DNA Mutations Mediate Microevolution between Host-Adapted Forms of the Pathogenic Fungus Cryptococcus neoformans

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Abstract

The disease cryptococcosis, caused by the fungus Cryptococcus neoformans, is acquired directly from environmental exposure rather than transmitted person-to-person. One explanation for the pathogenicity of this species is that interactions with environmental predators select for virulence. However, co-incubation of C. neoformans with amoeba can cause a “switch” from the normal yeast morphology to a pseudohyphal form, enabling fungi to survive exposure to amoeba, yet conversely reducing virulence in mammalian models of cryptococcosis. Like other human pathogenic fungi, C. neoformans is capable of microevolutionary changes that influence the biology of the organism and outcome of the host-pathogen interaction. A yeast-pseudohyphal phenotypic switch also happens under in vitro conditions. Here, we demonstrate that this morphological switch, rather than being under epigenetic control, is controlled by DNA mutation since all pseudohyphal strains bear mutations within genes encoding components of the RAM pathway. High rates of isolation of pseudohyphal strains can be explained by the physical size of RAM pathway genes and a hypermutator phenotype of the strain used in phenotypic switching studies. Reversion to wild type yeast morphology in vitro or within a mammalian host can occur through different mechanisms, with one being counter-acting mutations. Infection of mice with RAM mutants reveals several outcomes: clearance of the infection, asymptomatic maintenance of the strains, or reversion to wild type forms and progression of disease. These findings demonstrate a key role of mutation events in microevolution to modulate the ability of a fungal pathogen to cause disease.

Introduction

Pathogens across all major microbial groups – viruses, bacteria, fungi and protists – have representative species that owe their success to rapid change during infection or within a population. Microevolution is thus essential to pathogenesis, yet due to its stochastic nature it can be difficult to study and the underlying mechanisms challenging to elucidate.

Cryptococcus neoformans is a fungal pathogen that is acquired directly from the environment through inhalation of desiccated yeast cells or the sexual basidiospores. The fungus is found world wide and it causes disease predominantly in immunocompromised individuals, especially AIDS patients [1,2]. The global mortality rate is estimated at 624,000 per annum [3]. The closely-related species C. gattii causes disease mostly in healthy individuals, and is responsible for an ongoing and expanding outbreak of cryptococcosis in the Pacific Northwest of Canada and the United States [4,5,6]. Both Cryptococcus species are extensively studied, have a suite of experimental resources, and serve as general models for understanding pathogenesis and its evolution in pathogenic eukaryotes [7,8]. Cryptococcus species undergo microevolution in vitro, within animal models, and during the course of disease in humans [9,10,11,12].

A current hypothesis is that the Cryptococcus species are pre-selected for virulence within mammalian animals because of interactions with predatory microbes like amoeba or nematodes [13,14,15,16]. Evidence for this comes from studies on interactions with non-mammalian hosts. C. neoformans has been co-isolated with three different Acanthamoeba species [15,17,18] and these amoebae can take up the fungus by phagocytosis [19,20]. Genes that are essential for mammalian virulence are also required for virulence in non-mammalian models [21]. Furthermore, screens of insertional mutants of the fungus with the nematode Caenorhabditis elegans identified fungal genes required for nematode viability: deletion of these genes also reduces virulence in mouse models of cryptococcosis [22,23]. Additional support for the hypothesis comes from passage of C. neoformans through a slime mold host, Dictyostelium discoideum, since this produces strains with increased virulence in mice [24]. However, one caveat is that Acanthamoeba species ingest and kill C. neoformans. Surviving subpopulations can be isolated, including one common class that has pseudohyphal cells rather than the normal yeast shape [17]. The pseudohyphal strains were avirulent in animal models [17,25,26]. Strikingly, the pseudohyphal phenotype is not always stable. One of the eight pseudohyphal strains originally isolated, after wild
Author Summary

Many diseases are contracted from the environment, rather than from sick people. It is unclear why those species are able to cause disease, since the selective pressures in the environment are presumed to be very different from those found within the host. Cryptococcus neoformans is a fungus that causes life-threatening lung and central nervous system disease in approximately one million people each year. The fungus is inhaled from environmental sources. One hypothesis to account for C. neoformans virulence is that amoeba are predators for this fungus, and surviving strains are pre-selected to be virulent in the human host. On the other hand, experiments have found that amoeba eat C. neoformans. A pseudohyphal cell type can survive, and while protecting against amoeba these cells are unable to cause disease in mouse models. We predicted that the pseudohyphal morphology reflected a change in function of a pathway of genes, and that all pseudohyphal isolates contain mutations within genes for this pathway. The pseudohyphal trait is unstable, with reversion to normal yeast growth by counter-acting mutations. These mutations can occur during the course of mammalian infection. Our results show that mutation events account for a microevolution system currently described as phenotypic switching, and that mutations, at least under experimental conditions, can regulate pathogen adaptation and influence its host range.

type strains were exposed to amoeba, was inoculated into mice and it demonstrated wild type virulence [17]. Closer examination revealed, to quote directly, that “a high percentage of the cells in the inoculum of this isolate had reverted to the encapsulated yeast form” [17], a description of an unstable trait governed by epigenetics or microevolution. In repeat experiments, including use of different wild type strains of C. neoformans and a different species of Acanthamoeba, pseudohyphal isolates were again obtained: the phenotype exhibited instability in some, but not all, strain backgrounds [15, 25]. More recently, a similar pseudohyphal morphology was reported from in vitro experiments, and that it too could revert back to wild type at a high frequency (e.g. an average of 1 revertant per 1600 colonies) [27]. This high frequency of phenotypic change is referred to as phenotypic switching. The basis for this rapid evolution in C. neoformans remained unknown. The pseudohyphal morphology of strains from amoeba or phenotypic switching appear similar to those of C. neoformans strains with loss-of-function mutations in the RAM/MOR pathway of genes [28]. This pathway is conserved in eukaryotes and characterized primarily in Saccharomyces cerevisiae where the abbreviation is from Regulation of Ace2p activity and cellular Morphogenesis [29, 30]. In Schizosaccharomyces pombe the pathway is known as MOR, for the Morphogenesis-related NDR kinase network. The pathway comprises six components, centered around the action of two protein kinases Cbk1 and Kic1 and the accessory proteins Sog2, Hym1 and Mob2. The large size and physical interaction of Tao3 with Cbk1 and Kic1 suggests that Tao3 may act as a scaffold protein [29, 31]. Within the fungi, the pathway can have dramatically different effects on cell type, for instance promoting cell polarity in the ascomycete yeast S. cerevisiae whereas inhibiting it in the basidiomycete yeast C. neoformans [28, 29]. It is required for virulence in plant and human pathogenic fungi [32, 33, 34, 35], and polymorphisms in KIC1 recently emerged from genome wide association studies between clinical and non-clinical isolates of S. cerevisiae [36]. The role of the RAM pathway in pathogenesis has been most thoroughly analyzed in Candida albicans, in which components mediate cell separation and polarity, and thus mutations block filamentation, impair biofilm formation and surface adhesion, and reduce virulence [34, 37, 38, 39, 40, 41, 42]. Mutation of the RAM pathway in C. neoformans causes a pseudohyphal morphology and other phenotypic changes [28], although effects on virulence had not been tested.

We hypothesized that the RAM pathway is affected in strains that “switch” morphology either in vitro or upon exposure to amoeba, and that the analysis of this process would provide insight into the mechanism of microevolution. In this study, we identify mutations within a RAM pathway gene in original pseudohyphal mutants derived from amoeba and phenotypic switching. We recapitulate the isolation of pseudohyphal strains by both means, and find that these strains all have mutations in the RAM pathway. A strain derived from amoeba with a point mutation in the MOB2 gene was unable to cause disease in a murine model, unless during the course of infection the mutation reverted to wild type. Switching back to yeast morphology relies on a multifactor system for microevolution that is also driven by DNA mutations. These findings demonstrate that DNA mutation contributes to fungal pathogenesis.

Results

Pseudohyphal isolates of C. neoformans of different origins have similar phenotypes

The C. neoformans species consists of two varieties, var. grubii (serotype A) and var. neoformans (serotype D). Pseudohyphal strains have been isolated from both varieties (table S1). Three isolates of C. neoformans var. grubii from the 1970s that originated after exposure to amoeba (strains C, D and E; ATCC 42343–42345 [17]) and one C. neoformans var. neoformans isolate from the 1990s that originated from a phenotypic switching study (strain F7 [27]) were compared to defined deletion strains of the RAM pathway genes. The strains had similar pseudohyphal cell morphologies distinguishing them from wild type yeast cells (Fig. 1A). Most

Figure 1. Pseudohyphal strains derived from three origins share similar characteristics. The tao3Δ strain is in the KN99α background. Strains C, D and E were isolated after exposure to amoeba, probably in the strain G background. All three strains derived from exposure to amoeba exhibited identical phenotypes, so just D is illustrated. F7 is a phenotypic switching isolate in the ATCC 24067A background. WT = wild type. A. Light microscopy of cells (bar = 50 μm). B. Growth under different conditions. Cells were 10-fold serially diluted and spotted onto YPD medium with or without FK506 (1 μg/ml), and grown for two days at 30°C or 37°C. doi:10.1371/journal.ppat.1002936.g001
strains showed decreased growth at mammalian body temperature (Fig. 1B). Consistently, all were highly sensitive to the immunosuppressive chemical FK506 (Fig. 1B). This drug inhibits the calcineurin pathway, the impairment of which is synergistically lethal with RAM pathway mutation in *C. neoformans* [28]. The similarity in phenotypes between the strains isolated from amoeba and phenotypic switching with defined deletion strains suggests the same genes or pathways are affected, and that this could be the RAM pathway.

**Pseudohyphal isolates derived from amoeba or phenotypic switching have premature stop codons in the RAM pathway TA03 gene**

We hypothesized that the historical pseudohyphal strains represent loss of the RAM pathway. The nature of the deficiency was sought in the strains that had been isolated from amoeba and the phenotypic switched isolate. Epigenetic or microevolutionary changes can arise from a suite of different causes. As a first approach towards gene identification, constructs containing wild type copies of four of the genes in the pathway (*MOB2*, *CBK1*, *KIC1* and *SOG2*) were introduced into strains C, D, E and F7 with the endeavor to identify the affected gene by expressing additional copies. None of the four genes restored growth to the wild type yeast form, whereas these constructs complemented deletion strains. A fifth RAM pathway gene, *HIM1*, has not emerged as a RAM component in *C. neoformans* from random mutant screens, and has thus far eluded gene replacement experiments. The sixth component of the pathway, *TAO3*, is large and it was a challenge to generate a vector for this gene for transformation experiments. However, the lack of evidence for a direct role by the other members of the RAM pathway provoked closer examination of the *TAO3* gene in the pseudohyphal strains.

The *TAO3* gene was sequenced from the four historical pseudohyphal strains. The three strains isolated after exposure to amoeba (strains C, D and E) all contained an identical predicted G-T base pair substitution, that results in a codon for glutamic acid being substituted for a premature stop codon (Fig. 2A; dataset S1).

![Figure 2. Pseudohyphal strains of *C. neoformans* derived from independent sources bear RAM pathway mutations.](image)

**Figure 2. Pseudohyphal strains of *C. neoformans* derived from independent sources bear RAM pathway mutations.** The TA03 gene is most commonly mutated in pseudohyphal strains. Mutations in “historical” isolates are in black, spontaneous mutants in blue, T-DNA insertion mutants in brown, and from amoeba in red. **A.** *C. neoformans* var. *grubii*, in the strain G or KN99 backgrounds. The strains C, D and E were isolated from exposure to amoeba in the 1970s. **B.** *C. neoformans* var. *neoformans*, all in the ATCC 24067A strain background. Strain F7 is from a phenotypic switching study [27]. Sequence information for these mutations is provided in dataset S1.

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Figure 3. A tao3 point mutation can be reconstituted by homologous recombination with a wild type fragment of TAO3.

Strain D was transformed with a modified fragment of TAO3 by biolistic bombardment. Strains were plated on FK506 to select for wild type growth. A. and B. To ensure gene replacement rather than a reversion event, a BglII site (grey, k-g mutation in bold) was engineered near the affected codon. C. Morphology of strain D and two FK506 strains (bar = 50 μm). D. Amplification by PCR and restriction digests with BglII. Size markers = Invitrogen 1 kb+ ladder. Strain AI210 has undergone homologous recombination, while strain AI221 is a spontaneous revertant.

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Isolation of new pseudohyphal strains and their characterization reveals TAO3 as the primary target for mutation, although multiple genes of the RAM pathway can be affected

Additional pseudohyphal strains were sought in order to explore the basis of this trait and whether or not it was specific to the TAO3 gene. Two C. n. var. grubii candidate TAO3 mutant strains isolated in a previous study [28], but not further characterized, were examined through PCR and DNA sequencing (Fig. 2; dataset S1). One has a T-DNA insertion in the promoter of the TAO3 gene, and the other has a deletion of 7 bp (GCGTAGC). Two pseudohyphal T-DNA mutants were isolated during other experiments. One contains an insertion in the KIC1 gene and the other in TAO3. In addition, strains with complete deletion of TAO3 were generated in three backgrounds (C. n. var. grubii KN99α and strain G, and C.n. var. neoformans ATCC 24067A). Overlap PCR products were created to replace the TAO3 ORF with the nourseothricin acetyltransferase cassette via biolistic transformation and homologous recombination. The tao3 point mutant strains have the same phenotype as complete deletion or T-DNA insertion alleles, consistent with complete loss-of-function.

We screened and isolated 13 spontaneous pseudohyphal strains in the C.n. var. neoformans ATCC 24067A strain, six using a UV-induction method [27] and seven by plating colonies for random mutation events (table S1). The strains were transformed with the wild type copies of CBK1, KIC1, MOB2 or SOD2 to test for complementation and/or the TAO3 gene from these strains was sequenced. Eight contained changes in the TAO3 sequence, as illustrated in Fig. 2. Complementation experiments implicated mutations in the KIC1, MOB2, SOD2 and CBK1 genes in the remaining five, and these mutations were identified by sequencing those genes (Fig. 2 and dataset S1). The SOD2 gene in one had a mutation in which a stretch of 16 bp (TGCGAAGGGAATCC) in the fifth exon was duplicated and inserted adjacent to the original sequence. This insertion would result in a frame shift mutation. The cbk1 mutant bears an a-g mutation in the 3' splicing site of an intron. Likewise, the two kic1 mutants are both bp substitutions within splice sites. MOB2 was sequenced from the mob2 mutant, and has a bp deletion that will cause a translational frameshift. In summarizing, TAO3 is most often mutated in spontaneous pseudohyphal strains but other genes in the RAM pathway can also be affected.

The provenance of the C, D and E strains with pseudohyphal morphology that were isolated in the 1970s by exposing C. neoformans to amoeba is not well documented. Isolating RAM mutants using amoeba was tested. The original Acanthamoeba polyphaga strain from mouse feces and the A. palestinensis strain from pigeon guano, used previously to isolate pseudohyphal colonies, were not saved in a culture collection. The ATCC 30234 strain of A. castellanii was co-isolated with C. neoformans by Aldo Castellani [18], and is commonly used to study the interactions of amoeba with other pathogenic microbes. First, C. neoformans wild type and RAM mutants were exposed to this amoeba in the expectation that a similar interaction would occur as had been described in the 1970s. The outcome of the fungus-amoeba interaction depended on strain background and medium type. In some combinations, such as that illustrated in Fig. 4, the amoeba consumed the wild type strain whereas the RAM pathway mutants were resistant. Second, the wild type strains G (C.n. var. grubii) and ATCC 24067A (C.n. var. neoformans) were exposed to the amoeba on proteose peptone agar, and examined 2–3 weeks later for the presence of surviving colonies. A subset was of colonies comprised of pseudohyphal cells. The nature of the mutation within the RAM pathway for those strains was sought through complementation experiments and gene sequencing. Mutations were identified again in the TAO3 gene, as well as MOB2 (Fig. 2, dataset S1). These findings thus extend the diversity of the C. neoformans/amoeba interaction to include both C. neoformans varieties and a third Acanthamoeba species. In doing so, the results provide further evidence that the RAM pathway is integral to pseudohyphal morphology, and the isolation of these mutants reflects a common underlying ability across divergent C. neoformans strains.

Multiple mechanisms exist for reversion to wild type morphology in RAM pathway mutants

RAM pathway mutants are sensitive to FK506, providing a simple means to select for strains that revert to wild type. Revertants were sought from tao3 mutant strains D and F7. A high rate of spontaneous resistance to FK506 was observed for strain F7, but not all of these FK506 resistant strains had reverted to the wild type yeast cell morphology. Rather, the strains had acquired FK506 resistance through some other means, possibly through mutation of the gene encoding the FKBP12 protein to which
FK506 physically binds [43]. The frequency of reversion and FK506 resistance was noticeably lower in strain D than F7. Part of the TAO3 gene was sequenced from 50 revertants derived from F7 with the wild type yeast morphology: four types of changes were identified (Fig. 5A). In the first, an A-T bp mutation had restored the original lysine codon. In the second, an A-C bp change replaced the stop codon with a glutamine codon. In the third, the adjacent nucleotide had mutated (A-T) to change the stop codon to a leucine codon. In the fourth, the original stop codon was still present. In addition, another reversion event was also common, leading to a suppression of the RAM phenotype and partial return to the wild type phenotype. These strains were distinctive because of the yellow-colored colonies, with cells exhibiting a lemon-shape and inefficient cell separation. Sequence analysis revealed that the stop codon mutation was still present in TAO3 in these types of strains. In contrast to the revertants isolated from strain F7, when the region containing the stop codon in TAO3 was sequenced from 24 revertants of strain D, all 24 still contained the stop codon. Thus, mutation within TAO3 allows reversion back to wild type, although another mechanism(s) can account for some reversion events.

The pseudohyphal strain DM03 contains a 16 bp duplicated region within the SOG2 gene, and as such is a different type of mutation compared to the bp substitutions in the TAO3 gene described above. After selection on FK506 for wild type colonies from strain DM03, three reversion types were observed (Fig. 5B). In one, the duplicated piece of DNA was excised. In a second case, 4 bp were deleted downstream of the insertion event, returning the gene to the correct reading frame and producing an allele encoding 45 different amino acid residues. In the third, the original mutation was still present. Thus, excision of a duplication region or insertions or deletions correcting the open reading frame provide yet additional mechanisms to revert RAM mutants.

Northern blot analysis was used to examine changes in transcript levels or sizes in RAM pathway genes in response to mutation or FK506. C. neoformans RAM pathway mutants are resistant to Acanthamoeba castellanii. A. C. neoformans var. grubii wild type (KN99a) and tao3Δ deletion (AI235) strains grown on 5% V8 juice agar on 10 cm diameter Petri dishes. Amoeba were dropped at the intersection of the cross and plates incubated for 14 days at room temperature. B. Interactions between a mixture of wild type and tao3 mutant with amoeba, illustrating the size difference between amoeba and pseudohyphal cells (left panel) and internalization of yeast or occasional pseudohyphal cells into amoeba (right panel). Scale bar = 50 mm.

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reversion in a selection of var. neoformans and var. grubii strains (Fig. S1). The KRC1 and TA03 transcrans were below detectable levels. CBK1 and MOB2 have overall constitutive transcript levels. HIM1 and S0G2 showed variation in transcript levels, although there was no perfect correlation between loss of a RAM gene and upregulation. Of note, the mob2 mutant DM09 used in the virulence analysis described below exhibited altered transcript sizes, consistent with a mutation in a predicted intron splice site (Fig. S1).

The strain used for phenotypic switching studies is a hypermutator

If mutation is the primary source of pseudohyphal strains, it was surprising that they arise at such a high frequency. This together with the observation of high rates of spontaneous resistance to FK506 in the strains in the ATCC 24067A strain background used in phenotypic switching experiments [27,44], led us to test the mutation rate in this strain. ATCC 24067A is derived by laboratory passage from strain ATCC 24067 [45]. 20 separate cultures of strains ATCC 24067 and ATCC 24067A were plated onto medium to select for spontaneous uracil auxotrophy. The mutation rate for ATCC 24067 was 2.66 per 10^6 (95% confidence interval 1.78–4.75). In contrast, in ATCC 24067A the rate was 67.88 per 10^6 (95% CI 57.06–79.38). Thus, ATCC 24067A has greater than 25 fold higher mutation rate that its progenitor parent ATCC 24067.

To ensure that the uracil auxotrophs were due to mutations in the same gene, the URA5 gene encoding orotate phosphoribosyltransferase was amplified from 15 5-FOA resistant strains derived from separate starting colonies, and sequenced (Fig. 6A; dataset S2). 5-FOA resistance in fungi can result from mutation of either URA5 or URA15 homologs; prior studies suggest that URA5 is the main target in C. neoformans [46]. All 30 strains had mutations in URA5. Comparing the mutation profiles for ATCC 24067 and ATCC 24067A revealed similarities, e.g. three in each strain background had the same t-c mutation. A main difference was in mutations involving more than one base pair. Two indels for ATCC 24067A were single bp. In contrast, for ATCC 24067 three alleles have large insertions or rearrangements identified by altered or absent PCR products (data not shown). Another three uracil auxotrophs have insertions or deletions between 2 and 18 bp (dataset S2). We interpret this to imply a higher level of bp substitutions in the ATCC 24067A strain. Comparison of the ATCC 24067 and ATCC 24067A strains under stress conditions also revealed altered response to stress agents, especially oxidative stress agents and ethidium bromide (Fig. 6B). We hypothesize that during laboratory passage ATCC 24067A acquired a mutation in a DNA repair pathway gene.

The RAM pathway itself could potentially influence mutation rates, thereby enhancing the rate of reversion. Mutation rates were compared between 15 cultures each of ATCC 24067A and the ta03 point mutant strain F7. Uracil auxotrophs were isolated at a rate of 42.00 per 1 x 10^6 (CI 34.30–53.11) for the ATCC 24067A strain, and 15.74 per 1 x 10^6 (CI 13.37–18.93) for the F7 strain. These results suggest that there is no increase in mutation rate in the RAM mutants, since the wild type strain had 2.6 times higher frequency for isolation of uracil mutants compared to the F7 strain. A caveat in this comparison is the challenge of accurate quantification of viable cells for RAM pathway mutants.

Additional evidence was sought that pseudohyphal strains and reversions are due to mutation events. A “yellow” suppressor or partially-reverted strain was examined by Mendelian genetic analysis. The strain was isolated in the ta03::NAT deletion strain background (strain AI235) by selection on FK506. The AI235ya strain was crossed to a wild type of opposite mating type. 20 progeny were obtained: three pseudohyphal NAT^R FK506^R, ten wild type yeast NAT^R FK506^R, and seven “yellow” NAT^R FK506^R (Fig. 7). These results show that the suppression phenotype is meiotically stable, and the progeny ratio is consistent with its segregation as a single genetic locus. This finding further illustrates that mutation leads to some types of reversion events that are yet to be defined.

Mutation rates within the RAM pathway genes are similar to those in URA5

An alternative hypothesis for the high frequency of isolation of RAM mutants would be if the pathway or parts of it were hot spots for mutations. To test this, aliquots from the identical 20 cultures of strain ATCC 24067A used to isolate uracil auxotrophs were plated onto YPD. Six RAM mutants were identified from ~143,000 colonies. These six came from four starting cultures. In two examples, pairs on strains were isolated from the same plate. Characterization of the pairs (AI273–AI274 and AI275–AI276) revealed that they shared the identical mutation within TA03, reflecting an attached pair of pseudohyphal cells that were physically separated when spread on the plate. There are 225 codons in URA5 vs. 5750 codons combined for the six RAM pathway genes. Based on size alone we expected a 25.6 fold higher

Figure 6. The ATCC 24067A strain used in phenotypic switching is a hypermutator strain. A. Profile of ura5 mutations in strains ATCC 24067 and ATCC 24067A. Orange lines are positions of transversions, green are positions of transitions, and black indicates indels. The black line under ATCC 24067 represents three ura5 mutants with rearrangements. B. Ten-fold serial dilutions of ATCC 24067 and ATCC 24067A plated on different media types, and grown for two days. BHP is t-butyl hydroperoxide and EtBr is ethidium bromide.

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frequency of isolation of pseudohyphal strains compared to 5-FOA resistant strains, or 1 in every 57,546 colonies. The isolation of six mutants from 143,000 screened would be unlikely (P<0.03; Poisson distribution). However, if the pairs of identical tao3 mutants are considered as one event, then four from 143,000 is not statistically significant (P<0.24).

To circumvent bias due to cell separation in the RAM mutants, an alternative measure of mutation was taken. Uracl auxotrophs were isolated in the A1228 mutant, and the URA5 gene sequenced. A1228 has an α-α transition in an intron splice site in CBK1. Strain A1228 ura3 was isolated with a α-α transition in a highly-conserved glycine codon. We reasoned that the only way to revert the strain to wild type would be the perfect reversal of the mutation. 15 separate colonies were inoculated into liquid medium, cultured overnight, and from each 2.5×10^7 cells plated onto media supplemented with FK506, to select for a mutation in URA5. No wild type yeasts were obtained. In contrast, nine uracil prototrophs were isolated, reflecting reversion in URA5. These results indicate that at least one gene in the RAM pathway, CBK1, is not a general hot-spot for mutation.

**RAM pathway mutations are associated with phenotypic changes that can be either beneficial or detrimental to survival in the wild**

If amoeba select for pseudohyphal strains of *C. neoformans* in the wild, then why are pseudohyphal strains not isolated on a regular basis? We explored this question by testing isolation methods for *C. neoformans* and the fitness of the RAM pathway mutants under different growth conditions.

First, we explored the ability of RAM pathway mutants to grow on medium that mimics an environmental substrate, pigeon guano, with which *C. neoformans* is associated in nature [47]. Both wild type and RAM mutants grew equally well on pigeon guano medium, suggesting that RAM mutants have equivalent growth as wild type on this substrate (Fig. 8A).

Second, RAM mutants were grown on bird seed agar. This medium is made from *Guizotia abyssinica* seed and is a standard medium for isolation of *C. neoformans* from environmental sources, aided by melanization of *C. neoformans* colonies [48,49]. In two serotype A genetic backgrounds, the RAM mutant strains were delayed in pigmentation and produced smaller colonies compared to wild type (Fig. 8B). Melanin is a well-established virulence factor for *C. neoformans*. Another virulence trait is the biosynthesis of a polysaccharide capsule, which was found previously to be produced like wild type in RAM pathway mutants [28].

Third, phenotypes were explored under various stress conditions. No visual differences were observed between wild type and pseudohyphal strains growing on YPD at pH 4.5 or pH 8, or on YPD supplemented with high levels of salt NaCl, detergent sodium dodecyl sulfate, antifungal flucoazole, or oxidative stress agents (H₂O₂ and dodecyl sulfate). One phenotype identified that did differ is altered colony integrity. The RAM pathway mutant cells were easily dispersed by washing (Fig. 8C), possibly a detrimental trait leading to reduced protection of the cells within a structured colony in the wild. Mutation of the CBK1 homolog in the basidiomycete fungus *Ustilago maydis* causes sterility [33]. *C. neoformans* RAM mutants also have reduced fertility in crosses in which both parents bear mutations, since no filaments, basidia or basidiospores are produced in crosses on V8 juice or Murashige-Skoog medium (Fig. 8D).

Thus, explanations for the lack of pseudohyphal *C. neoformans* isolated from the wild could include their discard due to reduced melanization, reduced growth on bird seed agar or at elevated temperatures, and unconventional cell morphology. Alternatively, while it is possible that RAM mutation confers benefits under some environmental conditions, under others the pseudohyphal strains are less fit thereby countering the advantages gained in avoiding predation by amoeba.

The mechanism by which the RAM mutants of *C. neoformans* evade amoeba was explored using light microscopy, with confocal microscopy of GFP-expressing fungal strains and amoeba stained with FM4–64 used to confirm internalization of the fungal cells (data not shown). These results indicated that one possible mechanism of action is less efficient phagocytosis of RAM pathway mutants by amoeba. The pseudohyphal strains, especially when in clusters of cells, are larger than the amoeba thereby forming a physical impediment to phagocytosis (Fig. 4B). Consistent with this hypothesis, pseudohyphal cells are less frequently found inside amoeba. For instance, a mixture of wild type and tao3 mutants was exposed to amoeba. Only 3% of amoeba that
A RAM pathway mutant isolated by exposure to amoeba is attenuated for virulence, unless it reverts to wild type during the course of the infection

Based on previous observations of the virulence of pseudohyphal C. neoformans and the role of the RAM pathway in virulence in other fungi, we hypothesized that our mutants would be attenuated or avirulent. Wild type strain G, the mob2 mutant strain DM09 that was isolated by exposing strain G to amoeba, and a complemented strain AI255 (DM09+MOB2-NEO) were used to test the role of the RAM pathway in virulence in wax moth larvae and mouse models.

The mob2 mutant strain DM09 exhibits reduced growth at 37°C, which is predicted to influence virulence in mammalian hosts. To address virulence at a more permissive temperature, the three strains were inoculated into wax moth (Galleria mellonella) larvae and the larvae maintained at 30°C. 1 x 10^5 cells of wild type and complemented strains were used as inocula. Two inocula were used for DM09: 1 x 10^5 cell-clusters and one at 1/10 that concentration. Microscopic analysis of the cultures of the mob2 mutant and accompanying plating assays indicated that approximately 10 pseudohyphal cells formed the equivalent of one colony forming unit, due to the cell separation defect of the strain.

The larvae inoculated with wild type or complemented strains started dying five days after inoculation, and 22 of 23 were dead by day nine (Fig. 9). In contrast, the larvae inoculated with the mob2 mutant strain survived longer, e.g. on day nine only two of the 21 larvae had died. The experiment was terminated when the surviving larvae, including the control group inoculated with PBS, formed cocoons. Log-rank statistical comparisons indicated that the differences in survival between wild type or complemented strains with the mob2 mutant were significant (P<0.0001). Thus, the RAM pathway controls fungal virulence in an insect model and reduced virulence is independent of temperature.

Next, the three strains were tested in a mouse inhalation model of cryptococcosis. A subset of mice were sacrificed 24 and 96 h post-inoculation, and colony counts measured and lung tissue prepared for histology. The wild type proliferated during the three day interval, while the mob2 mutant strain maintained a level of approximately 1 x 10^5 cfu per gram (Fig. 10A). The colonies isolated from mice inoculated with the wild type were smooth and comprised of yeast cells. Colonies from mice inoculated with the mob2 mutant were all wrinkled and comprised of pseudohyphal cells. Yeast or pseudohyphal cells were evident in histological samples of the infected lungs.
lungs at both 24 and 96 h from mice infected with the wild type and mob2 mutant, respectively (Fig. 10B, C). These results show that C. neoformans pseudohyphal cells can penetrate the lung and survive at least four days.

The remaining sets of inoculated mice were monitored daily for signs of cryptococcal disease. The mice infected with the wild type or complemented strains succumbed to disease and were sacrificed by day 26, a time at which all of the mice inoculated with the mob2 mutant strain were alive and healthy. Interestingly, four of these mob2-inoculated mice developed symptoms of cryptococcosis and were sacrificed between days 38 and 49 post-inoculation (Fig. 11A). Sequence of MOB2 from these cells showed the wild type gene sequence. Two mice (numbers 7 and 10) had pseudohyphal strains only in the lung, and the brains were free of fungal cells (Fig. 11B,C). When the MOB2 alleles in the pseudohyphal strains were examined by DNA amplification and sequencing, those strains contained the original splicing mutation in MOB2 (Fig. 11C, dataset S3).

In summarizing the results from the mouse model, the mob2 mutant was attenuated for virulence (Log-rank test \( P<0.0001 \)). However, the pseudohyphal cells can persist during infection, and reversion mutations occur stochastically over time to restore cell shape to the wild type and fully pathogenic form.

**Discussion**

In this study we describe a new mechanism for microevolution in the human pathogenic fungus C. neoformans that controls the host range of the organism. Cryptococcus species have plastic genomes, with experiments showing changes in chromosome length over time, microevolution during human infection and in culture, and changes in chromosome numbers conferring azole drug resistance [9,10,45,50,51]. Microevolution modulates the polysaccharide capsule composition that surrounds the cell: this is the best-studied aspect about microevolution in the fungus [44,52,53].

The Cryptococcus genus is found in association with trees, soil, bird excreta and additional environments that are also homes to other microbial species [1,14]. One hypothesis is that selection for traits that defend against small predators, such as amoeba or nematodes, has led to species capable of causing disease in humans [13,14,15,16]. The evidence for amoeba-C. neoformans interactions date to over half a century ago with the work of Castellani, who showed that the species later named Acanthamoeba castellanii could kill C. neoformans cells [20]. In the 1970s, amoeba were again co-isolated with C. neoformans [15,17]. Subsequent studies found that a subset of C. neoformans colonies changed cellular morphology after exposure to Acanthamoeba species, from yeast to pseudohyphal cells, and that some isolates reverted rapidly to wild type yeast. Based on the more recent observation of phenotypic switching between pseudohyphal and yeast forms [27] and the discovery of a set of genes that produces a pseudohyphal phenotype when mutated [28], we hypothesized that phenotypic switching involved the RAM pathway.

Here we show that the RAM pathway is the integral component of “switching” in C. neoformans because it is mutated in pseudohyphal strains isolated from amoeba and spontaneously in culture. The largest gene in the pathway, TAO3, most commonly bears point mutations leading to the introduction of premature stop codons. Some strains like F7 revert to wild type at a high frequency in vitro. Analysis of the mutated region in those strains revealed that there are multiple ways in which the strain can revert (Fig. 5). This may be through a bp substitution leading to a coding triplet being reformed. Alternatively, the stop codon may still be present. The basis for the latter situation is unknown. It could be mediated by stop codon read through, tRNA suppressor
isolated from mice infected with the
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morphology and MOB2 mice were used. For the mob2 mice when sacrificed. For the wild type and complemented strain, three
Four (\(n = 9\)), mob2 mutant DM09 (\(n = 10\)) and complemented strain AI255 (\(n = 9\)). B. Colony forming units measured from brain and lung tissue of mice when sacrificed. For the wild type and complemented strain, three mice were used. For the mob2 mutant, all ten mice were examined. Four (\(#1–#4\)) that caused disease symptoms were sacrificed. The other six were sacrificed at day 70. Three had cleared the infection (not on graph), while three had the fungal burdens indicated. C. Cell morphology and MOB2 chromatograms and sequences from strains isolated from mice infected with the mob2 mutant strain (bar = 50 μm).

Figure 11. RAM pathway mutants are attenuated for virulence, and can only cause disease if they revert to wild type in a mouse model. A. Survival of mice infection with wild type strain G (\(n = 9\)), mob2 mutant DM09 (\(n = 10\)) and complemented strain AI255 (\(n = 9\)). B. Colony forming units measured from brain and lung tissue of mice when sacrificed. For the wild type and complemented strain, three mice were used. For the mob2 mutant, all ten mice were examined. Four (\(#1–#4\)) that caused disease symptoms were sacrificed. The other six were sacrificed at day 70. Three had cleared the infection (not on graph), while three had the fungal burdens indicated. C. Cell morphology and MOB2 chromatograms and sequences from strains isolated from mice infected with the mob2 mutant strain (bar = 50 μm).

The mob2 mutant carries the G-A mutation that impairs splicing. Strains from mice #4 and 5 have a reversion mutation, while strains from mice #7 and #10 are pseudohyphal and maintain the original mutation. Sequence data from additional strains is provided as dataset S3.

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mutations, changes in downstream gene expression, or epigenetic phenomena. Mutation in another RAM pathway component may suppress the phenotype, as occurs with a specific residue in the Cbk1 kinase of \(S. cerevisiae\) to rescue mutations in other pathway components [54], or be modified by interacting pathways such as seen for the Neurospora crassa cot-1 suppressors [35,36]. An alternative mechanism of reversion is illustrated by strain DM03 bearing a mutation in SOG2. Excision of the duplicated region or deletion of another region downstream reverts Sog2 sequence back to wild type or in frame, respectively. Taken together, the conversion between yeast and pseudohyphal cells reported previously as a form of phenotypic switching is based on DNA mutations, rather than epigenetic changes.

The effect of a defective RAM pathway on mammalian virulence was tested. The mob2 mutant strain used carries a bp substitution mutation within an intron splice site, and is phenotypically stable. Three strains were inoculated into mice. The wild type and complemented strains caused cryptococcal disease. In contrast, different outcomes were observed for mice infected with the mob2 mutant (Fig. 11). Four mice succumbed to disease, albeit weeks after those infected with the wild type and mutant had been sacrificed, and when their organs were harvested only yeast cells were recovered rather than the expected pseudohyphal cells that were used to inoculate the animals. The MOB2 gene was sequenced, and now had the wild type sequence (Fig. 11C). Sacrifice of the remaining and asymptomatic animals at day 70 and characterization of fungal material in lung and brain tissue shows that three mice had cleared the infection, one carried wild type cells, and the other two still maintained the original mob2 mutant phenotype and genotype. These results are consistent with previous virulence studies using pseudohyphal strains selected by amoeba in which reversion back to wild type for some occurred at a high frequency within mouse models [17,25,26]. A third animal experiment has been performed with pseudohyphal strains, presumably also RAM mutants, in a rat tracheal model [27]. In this experiment, two of the four animals cleared the infection while the other two did not, potentially representing another case of reversion within the host.

Morphological differentiation is important for Cryptococcus pathogenesis. Recently, a role has been assigned for a giant cell form during disease development [57,58], while constitutive filamentation by altered regulation of the \(ZNF2\) gene impairs virulence [59]. There are reports of pseudohyphal cells in histopathological samples of patients infected with \(C. neoformans\) [60,61]. One speculation is that pseudohyphal forms could allow escape of the fungus from mammalian or amoeba cells, in addition to escape of yeast cells from macrophages or \(A. castellani\) by exocytosis [62,63,64].

How can “switching” occur at high frequency? The formation of pseudohyphal strains relates to mutation rates in cells; switching also increases upon exposure to UV light [27]. Two factors influence frequency. The first is that the strain used in phenotypic switching studies has a 25 times higher mutation rate compared to a standard wild type strain. Second, because there are six genes in the RAM pathway there is a large amount of target DNA available for spontaneous mutation. Protein-coding sequence alone, the six genes comprise more than 17 kb of DNA, or ~0.1% of the genome (Fig. 2). \(TAO3\), as the largest member (42% of total cumulative size), is therefore the most likely gene to be hit. The original historical isolates bear mutations in this gene. Of 14 unique mutations that we
defined in the ATCC 24067A background, eight are in TAO3, supporting this hypothesis. One challenge for quantitative analysis of the pseudohyphal strains is their defect in cell separation. Reversion to wild type has been estimated as high as 1.6×10⁻¹ [27], but this may be an over-estimate by up to an order of magnitude if the colony forming units were derived from attached cells. It is unknown why an organism like Cryptococcus, which is normally found in the environment, can cause disease in humans or other mammals. Further, the fungal behavior upon entering the human host that ends in life-threatening disease remains unclear. Three points are worth raising. First, people are exposed to C. neoformans during their lifetimes yet most do not develop disease. For instance, children in city environments where there is a high prevalence of pigeons as sources of C. neoformans become antigen positive at an early age [65]. Second, cases of reactivation from quiescent infections brought about by immunosuppression supports a hypothesis that the fungus enters a latent state [66]. Third, in comparing virulence of strains derived directly from the environment vs. a human host, many environmental strains do not cause disease in animal models although they persist in the lungs [67,68,69,70]. Collectively, a high rate of exposure to C. neoformans, to strains that may not necessarily be able to cause disease immediately, and the potential for latency provide the scenario in which microevolution of C. neoformans by DNA mutations could influence clinical outcomes.

Multiple mechanisms can facilitate microevolution in pathogens. Among the fungi, mutations during infection lead to the emergence of antifungal drug resistance. However, these arise under conditions with a high fungal burden in the host, promoting the generation of strains from rare events. More broadly, there is evidence from diverse microbes that DNA is mutated to generate phenotypic variation. In the protist Trypanosoma brucei, mutation is required for evasion of the host immune response whereby double stranded breaks are generated and then repaired to generate antigenic diversity via the FSG genes [71]. Adaptation via mutation is implicated in bacterial disease progression. For example, 20% of Pseudomonas aeruginosa isolates from cystic fibrosis patients are “hypermutators” compared with 0% of environmental isolates [72]. Nevertheless, this trend in bacteria is not universal, as correlations between clinical isolates of E. coli and higher mutation rates have been found in some, but not all, studies [73,74]. A very different system to develop variation is the low fidelity of the human immunodeficiency virus’ reverse transcriptase: along with other factors this results in rapid genetic changes within the human host and reduces immunological recognition of the virus. In contrast, evidence for mutations affecting the pathogenicity of C. neoformans are rare at present. Curiously, a C. neoformans mutant in the MSH201 gene predicted to function in mismatch repair has a competitive advantage in mouse lungs compared to control strains [75].

This research points towards future directions into investigating the contribution mutation and mutation rates play in the ability of C. neoformans and other pathogenic fungi to adapt and cause disease. Specific directions are to assess mutation rates within the host and test if mutations that arise in the host result in more virulent strains. Second is to test for correlations between clinical and environmental isolates and rates of mutation. A third direction is to explore what role, if any, transcriptional, translational or epigenetic regulation of the RAM pathway plays in the interaction of C. neoformans with amoeba and mammalian cells.

Materials and Methods

Strains and growth conditions

Cryptococcus neoformans wild type strains used were KN99a (var. grubii), G (var. grubii, ATCC 42347), ATCC 24067, ATCC 24067A (var. neoformans), and JEC21 (var. neoformans). ATCC 24067A is derived from laboratory passage of ATCC 24067. Historical pseudohyphal strains were F7 (var. neoformans), and C, D, and E (var. grubii; ATCC 42345-5). Strains were kindly provided by Dr. Joseph Heitman and Dr. Bettina Fries. C. neoformans strains were cultured on yeast extract-peptone dextrose (YPD) ±2% agar medium, and stored as glycerol stocks at -80°C. The A. castellanii strain was obtained from the American Type Culture Collection (ATCC 30234) and maintained according to ATCC instructions and stored at 4°C [76]. To isolate new pseudohyphal strains, ATCC 24067A was grown in overnight YPD cultures, then spread on YPD plates. A subset of plates were subject to a low dose of UV light in a UV transilluminator. Colonies were screened by eye for those with a dry appearance, which were streaked to isolate single colonies. To isolate RAM mutants using the A. castellanii amoeba, C. neoformans strains ATCC 24067A and G were inoculated in a cross pattern on a selection of different agar medium types (potato dextrose, proteose peptone, YPD, V8 juice, Murashige-Skoog and trypan blue), and a drop of amoeba placed at the intersection, following the original protocol [17]. T-DNA insertional mutants were generated as described previously and RAM pathway mutants isolated based on colony morphology [28]. Wild type revertants were selected from RAM pathway mutants by plating on YPD agar supplemented with FK506 (1 μg/ml). Pigeon guano medium was 10% w/v of unfiltered pigeon guano (collected under the I-35 overpass of Southwest Blvd, Kansas City, MO) that had been homogenized in a coffee grinder and autoclaved with 4% agar. Bird seed agar was prepared as described [48]. Crosses were set up on 5% V8 juice or Murashige-Skoog agar [77]. Strains used and generated during this study are listed in Table S1.

Sequence analysis to identify point mutations

Genomic DNA was extracted using a CTAB buffer [78] from 50 ml overnight cultures of strains. The TA03 gene was amplified by PCR with two primer sets for each variety: ALID0013–ALID0061 and ALID0014–ALID0060 for var. grubii strains, and ALID0127– ALID0128 and ALID0129–ALID0138 for var. neoformans strains. SOG2 was amplified with primers ALID0123–ALID0162. MOB2 was amplified with primers DM062–DM063. CBK1 was amplified with primers ALID0977–ALID0978. Part of the KIC1 gene was amplified with primers ALID1681–ALID1682. The PCR products were sequenced with the primers used for amplification and additional internal primers. Primer sequences used for amplification are listed in Table S2.

Gene complementation

Tests were performed on pseudohyphal strains using vectors that complement the deletion mutants of mob2, cbk1, kic1, and sog2. The first three plasmids were generated in a previous study on the RAM pathway [28]. The SOG2-NEO1 construct was generated by amplification of SOG2 from strain JEC21 with primers ALID0123–ALID0162, cloning into TOPO pCR2.1 (Invitrogen, Life Technologies, Grand Island, NY), and a SpeI-XbaI fragment subcloning into the XbaI site of pZIP-NEO11. The four genes were in plasmids enabling their introduction into C. neoformans cells via Agrobacterium-mediated transformation [79]. Transformants were selected on YPD medium containing cefotaxime (200 μg/ml) and either nourseothricin (100 μg/ml) or neomycin (200 μg/ml). The TA03 gene in strain D was reconstituted by homologous recombination. A construct with an engineered BglII restriction enzyme site was generated by overlap PCR using primers ALID0061–ALID0227 and ALID0111–ALID0228, and introduced into strain D cells plated on YPD+1 M sorbitol by biolistic transformation with a PDS-1000/He Particle Delivery System.
Deletion of SOG2 and TAO3

The TAO3 gene was deleted in the KN99a, ATCC 24067A and G strains. For var. *gabii* strains, the 5’ and 3’ flanks were amplified from genomic DNA of strain KN99a using primers ALID0013 and dAMP6-dAMP7, respectively. For var. *neoforans*, the 5’ and 3’ flanks were amplified from strain ATCC 24067A using primers dAMP1-dAMP2 and dAMP3-dAMP4, respectively. Nourseothricin acetyltransferase (NAT) was amplified from plasmid pAI3 using primers ai006-ai2290 [79]. The primers ALID0013-dAMP7 or dAMP1-dAMP4 were used for overlap PCR. The SOG2 gene was deleted in strain KN99a. Primers ALID0483-ALID0484 and DM036-DM043 were used to amplify the 5’ and 3’ flanks, and ALID0483-DM036 used for overlap PCR with these and the NAT cassette. The DNA molecules were transformed into *C. neoformans* cells using the biolistic apparatus, cells allowed to recover for 3 h, and transferred to YPD medium containing nourseothricin (100 µg/ml). Correct gene replacement was confirmed by PCR analysis and Southern blotting with [³²P]-dCTP-labelled fragments of the genes.

Mutation rate analysis

Isolation of spontaneous uracil auxotrophs was used to measure mutation frequency. For comparison between strains ATCC 24067, which was acquired from the ATCC, and ATCC 24067A, strains were grown on yeast nitrogen base (YNB) medium, then 20 separate cultures of each strain established at 1×10⁶ cells/ml in YPD medium. After overnight culture in a roller drum incubated at room temperature, 5×10⁶ or 1×10⁷ cells were plated onto YNB supplemented with uracil (20 mg/L) and 5-fluoroorotic acid (5-FOA; 1 g/L) medium. The resulting colony numbers were analyzed to determine the mutation rate (Lea-Coulson method of the median) with FALCOR software [81]. To ensure mutations targeted the same gene in both strains, the URA5 gene was amplified with primers ALID0375-ALID0376 and sequenced.

To compare mutation rates in URA5 to the RAM pathway genes, aliquots from the same 20 ATCC 24067A cultures used to isolate URA5 mutants were diluted and plated onto ten YPD plates. Dry colonies were screened visually and pseudohyphal morphology confirmed by microscopy. The nature of the mutation in these strains was identified by complementation tests and DNA sequence analysis. Statistical analysis used the Poisson distribution, testing the probability of isolating n or more pseudohyphal strains. Strain AI228 ura#3 (dAI3 ura5) was inoculated into 15 YPD cultures, grown overnight, and 2.5×10⁶ cells plated onto YPD+2 µg/ml FK506 and YNB.

RNA purification and northern blotting

50 ml cultures in liquid YPD medium were incubated overnight at 150 rpm at room temperature. The cells were frozen and lyophilized. Total RNA was extracted with TRIzol (Invitrogen) or TRI reagent (Sigma-Aldrich, St. Louis, MO). For northern blots, 10 µg of RNA purified from each strain were resolved on 1.4% agarose/formaldehyde gels. RNA was blotted to Zeta-Probe membrane (Bio-Rad). [³²P]-labelled probes of the six RAM genes (the primers used for amplification are in Table S2) were hybridized to blots. Strips were stripped and reprobed with actin (ACT1) as a loading and RNA transfer control. RNA purified from wild type strain KN99a was also used to confirm the intron-exon boundaries of TAO3, by sequencing cDNAs reverse transcribed with Superscript III (Invitrogen).

Virulence studies in wax moth larvae and mice

Wax moth larvae (G. mellonella) were purchased from Vanderhorst Wholesale (Saint Mary’s, OH). Overnight cultures of *C. neoformans* grown in liquid YPD were washed in phosphate buffered saline (PBS), the concentration determined by counting cells with a hemocytometer, and diluted such that 1×10⁷ cells of wild type and the complementation mob2+MOB2 strain were injected into the larvae as described previously [82]. For mob2 mutant strain DM09, 1×10⁷ and 1×10⁸ cells were inoculated. Concentrations were confirmed by plating serial dilutions onto YPD agar plates.

Groups of female A/JCr mice (NCI-Frederick, MD) were infected intranasally with 10⁷ cfu of each strain, as previously described [83]. Inocula were confirmed by plating onto YPD agar. Animals that appeared moribund or in pain were sacrificed by CO₂ inhalation. For cfu assays, lungs and brain were dissected from animals, homogenized in PBS, and plated onto YPD medium containing ampicillin and chloramphenicol. Colonies were determined after incubation for 3 d at 30°C. For histology, lung and brain samples were fixed and hematoxylin and eosin (H&E) stained. Survival data from the murine experiments were statistically analyzed between paired groups using the log-rank test in the PRISM program 4.0 (GraphPad Software). P values of <0.01 were considered significant.
Figure S2  Histology samples of lung (A) or brain (B) from mouse #3 that was infected with mob2 mutant strain DM09 and sacrificed due to exhibiting signs of disease (H&E stained; bar = 50 μm). The pseudohyphal trait has reverted to the wild type yeast morphology. (TIFF)

Table S1  C. neoformans strains used in this study. A subset of the in vitro revertants is listed. (PDF)

References

25. Neilson JB, Fromtling RA, Bulmer GS (1981) Pseudohyphal forms of Cryptococcus neoformans revertants is listed. Table S1

Table S2  Oligonucleotide primers used for amplification of DNA. (PDF)

Author Contributions

Conceived and designed the experiments: DAM T-BL CX AI. Performed the experiments: DAM T-BL CX AI. Analyzed the data: DAM T-BL CX AI. Wrote the paper: DAM T-BL CX AI.

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A Microevolution Mechanism in C. neoformans


