Arp2/3 complex subunit *ARC19* and the nonessential proteinase B inhibitor *PIB2*. Based on the function of these genes, it is likely that *ARC19* is an essential gene in *C. neoformans*. In the other cases, the ascomycete orthologs of the identified *C. neoformans* genes are not required for viability, and consequently, it was not possible to determine whether one of the hit genes was responsible for the inviable phenotype or whether this was due to the chromosomal rearrangement.

The genes responsible for the inviable phenotypes of strains 2aGI17H3, which is aneuploid because it is missing the *MAT***a** allele, and 2aBIO1F11, generated by biolistic transformation, could not be identified by inverse PCR or splinkerette PCR.

Four other strains that produced inviable progeny were characterized by two T-DNA insertions. For strain 2aPlate2D9, only one hit gene was identified. The T-DNA insertion caused a 2,005-bp deletion involving the 3′ region (deletion of 1,108 bp of 3,906 bp) of the gene *ECM1* and the majority (deletion of 1,053 bp of 1,625 bp) of the gene *CET1*. In the model ascomycete yeast, *ECM1* encodes a nonessential endoplasmic reticulum (ER) protein and it is a member of a transmembrane complex required for efficient folding of proteins in the endoplasmic reticulum (29). *CET1* encodes the beta subunit of the essential enzyme that adds the 5′ cap to mRNA (30). The gene *CET1* was also selected for targeted gene replacement following the bioinformatic approach (see below), and no basidiospores isolated after sporulation of the *CET1/cet1*Δ heterozygote were able to grow on medium containing nourseothricin. This indicates that *CET1* is essential in *C. neoformans* and it is most likely responsible for the inviable phenotype observed for the progeny derived from strain 2aPlate2D9.

In strain 2aPlate4C11, only one T-DNA insertion was identified. The hit gene is CNAG_03105, and it encodes a hypothetical protein with no known conserved domains. This protein shares homology with the *S. cerevisiae* gene *ALB1*, a shuttling pre-60S factor involved in the biogenesis of the ribosomal large subunit, and with *S. pombe dhp1*, an essential 5′-3′ exoribonuclease involved in mitotic sister chromatid segregation and rRNA processing. However, similarity with the model yeast best hits is very low (E values of 0.96 for *S. cerevisiae* and 1.8 for *S. pombe*), and BLAST analyses on GenBank revealed that CNAG_03105 is specific only for *C. neoformans* var. *grubii*, and no homologs in *Cryptococcus neoformans* var. *neoformans* and *C. gattii* are present. Even though this finding is of interest, strain 2aPlate4C11 had 2 T-DNA insertions, and therefore, further experiments would be needed to assign a function to this specific *C. neoformans* var. *grubii* protein.

For strain 2aGI646, one of the T-DNA insertions was found in the 5′ UTR of CNAG_03404, a gene encoding a protein of unknown function (Fig. 3D.1). Strain 2aGI646 is an *ade2* adenine auxotroph (Fig. 3D.2), and PCR revealed that the *MAT*α allele is missing (Fig. 2B, lane 2). This indicates that 2aGI646 is aneuploid with one copy of chromosomes 6 and 4 missing. Since only *MAT***a**, the strain was unable to produce haploid basidiospores, but sexual reproduction was restored by crossing it with the congenic strain KN99α. All dissected basidiospores that germinated were NAT^S (Fig. 3D.3). The gene CNAG_03404 is annotated as a hypothetical protein, and no known conserved domains are present. In *S. cerevisiae*, it corresponds to the flocculin-encoding gene *FLO11*, and in *S. pombe*, it corresponds to the nonessential gene *pfl2*, an orphan sequence. In both cases, the similarity is very low, and further BLASTp searches on GenBank revealed that homologs of CNAG_03404 are present in *C. neoformans* var. *neoformans* and *C. gattii*, and in three Basidiomycotina belonging to the Agaromy-

cotina (*Trichosporon asahii*, *Tremella mesenterica*, and *Rhizoctonia solani*). Attempts to complement the phenotype using the wild-type copy of the CNAG_03404 gene were performed, and although viable progeny were obtained, an irregular segregation of the gene markers was observed (data not shown). Therefore, also for CNAG_03404 further experiments are needed to assign a function to this gene.

One insertion site was identified in strain 2a13, revealing a chromosomal rearrangement between chromosomes 2 and 8 involving two nonessential genes (*YTP1* and *HCM1*) in *S. cerevisiae*.

For 41 heterozygous insertional mutants selected in the primary screen as potentially bearing an insertion in essential genes, viable haploid progeny were obtained after Mendelian analysis of a single mutant sporulated on MS medium (see Table S2 in the supplemental material). Of note, 9 strains had a *NAT* insertion in *S. cerevisiae* orthologs required for viability, and 8 strains were unable to produce basidiospores. In addition, 6 other strains had insertions close to or in regions designated as miscellaneous RNA (miscRNA). The extensive presence of miscRNAs in the genome was recently revealed in transcriptome sequencing (RNA-seq) analyses (23). miscRNAs are spliced and polyadenylated in the same way as are other genes, but their function in the cell is unknown. A mechanism of regulation of gene expression seems to be the most plausible hypothesis, since the majority of them have opposite orientations from the closest coding gene. Our results revealed that their inactivation by the T-DNA does not lead to an inviable phenotype, and the mutants isolated in the present study could be used to investigate the role of these miscRNAs in the cells.

Overall, of 127 insertional mutants isolated in the primary screen, 56 were analyzed in further detail and are reported in Table 1 and in Table S2 in the supplemental material. The remaining 71 were excluded, with more than 20 characterized by chromosomal rearrangements, mainly translocations. While in other organisms reciprocal translocations yield a possible 50% viable progeny, in *C. neoformans* these events result in lower basidiospore germination rates, as reported in some examples in the text above. Moreover, in these strains at least two genes are involved in the chromosomal rearrangement, which makes it impossible in most cases to assign any observed phenotypes to either one of the two genes. Other excluded strains were those characterized by multiple T-DNA insertions, strains whose hit gene was already characterized in *C. neoformans* (*MAN1* [31], CNAG_03090 [32], and *UPC2* [33]), and strains assessed as having viable phenotypes before the PCR for the identification of the hit gene was performed.

Identification of suitable drug targets and reverse genetics to test gene function. A set of 35 genes was selected using bioinformatics approaches for target mutagenesis in the diploid strain of *C. neoformans* (see Materials and Methods). These genes share common features, such as being present as a single copy in the genome of *C. neoformans*, being common in a diversity of fungi, and having low or null similarity with human orthologs. The genes have roles in mitochondrial functions, metabolic and biosynthetic processes, RNA-related functions, and others (Table 2).

Mutants were generated by biolistic transformation, and correct homologous recombination events were confirmed by PCR and Southern blotting (see Fig. S1 and S2 in the supplemental material). Mutants were then transferred on MS medium to undergo meiosis, sporulation, and basidiospore production. Spores were micromanipulated on YPD agar and tested for the segregation of the four markers as described above.

The haploid progenies derived from 21 of the 35 mutants were unable to grow on selective medium containing nourseothricin, indicating that the inactivated genes in these strains are essential for *C. neoformans* viability (Table 2).

Eleven genes involved in mitochondrial functions were selected for gene replacement, and seven (*BOT1*, *MGM101*, *MRPL31*, *MRPS18*, *NAM9*, *RSM18*, and *TIM54*) were found to be essential for viability in *C. neoformans*. As an example, the segregation analysis of the *BOT1/bot1*Δ mutant is shown (Fig. 4A). This mutant was found as only *MAT*α and thus was likely aneuploid. Sexual fertility was successfully restored to the *BOT1/bot1*Δ mutant after crossing with the KN99**a** strain, and the haploid progenies derived from dissected tetrads were all NAT^S, indicating that *BOT1* is required for viability in *C. neoformans*. Genes *BOT1*, *MRPL7*, *MRPL31*, *MRPS18*, *NAM9*, and *RSM18* were grouped with Gene Ontology (GO) in mitochondrial translation. *MRPL7* was the only gene grouped in mitochondrial translation that was not required for viability, as confirmed by backcross and genetic analysis of a NAT^R progeny (Fig. 4B and C). Figure 4D represents the genetic analysis of progeny isolated from the heterozygous mutant *TIM54/tim54*Δ#1 strain. For *TIM54*, two independent heterozygous mutants were isolated and tested (*TIM54/tim54*Δ#1 and *TIM54/tim54*Δ#7 strains) and the two gave the same outcome.

The genes *MDM10*, *MDM34*, and *MMM1*, which are all ER components of the ERMES and SAM complex involved in the establishment of mitochondrion localization and organization, are not essential in *C. neoformans*. Conversely, the gene *MGM101*, a nonessential gene in *S. cerevisiae* involved in mitochondrial genome maintenance and mitochondrial DNA recombinational repair, was found to be required for viability in *C. neoformans*. This is because no nourseothricin-resistant haploid progeny were obtained after genetic analysis from three independent gene replacement heterozygous mutants that were tested.

Two genes encoding proteins predicted to be constituents of the cell wall were selected for targeted gene replacement. The rationale behind this is that the cell wall is a known attractive drug target for treating fungal infections. The first gene, *KTR3*, encodes an alpha-1,2-mannosyltransferase for synthesis of cell wall mannoprotein. NAT^R progeny derived from the *KTR3/ktr3*Δ mutant were viable, and the nonessential nature of this gene was confirmed by the generation of targeted mutant *ktr3*Δ directly in the KN99α haploid strain of *C. neoformans* (data not shown). In addition, a recent independent investigation of *KTR3* showed that the gene is required for virulence (34). The second gene, *PWP1*, has no orthologs in *S. cerevisiae* but encodes a nonessential cell wall protein in *S. pombe*. The *C. neoformans* ortholog has a Ser-Thr-rich glycosylphosphatidylinositol-anchored membrane family domain, which in *A. fumigatus* plays a role in cell wall organization (35). For *PWP1*, two independent *C. neoformans* heterozygous mutants were isolated, and both of them were unable to form basidiospores (Fig. 4F). For this gene, other strategies such as the use of a conditional promoter are necessary to establish whether or not *PWP1* is required for viability.

Genes involved in the biosynthesis of ergosterol have been studied for years as current and potential drug targets. In the present study, the genes *ERG8* and *MVD1* were selected for gene replacement in *C. neoformans*. *ERG8* encodes a phosphomevalonate

TABLE 2 Essential *S. cerevisiae* and/or *S. pombe* orthologs selected for targeted replacement in *C. neoformans* and genetic analysis of the heterozygous mutants generated

Gene name	Locus (H99 database)	H99 Broad description	No. of germinated basidiospores/total no. dissected	NAT^R haploid progeny	*C. neoformans* phenotype	GO biological process
BOT1	CNAG_07884	Hypothetical protein with eukaryotic mitochondrial regulator protein domain	9/40	0	Inviable[a]	Mitochondrial translation
BYR4	CNAG_06325	GTPase activator	31/37	0	Inviable[b]	Mitotic spindle orientation checkpoint
BUR6	CNAG_02034	Hypothetical protein with a histone-like transcription factor (CBF/NF-Y) and archaeal histone and core histone H2A/H2B/H3/H4 domains	9/39	0	Inviable	Regulation of transcription from RNA polymerase II promoter
CDC14	CNAG_04939	Hypothetical protein with cell division control protein 14 domain, SIN (septation initiation network) component	9/34	0	Inviable	Septation initiation network
CET1	CNAG_06549	mRNA-capping enzyme subunit beta	10/48	0	Inviable	7-Methylguanosine mRNA capping
ERG8	CNAG_06001	Phosphomevalonate kinase	17/44	0	Inviable	Ergosterol biosynthesis
FBA1	CNAG_06770	Fructose-bisphosphate aldolase 1	13/36	0	Inviable	Glycolysis
FOL1	CNAG_02786	Dihydropteroate synthase	10/56	0	Inviable	Tetrahydrofolate biosynthetic process
HYM1	CNAG_03305	Calcium binding protein 39	21/95	1[c]	Inviable	Component of the RAM pathway
HRB1	CNAG_05013	RNP domain-containing protein	25/48	0	Inviable	RNA-binding protein
KEI1	CNAG_00101	Hypothetical protein with inositolphosphorylceramide synthase subunit Kei1 domain	9/38	0	Inviable	Inositolphosphoceramide metabolic process
KTR3	CNAG_03832	Alpha-1,2-mannosyltransferase	24/34	13	Viable	Cell wall mannoprotein biosynthetic process
MDM10	CNAG_06345	Mitochondrial distribution and morphology protein 10	7/23	3	Viable	ER component of ERMES and SAM complex
MDM34	CNAG_02304	Mitochondrial distribution and morphology protein 34	21/38	9	Viable	ER component of ERMES and SAM complex
MGM101	CNAG_04267	Mitochondrial genome maintenance protein	10/40	0	Inviable[b]	DNA repair
MMM1	CNAG_05557	Mitochondrial outer membrane protein	33/48	12	Viable	ER component of ERMES and SAM complex
MRPL7	CNAG_01650	Large subunit ribosomal protein L7	25/39	6	Viable[b]	Mitochondrial translation
MRPL31	CNAG_04332	Large subunit ribosomal protein L31	9/37	1[c]	Inviable	Mitochondrial translation
MRPS18	CNAG_04334	Hypothetical protein with a ribosomal protein S11 domain	6/32	0	Inviable	Mitochondrial translation
MVD1	CNAG_05125	Diphosphomevalonate decarboxylase	12/39	0	Inviable	Ergosterol biosynthesis
NAM9	CNAG_03986	Nam9 protein	14/45	0	Inviable	Mitochondrial translation

(Continued on following page)

TABLE 2 (Continued)

Gene name	Locus (H99 database)	H99 Broad description	No. of germinated basidiospores/total no. dissected	NAT^R haploid progeny	*C. neoformans* phenotype	GO biological process
PWP1	CNAG_03223	Hypothetical protein with a Ser-Thr-rich glycosylphosphatidylinositol anchored membrane family domain	NA[d]	NA	NA	NA
PDC1	CNAG_04659	Pyruvate decarboxylase	24/39	9	Viable[b]	Pyruvate metabolic process
RIB2	CNAG_06467	Hypothetical protein with cytidine and deoxycytidylate deaminase zinc-binding region	25/50	10	Viable	Riboflavin biosynthesis
RIB3	CNAG_02506	3,4-Dihydroxy-2-butanone-4-phosphate synthase	34/70	13	Viable	Riboflavin biosynthesis
RSC9	CNAG_06744	Chromatin structure-remodeling complex subunit RSC9	11/29	6	Viable	Chromatin remodeling
RSM18	CNAG_04452	Small subunit ribosomal protein S18	15/37	0	Inviable	Mitochondrial translation
SAF2	CNAG_03338	Hypothetical protein with a WW domain	21/33	9	Viable	Unknown function
SEC5	CNAG_00736	Exocyst protein	31/44	18	Viable	Exocyst assembly and exocytosis
SEN54	CNAG_02564	tRNA-splicing endonuclease subunit Sen54	4/42	0	Inviable[a]	tRNA-type intron splice site recognition and cleavage
SFI1	CNAG_04247	Hypothetical protein with an Sfi1 spindle body protein domain	8/43	0	Inviable	Centrin-binding protein
SPAPB24D3.06c	CNAG_00592	Dolichol-phosphate mannosyltransferase	24/38	21	Viable	Unknown function
THP1	CNAG_01697	Nuclear pore	24/42	16	Viable	mRNA export from nucleus
TIM54	CNAG_05234	Mitochondrial import inner membrane translocase subunit TIM54	32/47 (strain #1)	0	Inviable	Protein import into mitochondrial inner membrane
TIM54			20/64 (strain #7)	0	Inviable	
TRL1	CNAG_01250	tRNA ligase	8/73	0	Inviable	tRNA ligase

[a] Mutant *bot1*Δ and *sen54*Δ strains were aneuploid, as PCR revealed the presence of only one *MAT* allele. In these mutants, sexual reproduction for genetic analysis was restored by crossing with the opposite mating type of the congenic strain KN99 pair.
[b] Phenotype assessed after backcross of a NAT^R progeny with the opposite mating type of KN99.
[c] For *mrpl31*Δ and *hym1*Δ heterozygous mutants, one NAT^R haploid progeny was obtained. PCR analysis revealed the presence of the wild-type copy of the genes of interest, suggesting that these NAT^R colonies are aneuploid and that both *MRPL31* and *HYM1* are required for viability in *C. neoformans*.
[d] NA, not available.

kinase that catalyzes the conversion of mevalonate-5-phosphate into mevalonate-diphosphate. The diphosphomevalonate decarboxylase *MVD1* catalyzes conversion of mevalonate-5-pyrophosphate to isopentenyl pyrophosphate. Both genes were found to be essential in *C. neoformans*.

The *C. neoformans* orthologs of five *S. cerevisiae* genes involved in RNA-related functions, *BUR6*, *CET1*, *HRB1*, *SEN54* and *TRL1*, were all found to be required for viability.

Other essential genes identified were *BYR4*, encoding a GTPase activator essential for viability in *S. pombe*; *CDC14*, encoding the cell division control protein 14, which is a component of the septation initiation network (SIN); *FBA1*, encoding a fructose-bisphosphate aldolase 1 that catalyzes conversion of fructose-1,6-bisphosphate to glyceraldehyde-3-phosphate and dihydroxyacetone-phosphate and is required for glycolysis and gluconeogenesis; *FOL1*, encoding a multifunctional enzyme of the folic acid biosynthesis pathway; *KEI1*, annotated as encoding a hypothetical protein containing an inositolphosphorylceramide synthase subunit Kei1 domain; and *SFI1*, annotated on the H99 Broad database as encoding a hypothetical protein containing an

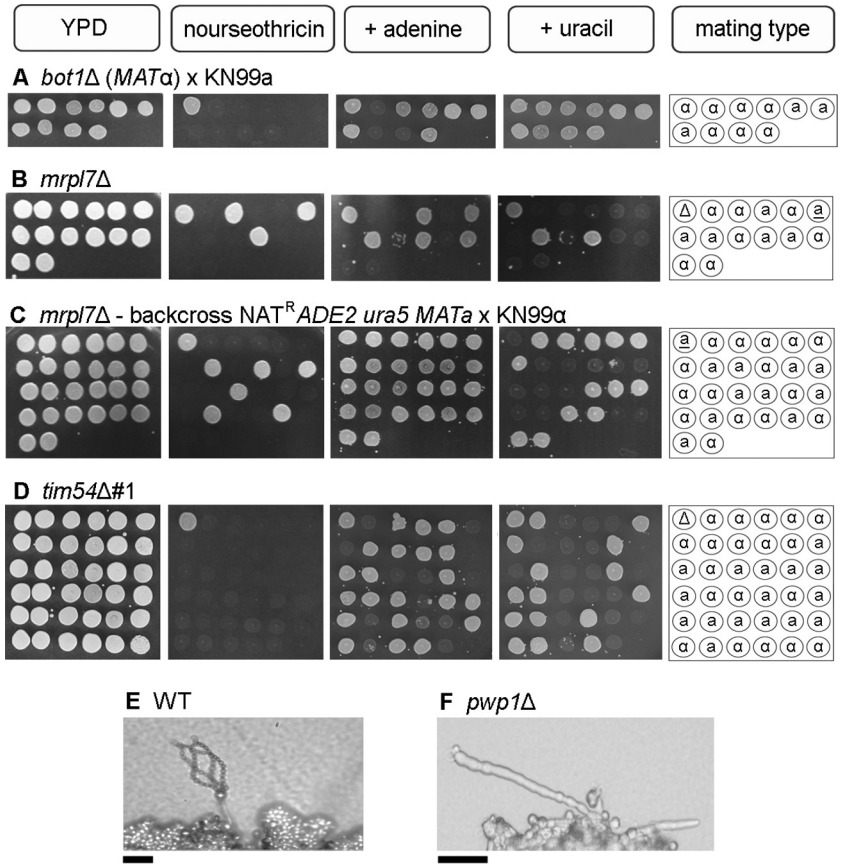

FIG 4 Representative examples of mutants generated through reverse genetics. In all cases, the first colony in the top left corner of all the panels represents the original heterozygous mutant from which the progeny analyzed originates. (A) Example of aneuploidy of mutant *BOT1/bot1*Δ strain for the *MAT***a** allele. Sexual reproduction in the heterozygous *BOT1/bot1*Δ strain was restored after the cross with KN99*α*, and resulting progeny were NAT^S, indicating that *BOT1* is an essential gene. (B) Segregation analysis of progeny derived from *MRPL7/mrpl7*Δ strain and example of backcross of a NAT^R colony performed to further assess the essentiality of *MRPL7*. Thirteen basidiospores germinated from the *MRPL7/mrpl7*Δ heterozygous mutant, and segregation analysis showed independent assortment for the four genetic markers. (C) Since only 3 NAT^R basidiospores were obtained, a number lower than the ~50% expected, one NAT^R *ADE2 ura5 MAT***a** progeny (underlined) was backcrossed with KN99*α*, and the segregation of the four markers was assessed again. Six NAT^R colonies were obtained, indicating that *MRPL7* is not essential for viability. (D) Segregation analysis of progeny derived from the *TIM54/tim54*Δ#1 heterozygous mutant; the generation of NAT^S progeny indicates that *TIM54* is an essential gene. (E and F) Lack of sexual structures for *PWP1/pwp1*Δ heterozygous mutant compared to the diploid AI187. Bars, 50 μM. WT, wild type diploid AI187.

Sfi1 spindle body protein domain that plays a role in spindle body duplication.

The *C. neoformans* orthologs of *PDC1*, *RIB2*, *RIB3*, *RSC9*, *SAF2*, *SEC5*, *THP1*, and *SPAPB24D3.06c* were replaced by targeted mutagenesis, and none were required for viability. The *S. pombe* gene *saf2*, which is absent in the *S. cerevisiae* genome, encodes a splicing-associated factor, and it contains a coiled-coil (Ncoils) domain (36). The *C. neoformans* homolog is annotated on the H99 database as a hypothetical protein with a WW domain. Despite the high similarity between Saf2 of *C. neoformans* and that of *S. pombe* (E value, 5.9e−13), this gene likely plays a different function in *C. neoformans*, since the WW domain is known to mediate regulatory protein complexes in various signaling networks. The case of the gene *THP1* is intriguing. In *S. cerevisiae*, *THP1* encodes a nonessential nuclear-pore-associated protein that is crucial for nuclear mRNA export and plays a role in mRNP biogenesis and genome maintenance (37). The *S. pombe* nuclear pore factor encoded by *SPBC1105.07* plays the same biological function as it does in *S. cerevisiae*, but it is required for viability. In *C. neoformans*, two independent *THP1/thp1*Δ heterozygous mutants were tested, and in both cases NAT^R haploid basidiospores were obtained, indicating that *THP1* is not an essential gene in *C. neoformans*. Lastly, CNAG_00592, which is the *C. neoformans* ortholog of another essential gene in *S. pombe* (*SPAPB24D3.06c*), was analyzed. CNAG_00592 is a dolichol-phosphate mannosyltransferase with three different domains: DUF1749, which plays an unknown function, and two alpha/beta hydrolases that are found in numerous enzymes. This gene is also not required for viability in *C. neoformans* since NAT^R progeny was isolated from the heterozygous mutant.

Attempts to generate targeted mutants for the genes *PHS1*, *TFAII*, *BRR6*, and *ROT1* were also performed, but for unknown reasons, homologous recombination was not successfully achieved.

Detection of "sick" strains: the component of the RAM signaling network *HYM1* as an example. The technique of germination of basidiospores offers advantages in examining essential gene functions over conditional promoters in questioning whether loss of a gene leads to no growth or a very poorly growing strain. The RAM pathway (from *r*egulation of *A*ce2 and cellular *m*orphology) is a conserved pathway in eukaryotes (38). There are

six characterized genes in the ascomycete yeasts *S. cerevisiae* and *S. pombe* (39). Mutation of the RAM pathway causes a pseudohyphal morphology in *C. neoformans* (40). Isolation of more than 36 pseudohyphal strains in two varieties and from different sources of mutations revealed mutations in five of these genes but never in *HYM1* (40, 41). Attempts to disrupt this gene in a haploid background were unsuccessful. One copy of the gene was therefore disrupted in the diploid strain, and the resulting heterozygous mutant sporulated. There was a low frequency (22%) of basidiospores yielding colonies, and only one colony was NAT^R (see Fig. S3A in the supplemental material). Further analysis showed that this colony was aneuploid for chromosome 8 and that it had both the wild-type and the mutated copies of *HYM1*; hence, it was able to grow under selective conditions. Examination of the basidiospores under the microscope revealed that many had germinated to produce small colonies with an elongated cell form reminiscent of the RAM mutants. Occasional cell rupture was also noticed. Thus, it appears possible that Hym1 is part of the RAM pathway in *C. neoformans* but has an additional role in the cell. This finding is consistent with studies in *Neurospora crassa*, in which the RAM pathway inputs and outputs have diverged from what is found in the ascomycete yeast species (42). The factor(s) in addition to the RAM pathway affected by loss of Hym1 currently remains unknown.

Bioinformatic comparisons for antifungal drug prioritization. The ideal protein target should be well conserved across the pathogenic fungi and lack mammalian orthologs, as inhibitors should not target human proteins. An example of a drug target with these features is the protein Fks1, which is the catalytic subunit of 1,3-beta-D-glucan synthase and is targeted by the echinocandins. To prioritize proteins for potential rational drug design, 56 genes of *C. neoformans* were subjected to BLASTp similarity searches against *S. cerevisiae* and *Homo sapiens* and against the human pathogens *C. albicans* and *A. fumigatus*. The 56 genes include 32 essential genes identified in the present study and 19 essential genes previously reported in the literature (see Table S1 in the supplemental material). As references, the genes of five known drug targets were included: (i) *FKS1*, which is targeted by echinocandins; (ii) *ERG1* and (iii) *ERG11*, both involved in ergosterol biosynthesis and targeted by terbinafine and by the azoles, respectively; (iv) *CDC60*, encoding an essential tRNA synthetase and targeted by AN2690, an emerging antifungal for topical application; and (v) *EFT2*, encoding elongation factor 2, which catalyzes ribosomal translocation during protein synthesis, targeted by sordarin.

To facilitate the interpretation of the BLASTp searches, two plots were generated in a similar way as previously done for *C. albicans* (17) (Fig. 5). To identify proteins that could be considered an ideal drug target (conserved in fungi but absent or having low similarity with human homologs), the plot of Fig. 5A shows the BLASTp outcome of the 56 *C. neoformans* proteins against the *S. cerevisiae* and *H. sapiens* protein set. The plot in Fig. 5B shows the conservation of the *C. neoformans* proteins with those in the ascomycete human pathogens *C. albicans* and *A. fumigatus*.

As expected, most of the genes are located on the left side of the plot in Fig. 5A, indicating higher homology of the *C. neoformans* proteins with *S. cerevisiae* than with their human counterpart. The genes for targeted replacement had been selected based on phylogenetic and functional analysis, and priority was given to those with low or null similarity to mammalian homologs. Therefore, most of the identified *C. neoformans* essential genes in this study are suitable candidate targets for a broad-spectrum antifungal drug. The essential genes *FBA1*, *TRR1*, *FOL1*, *TRL1*, and *MGM101* are the most conserved in ascomycete fungi, as validated also in Fig. 5B. Among the genes identified through insertional mutagenesis, *STU2*, *RRP40*, *ATG4*, and *RSA4* are well conserved in humans, and therefore, their encoded proteins are less likely to be candidate drug targets. Conversely, except *TRR1*, three other essential *C. neoformans* genes, CNAG_01800, CNAG_03404, and CNAG_03105, are all located in the lower left corner of the plots of Fig. 5A and B, indicating that they are not conserved genes and therefore are potentially suitable for narrow-spectrum *Cryptococcus* drug targets. Five other *C. neoformans* essential genes previously characterized, *FAS1*, *FAS2*, *HOM3*, *THR1*, and *IPC1*, also have excellent bioinformatic features as targets for broad-spectrum antifungal drugs, with *FAS2* overlapping the antifungal drug target *FKS1*. Notably, it is evident that two genes that are already targeted by antifungals, *CDC60* and *EFT2*, are located on the upper right corner of the plots of Fig. 5A and B and hence are conserved in fungi and humans. This indicates that antifungal drugs can be optimized to be selective despite their target being present in humans.

C. neoformans can gain resistance to antifungal agents through the process of heteroresistance, in which there is a duplication of chromosomes carrying the genes for the drug targets (10). Thus, it is important to understand the genome organization of the possible drug targets to aim for those on multiple chromosomes. All genes used for the scatter plot generation were mapped on the 14 *C. neoformans* H99 chromosomes. Essential genes known so far are located on all chromosomes with the exception of chromosome 10, with the majority located on chromosome 9 (see Fig. S4 in the supplemental material).

Chemical genetics and haploinsufficiency-induced profiling. Phenotypic analysis is a required step to understand the function of the genes in the cell. Heterozygous mutants, and some haploid progeny derived from the mutants, were tested for altered virulence traits and with a number of drugs and stressors. All the strains selected for analysis were initially tested as a single cellular spot (see Fig. S5 in the supplemental material). Several control conditions were tested in order to assess the phenomena of aneuploidy and/or auxotrophy, as it is known that these conditions might lead to altered phenotypes. All the selected strains showed nourseothricin resistance as expected. Diploid mutants were all prototrophs, and only in two cases (*BOT1/bot1*Δ and *SEN54/sen54*Δ strains) was a single *MAT* allele present, likely indicating aneuploidy for chromosome 5. Moreover, progeny isolated from heterozygous mutants for nonessential genes showed prototrophy and, as expected, the presence of only one *MAT* gene; only four mutants (*mdm34*Δ, *mmm1*Δ, *rib2*Δ, and *rib3*Δ strains) were uracil auxotrophs, as no prototrophic NAT^R progeny were produced from the respective heterozygous mutants. These four haploid mutants were sensitive to the drug 5-fluorocytosine (5-FC), but this is due to the uracil auxotrophy since the *ura5* mutant JF99 of *C. neoformans* is also sensitive to 5-FC (data not shown). Phenotypes obtained in the primary screen were confirmed by plating 10-fold serial dilutions of cultures. An exception is the insertional mutant 2aBIO2E9 that had the *NAT* insertion involving two genes (*asl1* and *ATG4*) and had a cold-sensitive phenotype (see Fig. S5 in the supplemental material). Heterozygous *BOT1/bot1*Δ and *TIM54/tim54*Δ and haploid *rsc9*Δ and *saf2*Δ strains were sensitive

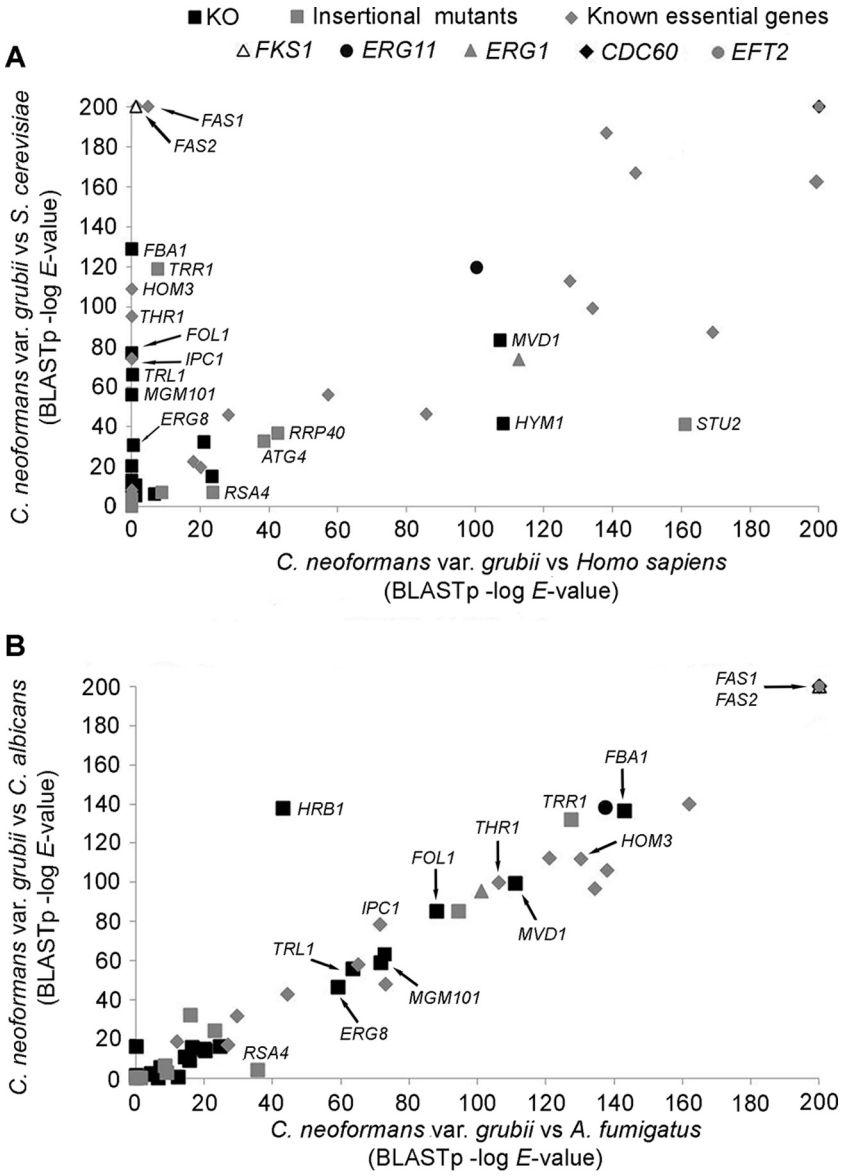

FIG 5 Scatter plot analyses showing the similarity between 56 *C. neoformans* defined or predicted essential genes against *S. cerevisiae* and *H. sapiens* (A) and the human pathogens *C. albicans* and *A. fumigatus* (B). The similarity is expressed as the negative logarithm of the E values obtained after BLASTp analysis. The higher the value, the higher the similarity, with 200 set as the maximum. 0 is the minimum value, indicating null or extremely low similarity.

to the cell wall-destabilizing agent sodium dodecyl sulfate (SDS). The haploid *sec5*Δ strain had impaired recovery after exposure at 37°C. The haploid *mrpl7*Δ strain was the only mutant that showed a partial inhibition of growth on the immunosuppressant drug rapamycin. The heterozygous *FOL1/fol1*Δ strain showed an expected sensitivity to the anticancer compound methotrexate, corroborating previous findings in *S. cerevisiae* (43). Among the antifungal drugs tested, phenotypes were obtained only for fluconazole (FLC). In particular, both heterozygous diploids and haploid mutants with mutations of *MRPL7*, *SEC5*, and *THP1* were hypersensitive. Note that the diploid strains of *C. neoformans* are more resistant to fluconazole than are the haploid strains, and this might be due to the presence of two copies of the fluconazole target gene *ERG11*. The *TIM54/tim54*Δ heterozygous mutant showed sensitivity to hydrogen peroxide, which caused a strong inhibition growth also for the heterozygous *HRB1/hrb1*Δ, *RSM18/rsm18*Δ, and *MRPS18/mrps18*Δ strains (Fig. 6).

DISCUSSION

The development of new drugs is the major challenge to decrease the morbidity and mortality caused by fungal infections. Fungal cells share many aspects of their basic biochemistry with mammalian cells, hence limiting the number of processes available for drug discovery and development. Despite the modest efforts in identifying attractive drug targets and repurposing existing medications, currently few drugs are broadly accepted, and therapy for fungal infections is still based on medications discovered decades ago (44, 45). The clinical arsenal available is limited to four different classes of systemic antifungals. Briefly, they include (i) amphotericin B, a broad-spectrum polyene targeting fungal ergosterols

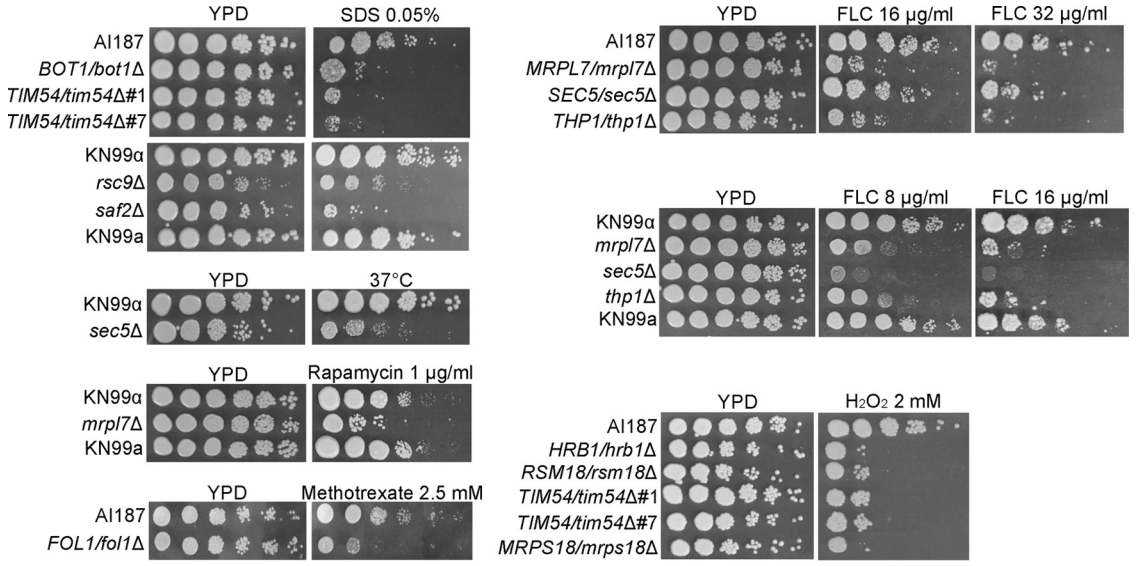

FIG 6 Phenotypes of *C. neoformans* heterozygous and haploid mutants. Tenfold serial dilutions of yeast cells were spotted on agar medium containing several stressors and drugs, as identified in Fig. S5 in the supplemental material. The concentrations of stressors and drugs that gave the phenotypes shown are indicated.

of the plasma membrane; (ii) triazoles, targeting the Erg11 enzyme involved in ergosterol biosynthesis, including fluconazole, itraconazole, and the more recently formulated posaconazole and voriconazole; (iii) 5-fluorocytosine (5-FC), which inhibits DNA synthesis and is mainly used in combination with the liposomal formulation of amphotericin B; and (iv) echinocandins, the most recently developed antifungals, which target Fks1 to inhibit the synthesis of 1,3-beta-D-glucan (46). In addition to the limited availability of drugs, treatments are often complicated by low tolerability, high toxicity, inefficacy, and the emergence of resistant strains. To overcome these deficiencies, combinations of available drugs having different mechanisms of actions have also been tested and evaluated in clinical trials (47). Nevertheless, their efficacy has yet to be proven, and there is desperate need of new and cheap fungicides that overcome the limits of the existing molecules.

Essential genes are attractive drug targets. The most widely used strategies for identifying essential genes in fungi include targeted gene replacement in a diploid background and phenotypic analysis of derived progeny, or the use of conditional promoters and analysis of the phenotype under repressive or inducible conditions. Other techniques also exist (13). In the present study, both reverse and forward genetics were used for the identification of essential genes in the human pathogen *C. neoformans* through the generation of mutants in a diploid background. Forward genetics performed through AMT or biolistic transformation have been largely used in *C. neoformans* serotype A or D for identifying genes involved in cellular processes (48–50). Nevertheless, those techniques have never been applied for large-scale identification of genes required for viability. In one case, Kraus et al. (51) identified a temperature-sensitive mutant of *C. neoformans* serotype D bearing a *NAT* insertion downstream of the calmodulin gene *CAM1*. Kraus et al. subsequently demonstrated that it was required for viability by generating a targeted mutant in a diploid background. In the present study, we sought to establish approaches that could yield the identification of the full set of *C. neoformans* essential genes. The advantages of a random mutagenesis approach over targeted mutagenesis are that it does not require knowledge of the function of the gene nor its structure and its location. This aspect is even more significant in the case of *C. neoformans*, since it belongs to the Basidiomycota, which as a group are less well studied than the Ascomycota.

To test the feasibility of the insertional mutagenesis approaches, more than 2,500 transformants were tested, and 127 were selected in the primary screen. The 127 selected strains were individually transferred on MS medium to undergo meiosis and basidiospore production for genetic analysis. Results obtained were surprising, as only 15 strains (~12%) were found to generate inviable progeny. The screen revealed interesting differences between basidiomycete and ascomycete essential genes. Six genes that were identified as essential in *C. neoformans* (*RRP40*, *TRR1*, *STU2*, *RSA4*, *ARC19*, and *CET1*) have orthologs that are also essential in the model yeast *S. cerevisiae*. One gene (*ATG4*) was found to be essential in *C. neoformans* but not essential in *S. cerevisiae*. Two genes (CNAG_01800 and CNAG_03404) found to be essential in *C. neoformans* are not present in *S. cerevisiae* but are found in other fungi. And finally, one gene (CNAG_03105) that was determined to be essential in *C. neoformans* was found only in *C. neoformans* var. *grubii* and has no orthologs. For the remaining strains selected from the primary screen, the gene responsible for the inviable phenotype could not be unequivocally assigned (Table 1). Strikingly, we also isolated nine *C. neoformans* strains that had insertions involving orthologs of *S. cerevisiae* that are all required for viability but that showed a clear viable phenotype after segregation analysis of dissected haploid progeny (see Table S2 in the supplemental material).

The ability to identify and distinguish genes uniquely essential in basidiomycete species or *Cryptococcus* from genes that are essential in *S. cerevisiae* but not in *C. neoformans* highlights the usefulness of an unbiased search for these genes using forward genetics. Similar findings were reported for the identification of essential genes in the human pathogen *A. fumigatus* (14). In that

study, the authors used the transposon *impala* to generate random mutations and identified 96 predicted essential genes, some of which had unknown function and/or whose orthologs were not present in *S. cerevisiae*. From this perspective, a random mutagenesis strategy had advantages over the one used by Hu et al. (15), in which the predicted *A. fumigatus* promoter of orthologs to previously characterized essential genes in *S. cerevisiae* and *C. albicans* was replaced with a regulatable promoter.

Despite its benefits, the insertional mutagenesis approach used in the current study has limits in application for large-scale identification of essential genes in *C. neoformans*. The genetic screen relies on the basidiospores to germinate on medium at pH 4 containing nourseothricin and 5-FOA. Thus, strains sensitive to low pH or a combination of nourseothricin and 5-FOA would be isolated in the primary screen. In addition, strains with impaired sexual fertility, reduced germination rates, and other events that influence the sexual cycle, such as chromosomal rearrangements or the lack of either one of the *MAT* genes, would also be isolated in the primary screen. For example, ~20 strains had chromosomal rearrangements that affected basidiospore germination in *C. neoformans*. The germination defect was characterized by the less than 30% germination achieved in four strains that were tested (2a63, 2a129, 2a46, and 2a13). Moreover, 27 other strains were unable to produce chains of basidiospores after growth on MS medium, and further analysis revealed that five of them were characterized by the presence of only one of the two *MAT* alleles (Fig. 2B). In two other cases (2aGIPlate18E5 and 2a295), the lack of germination was not due to the gene hit by the T-DNA (*RSA4* and *THP1*, respectively) because *RSA4/rsa4*Δ and *THP1/thp1*Δ targeted heterozygous mutants were both able to produce basidiospores. In this case, subsequent segregation analysis revealed that *RSA4* is essential for viability in *C. neoformans* (Fig. 3B) and *THP1* is not. Idnurm (52) and Ni et al. (53) demonstrated that improper segregation of genetic markers in *C. neoformans*, as well as low germination rates of dissected basidiospores, is mainly due to aneuploidy. Moreover, aneuploid basidiospores can be routinely isolated from basidia and can be responsible for altered phenotypes. Therefore, it seems likely that the majority of the insertional mutants isolated in the present study were characterized by aneuploidy, and this accounted for the isolation of a high number of potentially inviable strains in the primary screen and the inability to characterize several others in the subsequent Mendelian genetic analysis. However, aneuploidy was observed at a lower rate in the reverse genetic approach discussed below, suggesting that transformation using *Agrobacterium tumefaciens* may contribute to this phenomenon.

Using a reverse genetics approach, a set of 35 genes present as a single copy in the *C. neoformans* genome were mutated. This approach identified 21 genes required for viability, 13 nonessential genes, and one that could not be classified because the mutant was unable to produce haploid basidiospores (Table 2). Cases of aneuploidy were also observed for mutants generated through reverse genetics but with less frequency than for mutants generated with the T-DNA insertion. A reverse genetics strategy is more straightforward and likely more effective at resolving the complete set of essential genes in *C. neoformans*, especially if coupled with the creation of a complete gene deletion set of strains (32).

Our genetic analysis revealed that the basidiomycete *C. neoformans* has both common and specific features compared to the model ascomycetes *S. cerevisiae* and *S. pombe*. The majority of genes that we identified as essential in *C. neoformans* are essential in multiple fungi, such as *BUR6*, *CET1*, *ERG8*, *FBA1*, *KEI1*, *MVD1*, *TIM54*, etc. Moreover, we also identified genes that are essential in *C. neoformans* and *S. pombe* but absent or not required for viability in *S. cerevisiae*, such as *BYR4*, *BOT1*, *CDC14*, *MRPL31*, and *MGM101*. Furthermore, other genes like *RIB2*, *RIB3*, and *RSC9* are essential in the ascomycetes, and *MDM10*, *MDM34*, *MMM1*, and *MRPL7* are essential only in *S. pombe*. *HRB1* is the only gene selected for targeted replacement that is essential only in *C. neoformans*. A comparison of gene essentiality in *C. neoformans*, *S. cerevisiae*, and *S. pombe* is presented in Table S4 in the supplemental material.

One experimental benefit of the heterozygous mutants is that they are useful for assessing haploinsufficiency in *C. neoformans*. Haploinsufficiency is a condition in which the loss of function of one gene copy in a diploid organism results in an abnormal phenotype. Haploinsufficiency is a major field of research in humans as it is implicated in several diseases (54). In fungi, haploinsufficiency was pioneered in *S. cerevisiae* using a collection of heterozygous deletion strains, and it was proven to be effective in characterizing the mechanism of action and to identify targets of drugs and other bioactive compounds (43, 55). Subsequent haploinsufficiency assays were performed in *S. pombe* (20) and in the pathogen *C. albicans* (56, 57), both being ascomycetes fungi. In the present study, we report for the first time a screening for haploinsufficient phenotypes in a basidiomycete, *C. neoformans*. Strains subjected to analysis were the diploid mutants generated through reverse genetics and a subset of the diploid mutants generated through random mutagenesis. Excluded strains were those characterized by suspected aneuploidy, chromosome rearrangements, and multiple insertions, which were unlikely to give a correct phenotype. A classical chemical genetic screen was also performed using haploid mutants derived from heterozygous mutants of nonessential genes.

In the chemical-genetic experiments, both known and novel information was found (Fig. 6). Corroborating the results previously obtained in *S. cerevisiae* (43), the *C. neoformans FOL1/fol1*Δ strain showed sensitivity to methotrexate, indicating that the folic acid biosynthetic pathway is conserved across the fungal kingdom. This also highlighted the importance of Fol1 as a potential drug target since no ortholog of this essential protein exists in humans. Fluconazole caused growth inhibition both for heterozygous diploids and for *mrpl7*Δ, *thp1*Δ, and *sec5*Δ haploid mutants, which might indicate that these three nonessential proteins can operate in conjunction with sterol biosynthesis pathways. Moreover, the *mrpl7*Δ haploid mutant of *C. neoformans* was also sensitive to rapamycin, a finding that is in contrast to the *S. cerevisiae* mrpl7Δ mutant, which is rapamycin resistant (58). Thus, it is evident that Mrpl7 has different functions in *S. cerevisiae* and *C. neoformans*, despite their high sequence similarity (E value, 8.8e−32). It can be speculated that *C. neoformans* Mrpl7 is involved in drug metabolism and/or its functionality is required for buffering (i) the ergosterol biosynthesis pathway, targeted by fluconazole, and (ii) the TOR (target of rapamycin) pathway, involved in rapamycin resistance.

The analysis of stress responses of the *C. neoformans* strains also revealed additional cellular functions for some essential proteins. For example, Tim54, Hrb1, Rsm18, and Mrps18 are most likely involved in oxidative stress resistance, supported by the increased sensitivity to hydrogen peroxide when these genes are mu-

tated. Bot1 and Tim54 were found to be involved in cell wall maintenance, supported by the increased sensitivity to SDS in the heterozygous strains carrying *bot1* and *tim54* mutations. A similar function in cell wall maintenance has been reported for *S. pombe* Bot1 (59). The *C. neoformans* protein Saf2 is also involved in the cell wall regulatory network, as suggested by the sensitivity of the *saf2*Δ mutant to SDS. This supports the bioinformatic findings that suggested a difference in roles between *C. neoformans* and *S. pombe* Saf2, characterized by the presence of different functional domains: a WW domain in *C. neoformans* and a coiled-coil (Ncoils) domain in *S. pombe*. No mutants were defective in virulence attributes, with the exception of the *sec5*Δ mutant that showed weak recovery after exposure to human body temperature, corroborating previous findings (32).

In conclusion, the present study aims for the first time at the systematic identification of essential genes in fungi, using a basidiomycete fungus as a model organism. With both forward and reverse genetics, more than 30 essential genes were identified, significantly increasing the number of known genes required for viability in *C. neoformans*. The results of this study supplement the critical lack of information on essential genes in basidiomycetes and provide crucial insights that help to integrate the knowledge of ascomycetes for the development of novel drugs to treat fungal infections successfully.

MATERIALS AND METHODS

Strains and culture conditions. The diploid *C. neoformans* var. *grubii* strain AI187 (*ade2*/*ADE2 ura5*/*URA5 MAT***a**/*MAT*α; FGSC 10993) was used as the wild type (52). The strain was created through the fusion of strains JF99 (*ura5 MAT***a**) and M001 (*ade2 MAT*α), which are two haploid auxotrophs of opposite mating type, and maintained on yeast extract-peptone-dextrose agar medium.

Insertional mutagenesis. Insertional mutagenesis was performed using *Agrobacterium tumefaciens* and biolistic transformations. For *Agrobacterium*-mediated transformation (AMT), strain EHA105 containing the plasmid pPZP-NATcc was used; for biolistic transformation, the *NAT* marker was delivered using 1 μg of XbaI-linearized plasmid pAI3. Strains were transformed by standard methods (48, 49), and transformants were selected on YPD supplemented with nourseothricin (100 μg/ml), and with cefotaxime (200 μg/ml) in the case of AMT. Colonies were patched onto Murashige-Skoog (MS) medium at pH 5 (60) and incubated at room temperature in the dark to induce the meiotic cycle and sporulation to produce haploid basidiospores. Usually, this process took from 1 to 4 weeks. The sporulating strains were velvet replica plated onto YPD medium containing nourseothricin (100 μg/ml), 5-fluoroorotic acid (1 g/liter), and adenine (20 mg/liter); adjusted to pH 4.0; and grown for 4 to 7 days at 30°C. Strains selected in the primary screen were single colony purified and retested by genetic segregation analysis as described below.

Targeted gene replacement. A set of genes was identified for targeted disruption in *C. neoformans* by different bioinformatics approaches. First, a database of gene information on *C. neoformans* divided into classes (e.g., found in other fungi, not animals) was queried (61). A total of 509 *C. neoformans* genes were found in other fungi but not mammals. Equivalent analyses, such as that performed using five ascomycete species that identified 226 fungus-specific genes (62), provide different numbers depending on the BLAST cutoffs used. The literature was then examined for the functions of the 509 genes in *S. cerevisiae* and/or *S. pombe* to select those required for viability. Genes were further prioritized for those that are single copy in *C. neoformans* and found in the majority of fungi. Second, an analysis of essential genes present in *C. albicans* and *A. fumigatus* but not present in mammals provided 57 hits (63). Third, 91 genes were reported as essential in both *S. cerevisiae* and *S. pombe* but without mammalian homologs (20). Fourth, 175 genes specific to *C. albicans* and absent/diverged in humans were placed under the control of a regulatable promoter and the phenotypes of the strains were analyzed *in vitro* and in mice (64). From compiling this information and excluding the already-known essential genes of *C. neoformans*, 35 genes were selected for analysis in *C. neoformans* (Table 2).

Constructs for targeted gene replacement were generated by *in vivo* recombination in *S. cerevisiae* as previously described (65). The primers used to amplify the fragments are listed in Table S3 in the supplemental material. The constructs consisted of 1.5-kb flanking regions fused with the *NAT* cassette. The sites for the insertion of *NAT* were distant at least 200 bp from the start codon of the gene to be replaced and at least 500 bp from the start codon of the adjacent genes. This design was to avoid any interference with the adjacent genes. The replacement alleles were precipitated onto gold beads and transformed into *C. neoformans* using a Bio-Rad particle delivery system, which is the standard method for targeted mutagenesis in *C. neoformans* (66). Transformants were screened for homologous recombination events by PCR, and single-copy insertions were confirmed by Southern blot analysis using the *NAT* probe on DNA digested with EcoRV (one cut site in the middle of the *NAT* cassette yielding the expected two bands of sizes predicted from the genome sequence). For Southern blot analysis of transformants generated by insertional or targeted mutagenesis, ~2 μg of genomic DNA was digested with either ClaI or EcoRV, respectively; resolved on 0.8% agarose gels in 1× Tris-acetate-EDTA (TAE) buffer; and transferred to a Zeta-Probe membrane (Bio-Rad). The blots were probed with [^{32}P]dCTP-labeled DNA of the *NAT* open reading frame (ORF).

Genetic analyses. The strains generated with forward genetics selected in the primary screening and those generated with reverse genetics were individually streaked onto MS medium plates to induce meiosis and sporulation. Micromanipulation was carried out as previously described (52). For each strain, at least 30 haploid basidiospores from mixed populations were dissected onto YPD agar plates. Resultant colonies were grown for 3 to 4 days at 30°C, transferred to a 96-well plate containing 100 μl of YPD, and tested for the four genetic markers that segregate during meiosis (nourseothricin resistance [NAT^R] or sensitivity [NAT^S], *ura5/URA5*, *ade2/ADE2*, and *MAT***a**/*MAT*α) by spotting 3 μl of cell suspensions onto YPD plus nourseothricin (100 μg/ml), YNB plus adenine (20 mg/liter), or YNB plus uracil (40 mg/liter). The mating type marker was scored by crossing haploid progenies to strains JEC20 and JEC21 or KN99**a** and KN99α on V8 or MS medium supplemented with adenine and uracil and by evaluating the formation of sexual structures under a microscope.

Mutants unable to produce basidiospores were tested by PCR analysis for the presence of the mating type **a** and α alleles using primer pairs ai144-ai145 and ai150-ai151, respectively. When either one of the *MAT* alleles was absent, the strains were crossed with the opposite mating type of the congenic strain pair KN99**a** and KN99α (67).

In cases of atypical segregation of the *NAT* marker that prevented assignment of whether or not a gene was essential for viability, additional crosses were used. NAT^R progenies were either self-sporulated or backcrossed on MS medium with the opposite mating type of strain KN99**a** or KN99α, haploid basidiospores were micromanipulated, and the segregation of the four markers was assessed again. In some cases, PCR was performed to assess either the absence of the replaced regions in targeted mutants or the presence of the T-DNA in insertional mutants.

DNA manipulations. Fifty-milliliter overnight cultures were grown in YPD liquid medium. DNA was extracted with a cetyltrimethylammonium bromide (CTAB) extraction buffer from lyophilized cells. For mutants generated using AMT or biolistic transformation, inverse PCR was used to identify the genes hit by the insertions; when this was unsuccessful, splinkerette PCR was used.

For inverse PCR, approximately 2 μg of DNA was digested with restriction enzymes ClaI, NcoI, and NdeI (6-bp recognition sites) or NlaIII and TaqI (4-bp recognition sites). The mix was purified through a Qiagen column or by precipitation with 1/10 volume of 3 M sodium acetate and 2 volumes of 100% ethanol. The DNA was eluted or resuspended in 30 μl of

water. An 8.5-μl amount was self-ligated with T4 DNA ligase (New England Biolabs) in a 10-μl volume overnight at 4°C, and 1 μl was used as the template for inverse PCR with primers ai076-ai077.

Splinkerette PCR was performed as previously reported (68). Briefly, genomic DNA was digested with a restriction enzyme producing GATC sticky ends (BamHI, BglII, BstYI, or BfuCI) and ligated with splinkerette adaptors (ALID1809 and ALID1810) overnight at 4°C using T4 DNA ligase. The first-round PCR was performed using the primer ALID1811-S1 in combination with ALID1813-T1 (for identifying junctions at the left border) or ALID1814-T4 (for junctions at the right border). The second-round PCR was performed with the primer ALID1812-S2 in combination with ai077 (for identifying junctions at the left border) or ai076 (for junctions at the right border) using as the template the PCR products obtained in the first round.

PCR products were resolved on agarose gels, excised, and purified, and DNA was sequenced. Sequencing results were compared to the H99 genome database at the Broad Institute. The predicted *C. neoformans* proteins encoded by the genes bearing the *NAT* insertions were compared by BLASTp analysis to the *Saccharomyces* genome database to identify orthologs and to predict their function. In the case of no or low similarity, BLASTp was performed on the *S. pombe* genome database PomBase and on GenBank. The gene nomenclature followed guidelines proposed by Inglis et al. (69).

Bioinformatic comparison of *C. neoformans* essential genes. Fifty-six *C. neoformans* genes were used for bioinformatic analysis. These include 32 essential genes identified in the present study with forward and reverse genetics approaches (Tables 1 and 2), 19 known essential genes previously reported in the literature (*TRR1* was included in the genes identified in this study and *FKS1* in the drug targets [see Table S1 in the supplemental material]), and 5 known drug targets (*FKS1*, *ERG1*, *ERG11*, *CDC60*, and *EFT2*), which were identified in the *C. neoformans* H99 database through BLASTp analysis of *S. cerevisiae* orthologs obtained from the SGD (http://www.yeastgenome.org/). The translated sequences of the 56 *C. neoformans* genes were retrieved from the H99 database and used for BLASTp similarity searches against *S. cerevisiae* on SGD, *C. albicans* on CGD (http://www.candidagenome.org/), *A. fumigatus* on AspGD (http://www.aspergillusgenome.org/cgi-bin/compute/blast_clade.pl), and *Homo sapiens* on GenBank. BLASTp was performed using default settings. The *E*-value of the best hits was recorded and converted into a negative logarithm used to express the similarity: the higher the −log value, the higher the similarity. The value of 200 was set as the maximum and includes values greater than 200; 0 is the minimum value, indicating absence or extremely low similarity (*E*-value of ≥1.00E+00). The 56 genes used for generation of the scatter plots were mapped on the 14 *C. neoformans* H99 chromosomes using the recent genome sequence release version CNA3 (23).

Chemical genetic screen and phenotypic analysis. Phenotypic analysis was performed using the heterozygous mutants and viable NAT^R haploid mutant progeny where nonessential genes were targeted. The phenotypes of selected strains were initially tested as single spots on different agar media, with the exception of the growth in low-iron medium (LIM). The control medium types were YPD, YPD plus nourseothricin, minimal medium (MM), MM plus uracil, and MM plus adenine. The mating type was scored by crossing the heterozygous mutants and the selected haploid progeny with JEC20 and JEC21. Several stressors dissolved in YPD agar at different concentrations were also tested: 1.5 mM NaCl, 0.5% (wt/vol) Congo red, 0.05% (wt/vol) sodium dodecyl sulfate (SDS), 10 μg/ml ethidium bromide, 0.4 mM *tert*-butyl hydroperoxide (*t*BOOH), 2 mM hydrogen peroxide (H_2O_2), 0.05% methyl methanesulfonate (MMS), 2.5 μg/ml benomyl. Strains were also exposed to UV light (120 J/m^2) using a UV cross-linker and for cold stress were incubated at 4°C for 2 months. Sensitivity drug tests were performed on YPD agar supplemented with different drugs at a range of sublethal concentrations identified where available through an Etest strip using the *C. neoformans* diploid strain AI187. Rapamycin and FK506 were used at 3 μg/ml, tunicamycin was used at 0.75 μg/ml and 1 μg/ml, 5-fluorocytosine (5-FC) was used at 100 μg/ml, fluconazole (FLC) was used at 32 μg/ml and 48 μg/ml, and amphotericin B was used at 0.75 μg/ml and 1 μg/ml in yeast nitrogen base (YNB) medium (since amphotericin was ineffective when dissolved on YPD). Altered virulence attributes were evaluated as growth of the heterozygous and haploid mutant strains at human body temperature (37°C) on YPD agar, melanin production on L-3,4-dihydroxyphenylalanine (L-DOPA) medium, and capsule formation through microscopic examination of cells stained with India ink after growth for 3 days in liquid LIM.

Strains that showed a phenotype were confirmed by 10-fold serial dilution in liquid YPD and by spotting 2.5 μl of cell suspension on the appropriate agar medium; in these experiments, different stressors and drug concentrations were retested, and the ones that gave the clearest phenotypes were photographed. The anticancer agent methotrexate was tested at a 2.5 mM concentration only for the *C. neoformans FOL1/fol1*Δ heterozygous mutant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02334-14/-/DCSupplemental.

Figure S1, TIF file, 0.1 MB.
Figure S2, PDF file, 2.3 MB.
Figure S3, TIF file, 1.5 MB.
Figure S4, TIF file, 0.1 MB.
Figure S5, PDF file, 1.8 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.
Table S3, XLSX file, 0.02 MB.
Table S4, DOCX file, 0.1 MB.

ACKNOWLEDGMENTS

We thank Suman Chaudhary, Karisa Deculus, Brian Foley, and Denise Magditch for their technical help and Brooke Esquivel for comments on the manuscript.

This research was supported by grants from the University of Missouri Research Board, the U.S. National Institutes of Health (NIAID R21 AI094364), and the Australian Research Council.

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