RESEARCH ARTICLE

Prezygotic and Postzygotic Control of Uniparental Mitochondrial DNA Inheritance in *Cryptococcus neoformans*

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ABSTRACT **Uniparental inheritance of mitochondrial DNA is pervasive in nonisogamic higher eukaryotes during sexual reproduction, and postzygotic and/or prezygotic factors are shown to be important in ensuring such an inheritance pattern. Although the fungus *Cryptococcus neoformans* undergoes sexual production with isogamic partners of opposite mating types a and α, most progeny derived from such mating events inherit the mitochondrial DNA (mtDNA) from the a parent. The homeodomain protein complex Sxi1α/Sxi2a, formed in the zygote after a-α cell fusion, was previously shown to play a role in this uniparental mtDNA inheritance. Here, we defined the timing of the establishment of the mtDNA inheritance pattern during the mating process and demonstrated a critical role in determining the mtDNA inheritance pattern by a prezygotic factor, Mat2. Mat2 is the key transcription factor that governs the pheromone sensing and response pathway, and it is critical for the early mating events that lead to cell fusion and zygote formation. We show that Mat2 governs mtDNA inheritance independently of the postzygotic factors Sxi1α/Sxi2a, and the cooperation between these prezygotic and postzygotic factors helps to achieve stricter uniparental mitochondrial inheritance in this eukaryotic microbe.**

IMPORTANCE **Mitochondrial DNA is inherited uniparentally from the maternal parent in the majority of eukaryotes. Studies done on higher eukaryotes such as mammals have shown that the transmission of parental mitochondrial DNA is controlled at both the prefertilization and postfertilization stages to achieve strict uniparental inheritance. However, the molecular mechanisms underlying such uniparental mitochondrial inheritance have been investigated in detail mostly in anisogamic multicellular eukaryotes. Here, we show that in a simple isogamic microbe, *Cryptococcus neoformans*, the mitochondrial inheritance is controlled at the prezygotic level as well as the postzygotic level by regulators that are critical for sexual development. Furthermore, the cooperation between these two levels of control ensures stricter uniparental mitochondrial inheritance, echoing what has been observed in higher eukaryotes. Thus, the investigation of uniparental mitochondrial inheritance in this eukaryotic microbe could help advance our understanding of the convergent evolution of this widespread phenomenon in the eukaryotic domain.**

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Organelle genomes are different from nuclear genomes in their ability to replicate multiple times per cell cycle, to segregate during both mitosis and meiosis, and to be inherited uniparentally (1, 2). Uniparental inheritance of organelle genomes is pervasive in sexual eukaryotes and is observed across a wide variety of organisms, including fungi, protists, plants, and animals (1–5). Uniparental inheritance of organelle genomes is shown to help prevent the spread of deleterious mutations present in organelle genomes as well as harmful parasites present in the cytoplasm (2). The discordance of mitochondrial and nuclear genomes could cause senescence in fungi (6) and male sterility in plants (7). Thus, uniparental mitochondrial inheritance has been a subject of intense research. However, despite the widely accepted view regarding the importance of uniparental mitochondrial inheritance, the timing and the underlying mechanisms of such an inheritance pattern during sexual reproduction are highly debated.

In the fungus *Cryptococcus neoformans*, bisexual reproduction commences when yeast cells of opposite mating types (**a** and α) are in close proximity under appropriate conditions. Mating in this fungus involves the formation of conjugation tubes by α cells in response to the pheromone produced by nearby **a** cells (8) (Fig. 1). Cytological studies showed that there is unidirectional nuclear migration from the α cell to the **a** cell during conjugation (8). The cell fusion product contains the nuclear genomes of both parents and is equivalent to the zygote of plants and animals (5,9). The two parental nuclei congress but do not fuse after the α-**a** cell fusion event. A dikaryotic mating hypha emerges from the zygote by the side of the original **a** parental cell, followed by septation between the zygote and the growing dikaryotic hyphae (8). The dikaryon can be maintained in the hyphal form indefinitely until the formation of basidia, where nuclear fusion and meiosis occur. Recombinant spores are generated subsequently from the basidia, forming four spore chains (Fig. 1). If the environmental temperature is high, nuclear fusion takes place in the dikaryon to form a

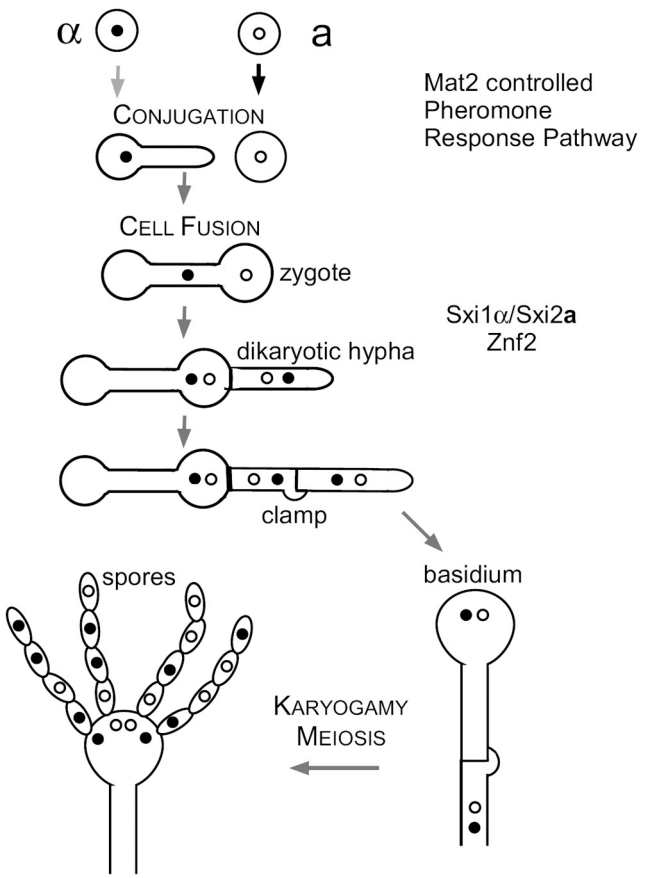

FIG 1 Bisexual mating of *Cryptococcus neoformans*. Under mating-inducing conditions, **a** and α cells undergo cell fusion. The early events during mating are controlled by the pheromone sensing and response pathway. Following cell fusion, the original zygote sends out the dikaryotic hypha, which grows filamentously as heterokaryotic dikaryons. Nuclear fusion and meiosis occur in the basidium formed at the tip of the aerial hypha. Four chains of spores are then generated from the basidium. Transcription factor Mat2 is essential for cell fusion (26), the mating-type-specific homeodomain complex Sxi1α/Sxi2**a** is specifically required for dikaryotic hypha formation and the completion of sexual development (15, 16), and the transcription factor Znf2 is essential for hyphal formation under all conditions (26, 27).

stable diploid, which can differentiate to form monokaryotic diploid hyphae and give rise to haploid spores after meiosis in basidia when the temperature decreases (10).

It has been well established that the progeny resulting from α-**a** sexual reproduction inherit mitochondrial DNA (mtDNA) predominantly from the **a** parent (11, 12). The uniparental mtDNA inheritance pattern appears to be established early during sexual reproduction, as intermediate cell types examined predominantly inherit the **a** parental mtDNA (11, 12). However, the timing of such an event has not been defined. The protein complex Sxi1α/Sxi2**a** formed after the cell fusion event was shown to control the uniparental mtDNA inheritance postzygotically (9, 13). This protein complex is specifically required for the production of dikaryotic hyphae from the original zygote and the completion of the subsequent sexual development (15, 16). Neither Sxi1α in α cells nor Sxi2**a** in **a** cells has any apparent role in the early mating events before the cell fusion. How the Sxi1α/Sxi2**a** complex that is formed in the zygote helps to control the uniparental mtDNA inheritance is currently unknown.

Based on these previous observations, three nonexclusive hypotheses can be envisioned to explain the uniparental mtDNA inheritance from the **a** parent in *Cryptococcus*: (i) blockage of the α mitochondria from entering the zygote (prezygotic control), (ii) incomplete cytoplasmic mixing and preferential mating hyphal formation from the **a** parental side (postzygotic control due to position effect), or (iii) selective degradation of α mitochondria and/or selective preservation of the **a** mitochondria in the zygote due to prezygotic marking (cooperation between the prezygotic and the postzygotic control).

In this study, we employed genetic approaches to examine the timing and genetic factors that control the mtDNA inheritance in *Cryptococcus*. We specifically focused on the key components that function during sexual development as uniparental mtDNA inheritance is observed only during **a**-α matings (9). Our findings argue against the hypothesis that postzygotic control due to position effect is important for the determination of the uniparental mtDNA inheritance pattern. The data suggest that the mtDNA inheritance pattern is determined in the original mature zygote and α mitochondria can efficiently enter the zygote but they are subsequently degraded during zygote maturation. Our observations support the role of the prezygotic factor Mat2 in the control of uniparental mtDNA inheritance. We propose that the **a** mtDNA inheritance in *Cryptococcus* is determined through selective preservation of **a** mitochondria and selective elimination of α mitochondria in the zygote. This is achieved by differential marking by Mat2 before the cell fusion event and the degradation of unpreserved α mitochondria in the zygote assisted by Sxi1α/Sxi2**a**. Thus, uniparental mtDNA inheritance in *Cryptococcus* is controlled both at the postzygotic level and at the prezygotic level.

RESULTS

Uniparental mitochondrial DNA inheritance is determined in the original zygote before the formation of mating hyphae. Previous studies on mtDNA inheritance in *Cryptococcus* demonstrated that intervariety matings (hybrid matings between strains of two different serotypes) or intravariety matings (matings between strains of the same serotype) both lead to uniparental mtDNA inheritance from the **a** parent (9, 11, 12, 17). Here, we also chose to use intervarietal matings between serotype A and serotype D strains to investigate the factors involved in determining mtDNA inheritance in this fungus for the following reasons. The intervarietal mating shows the same hallmarks as does the intravariety mating (see Fig. S1 in the supplemental material); the intervarietal mating also displays uniparental **a** mtDNA inheritance. The meiosis-associated problems due to genome divergence occur later at basidia during the late stage of sexual development, which is long after the mtDNA inheritance pattern is established (9, 11–13, 17). Hybrid matings occur commonly in nature (18–22), and studying mtDNA inheritance in hybrid matings would help our understanding of the genetic makeup of cryptococcal natural populations. Importantly, AD hybrid mating allows us to track the origin of the progeny's mtDNA based on the serotype-specific size polymorphism of the gene residing in the mitochondria that encodes cytochrome *b* subunit 1 (*COB1*) (17, 23). The serotype A congenic pair KN99**a** and KN99α are designated A**a** and Aα, and the serotype D congenic pair JEC20**a** and JEC21α are designated D**a** and Dα in this study. The A**a**/Aα strains do not self-filament, and they mate poorly with each other (Fig. 2). The Dα strain self-filaments sporadically after long incubation, and the D**a** strain

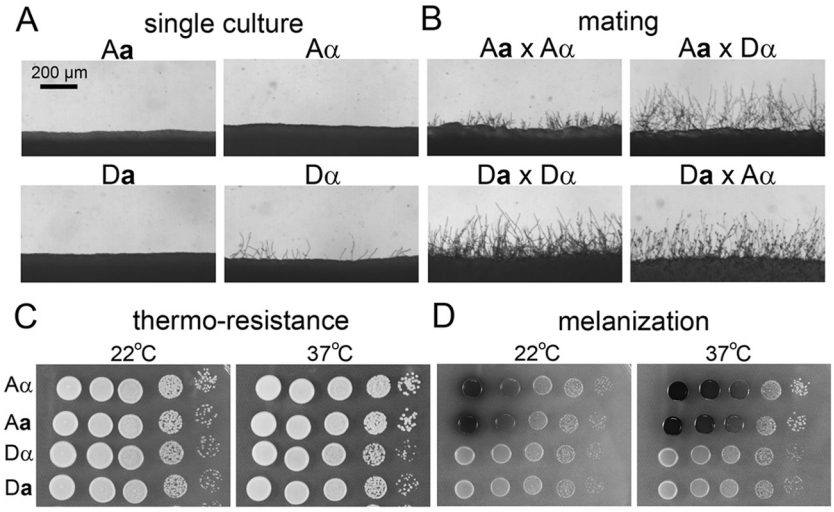

FIG 2 Phenotypic differences of the strains used. (A) Strains were incubated alone on V8 agar medium for 48 h. A and D represent variety serotype A and serotype D, respectively. **a** and α indicate the mating types of the strains. (B) Coculture of cells of opposite mating types on a V8 agar plate for 48 h to visualize filamentation, which reflects successful mating. (C and D) Strains with equal numbers of cells with serial dilutions were inoculated on YPD medium (C) or L-DOPA medium (D) and incubated at the indicated temperature for 48 h to analyze their thermotolerance and melanin production, respectively.

does not self-filament. However, the D**a** and Dα strains mate with each other robustly. Furthermore, they also mate with the Aα or the A**a** strain robustly (Fig. 2). These strains also differ in other phenotypes, such as thermotolerance and melanization (29) (Fig. 2).

Previous studies have shown that the mtDNA inheritance pattern is established at an early but unidentified stage of sexual development. Sexual spores, hyphae, cells budded from the hyphae, or diploids derived from dikaryons all inherit mtDNA predominantly from the **a** parent (9, 12, 13). Here, we used previously established methods and tested the mtDNA of cells derived from the hybrid crosses. As shown in Table 1 and also in Fig. S2 in the supplemental material, cells derived from the reciprocal hybrid matings showed mtDNA inheritance predominantly from the **a** parent (Table 1, crosses 1 and 4).

It has been proposed that the postzygotic control of uniparental mtDNA inheritance could be caused by the incomplete cytoplasmic mixing and subsequent filamentation from the **a** parental side of the original zygote (12). This hypothesis is reasonable given that in *Saccharomyces cerevisiae*, mtDNA inheritance in daughter cells depends on the budding position on the zygote. If the first bud arises from either end of the zygote, then those cells contain mtDNA from only one parent, depending on which parental side the bud arises from. If the first bud arises from the middle of the zygote, then it contains mtDNA from both the parents (24, 25). If such a position effect is critical in *Cryptococcus* mtDNA inheritance, one would predict that progeny would be more likely to inherit mtDNA from the parental strain with an enhanced ability to filament. Alterations of the ability to undergo filamentation in the parental strains would then affect the mtDNA inheritance pattern.

We decided to examine the impact of alterations in the ability to undergo self-filamentation on the mtDNA inheritance pattern through genetic mutations of the *ZNF2* gene. Znf2 is the master regulator of filamentation, and it does not control cell fusion events during mating (26, 27). Deletion of *ZNF2* completely abolishes the cryptococcal ability to filament (26, 27) (Fig. 3). To examine the position effect of mating hyphal formation, we analyzed mtDNA inheritance in crosses involving *znf2*Δ mutants in the **a** or the α mating partner. If the position effect controls the mtDNA inheritance, one would predict a decrease in the inheritance from the **a** parent when *ZNF2* is deleted in the **a** parental strain. However, blocking the ability to filament in the D**a** parental strain did not diminish the predominance of **a** mtDNA inheritance in the

TABLE 1 Mitochondrial inheritance from wild-type crosses and crosses involving *znf2*Δ strains[a]

Cross no.	Cross	% mtDNA from parent (no. positive/total no.)	
		α	**a**
1	A(α) × D(**a**)	4 (2/50)	82 (41/50)
2	A(α) × D(**a**) *znf2*Δ	0 (0/50)	100 (50/50)
3	A(α) *znf2*Δ × D(**a**) *znf2*Δ	2 (1/48)	87.5 (42/48)
4	D(α) × A(**a**)	4 (2/49)	92 (45/49)
5	D(α) *znf2*Δ × A(**a**)	2 (1/49)	98 (48/49)
6	D(α) *znf2*Δ × A(**a**) *znf2*Δ	13 (6/45)	87 (39/45)

[a] A minor portion of the progeny in certain crosses were found to inherit mtDNA from both the **a** and the α parent. It is also possible that recombination could also occur in these rare cases (43). Such a phenomenon was also observed previously (9, 13, 17). The number of progeny examined from each cross is indicated in parentheses. Inheritance of mitochondrial DNA from **a** and α parents in unilateral crosses or bilateral crosses involving *znf2*Δ mutant strains is not statistically different from that in the respective wild-type crosses.

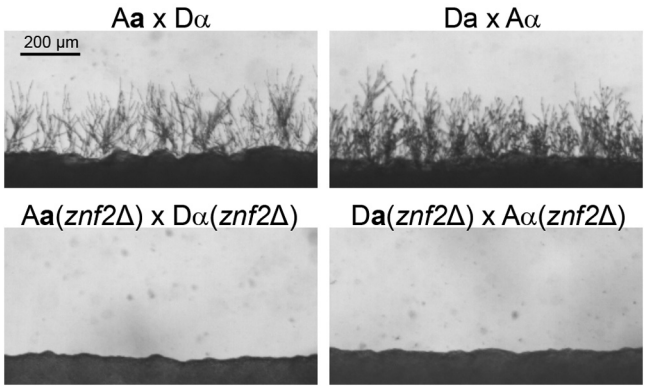

FIG 3 Deletion of *ZNF2* abolishes filamentation. Robust filamentation is produced in the control matings after 48 h. Production of filamentation is completely eliminated in the bilateral matings with the *znf2*Δ mutants.

unilateral cross A(α) × D(**a**) *znf2*Δ (Table 1, crosses 2 and 1; see also Fig. S2 in the supplemental material). Similarly, we did not observe any significant change in the mtDNA inheritance pattern in the unilateral cross D(α) *znf2*Δ × A(**a**), where the *ZNF2* gene was deleted in the α parent compared to the control cross (Table 1, crosses 5 and 4; see also Fig. S2). Thus, it appears that the position of hyphal formation does not affect the uniparental mtDNA inheritance pattern.

However, because the *ZNF2* gene is deleted in only one parent, the *ZNF2* gene from the other wild-type parent could compensate for the loss once the two parental cells are fused. It has been shown previously that the deletion of *ZNF2* abolishes mating hypha formation only in bilateral crosses (*znf2*Δ × *znf2*Δ), not in unilateral crosses (*znf2*Δ × wild type) (26). Thus, we decided to examine mtDNA inheritance in crosses where the *ZNF2* gene is deleted in both parental strains. If the position effect of mating hypha formation is responsible for the uniparental mtDNA inheritance from the **a** parent, abolishing the formation of mating hyphae from the zygote should result in a biparental mtDNA inheritance pattern. Interestingly, bilateral crosses with Znf2 disrupted in both parents yielded uniparental mtDNA inheritance from the **a** parent (Table 1, crosses 3 and 6; see also Fig. S2 in the supplemental material), a pattern similar to that for the wild-type crosses (Table 1, crosses 1 and 4). Again, the results suggest that the formation of mating hyphae is not important for the establishment of mtDNA inheritance pattern. Consequently, the position of mating hyphal formation does not determine the uniparental mtDNA inheritance in *Cryptococcus*.

Taken together, these results refute the original hypothesis and indicate that mtDNA inheritance is not dependent on the position of the mating hypha formation from the original zygotes. These results also suggest that the pattern of mtDNA inheritance is established in the original zygotes before the formation of mating hyphae. This new hypothesis offers a plausible explanation for the previous observations that various intermediate cell types obtained from bisexual matings predominantly inherited mtDNA from the **a** parent (9, 11, 12).

Prezygotic control of uniparental mitochondrial DNA inheritance. The results presented above indicate that a uniparental mtDNA inheritance pattern is established in the original zygote before the generation of dikaryotic hyphae. The formation of dikaryotic hyphae requires the homeodomain protein complex Sxi1α/Sxi2**a** (15, 16), which is demonstrated to affect the uniparental mtDNA inheritance in *Cryptococcus* (9, 13). Because this intact protein complex can form only after the cell fusion event, deletion of either the *SXI1*α gene from the α parent or the *SXI2***a** gene from the **a** parent (unilateral matings) has the same effect as does the deletion of both genes (bilateral matings). A biparental mtDNA inheritance pattern was observed in these unilateral or bilateral crosses, and surprisingly with modestly more progeny inheriting the α parental mtDNA (9, 13). We further confirmed these previous observations in our study (see below for details). These observations suggest that the original zygote contains ample mitochondria that originate from the α parent.

The observation that zygotes derived from crosses involving *sxi1*αΔ or *sxi2***a**Δ mutants are more likely to contain mtDNA from the α parent than the **a** parent makes it unlikely that uniparental mitochondrial inheritance in *Cryptococcus* is due to blockage of α mitochondria from entering the zygote. We envision that rapid and selective degradation of α mitochondria in the zygote could achieve the uniparental **a** mtDNA inheritance and be consistent with the presence of both **a** and α mitochondria in the newly formed zygote. The retention of **a** mtDNA in the zygote can be achieved by prezygotically marking either the **a** mitochondria for preservation or the α mitochondria for annihilation, followed by rapid degradation of the α mitochondria in the zygote after Sxi1α and Sxi2**a** form the functional complex.

Such a model predicts that efficient selective elimination of the α mtDNA from the zygote relies on selective marking of parental mitochondria (or parental mtDNA) before the cell fusion event. Thus, some prezygotic factors must be involved in the control of mtDNA inheritance in *Cryptococcus*. We decided to investigate the role of Mat2 in mtDNA inheritance, given its essential role in early stages of mating. Mat2 is the key transcription factor in the pheromone sensing and response cascade (26, 28). It directs the conjugation process, and it must be present in both mating partners for the cell fusion event during α-**a** mating (26, 27). The essential role of Mat2 in cell fusion presents a challenge, as no zygote can be generated even in unilateral matings where Mat2 is disrupted in only one parent. Hence, we decided to use *MAT2* overexpression (*MAT2*^{oe}) strains where the *MAT2* gene is placed under the control of the constitutively active promoter from the *GPD1* gene (27). We generated *MAT2*^{oe} strains in both Aα and A**a** backgrounds. As shown in Fig. 4A and B, overexpression of *MAT2* increases the competency of cells to mate as indicated by increased production of shmoo cells in both Aα and A**a** backgrounds. This is consistent with the established roles for Mat2 (26, 27): stimulating pheromone production and driving the formation of mating-competent shmoo cells.

Surprisingly, when *MAT2* was overexpressed in the α parent, a modest dominance of α mtDNA inheritance (60%) was observed [Table 2, cross 2, A(α) *MAT2*^{oe} × D(**a**); also see Fig. S2B in the supplemental material]. Thus, it appears that the overexpression of *MAT2* in the α parent was able to change the progeny inheritance pattern from predominantly **a** mtDNA to biparental with modest dominance by the α mtDNA. When *MAT2* was overexpressed in the **a** parent, mtDNA inheritance remained uniparental from the **a** parent (Table 2, crosses 5 and 6; see also Fig. S2B). To determine whether the dominance of the mtDNA originating from the parental *MAT2*^{oe} strain is caused by an increased number of copies of mtDNA in the *MAT2*^{oe} strains, we compared the ratio of mtDNA (the *COB1* gene) and nuclear DNA (the *TEF1* gene)

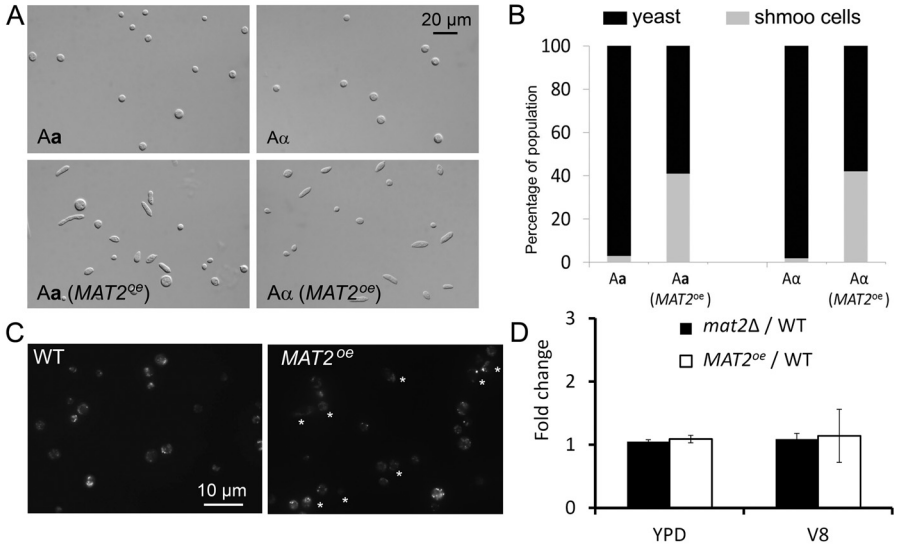

FIG 4 Overexpression of *MAT2* drives shmoo cell formation but does not alter mitochondrial morphology or the mtDNA copy number. (A) The images in the top panel show the round yeast cells of the wild-type (WT) A**a** and Aα strains after incubation in V8 medium for 48 h. The images in the bottom panel show the cell morphology (round yeasts and pear-shaped or elongated shmoo cells) of A**a** and Aα strains when *MAT2* is overexpressed. (B) Quantification of the distribution of yeast cell morphology and shmoo cell morphology in different populations as shown in panel A. (C) Mitotracker CMXRox-labeled wild-type cells and the *MAT2*^{oe} cells. Stars indicate shmoo cells. (D) The comparison of the *COB1*/*TEF1* ratios between the wild type, the *MAT2*^{oe} strain, and the *mat2*Δ mutant cultured in YPD and V8 media. All strains shown here were in the Aα background. Similar results were obtained for strains in the A**a** background (see Fig. S3 in the supplemental material).

between the *MAT2*^{oe} strains and the wild-type strains. No difference in the *COB1*/*TEF1* ratio was observed between the *MAT2*^{oe} strains and the wild-type strains when cells were cultured in either yeast extract-peptone-dextrose (YPD) medium or V8 juice medium (Fig. 4D; see also Fig. S3). Similarly, the deletion of the *MAT2* gene did not alter the ratio of *COB1*/*TEF1* either (Fig. 4D). Thus, mutations of Mat2, either disruption or overexpression, do not appear to alter the copy number of mtDNA per cell. Furthermore, we found no apparent alterations in mitochondrial morphology in the *MAT2*^{oe} strain compared to the wild type based on microscopic examination (Fig. 4C). Thus, Mat2 promotes the mtDNA inheritance through a means other than increasing the copy number of mtDNA in the cell.

Prezygotic control and postzygotic control cooperate to determine mitochondrial DNA inheritance. Next we decided to investigate whether the prezygotic control of mtDNA inheritance regulated by Mat2 is dependent on the postzygotic control regulated by Sxi1α/Sxi2**a**. As observed in previous studies (9, 13), we found that the deletion of either the *SXI1*α gene in the α parent or the *SXI2***a** gene in the **a** parent results in biparental mtDNA inheritance in the unilateral matings (Table 2, crosses 3, 7, and 9; see also Fig. S2B in the supplemental material). A modest domination by the α mtDNA inheritance was observed in all of these crosses (67%, 75%, and 69%, respectively). Modest dominance of α mtDNA among the progeny was also observed previously when either the *SXI1*α gene or the *SXI2***a** gene was disrupted in the parental strains. We found that this altered mtDNA inheritance pattern is independent of the serotype or the mating type where the mutation occurs. This is consistent with our hypothesis that the newly formed zygote contains both **a** and α mtDNA, with α mtDNA being modestly more abundant.

To examine whether mtDNA inheritance controlled by Mat2 is dependent on the Sxi1α/Sxi2**a** complex, we analyzed the mtDNA inheritance pattern of crosses involving one parent overexpressing

TABLE 2 Effect of mutations of *SXI1*α, *SXI2***a**, and *MAT2* on mitochondrial inheritance[a]

Cross no.	Cross	% mtDNA from parent (no. positive/total no.)	
		α	**a**
1	A(α) × D(**a**)	4 (2/50)	82 (41/50)
2	A(α) *MAT2*^{oe} × D(**a**)	60 (52/87)	35 (31/87)
3	A(α) × D(**a**) *sxi2***a**Δ	67 (20/30)	20 (6/30)
4	A(α) *MAT2*^{oe} × D(**a**) *sxi2***a**Δ	84 (36/44)	12 (5/44)
5	D(α) × A(**a**)	4 (2/49)	92 (45/49)
6	D(α) × A(**a**) *MAT2*^{oe}	2 (1/46)	98 (45/46)
7	D(α) *sxi1*αΔ × A(**a**)	75 (36/48)	25 (12/48)
8	D(α) *sxi1*αΔ × A(**a**) *MAT2*^{oe}	23.8 (10/42)	73.8 (31/42)
9	D(α) × A(**a**) *sxi2***a**Δ	69.4 (34/49)	24.5 (12/49)
10	D(α) × A(**a**) *sxi2***a**Δ *MAT2*^{oe}	6 (3/49)	83.6 (41/49)

[a] Effect of *MAT2*, *SXI1*α, and *SXI2***a** on mitochondrial inheritance. The control crosses 1 and 5 are the same as crosses 1 and 4 in Table 1. They are included here for easy comparison and are shown with shading.

MAT2 and the other parent disrupted with the homeodomain complex (Table 2, crosses 4 and 8). When *MAT2* is overexpressed in the **a** parent, crossing with the α *sxi1*$\alpha\Delta$ strain produced a modest dominance of **a** mtDNA inheritance among the progeny (74%), compared to the mere 25% **a** mtDNA inheritance in the cross between A**a** and D(α) *sxi1*$\alpha\Delta$ strains (Table 2, crosses 7 and 8). Thus, it appears that the overexpression of *MAT2* in the **a** parent partially restores the dominance of the **a** mtDNA inheritance pattern, although the restoration is not to the level comparable to that of the wild-type cross where 92% of the progeny inherited the **a** mtDNA (Table 2, cross 5). In contrast, the overexpression of *MAT2* in the α parent worsened the alteration caused by the deletion of *SXI2***a** and increased the dominance of α mtDNA inheritance (84%) in the cross A(α) *MAT2*^{oe} × D(**a**) *sxi2***a**Δ compared to 67% observed in the cross A(α) × D(**a**) *sxi2***a**Δ (Table 2, crosses 3 and 4). Thus, it appears that the parental origin of the *MAT2* overexpression determines the type of mitochondria to be preserved independently of Sxi1α/Sxi2**a**: active Mat2 in the **a** parent leads to the preservation of the **a** mtDNA in the progeny and active Mat2 in the α parent results in the preservation of the α mtDNA in the progeny. However, a stricter mtDNA inheritance is achieved when the Sxi1α/Sxi2**a** complex works together with Mat2.

To further confirm the cooperation between Mat2 and Sxi1α/Sxi2**a** in the control of mtDNA inheritance, we constructed a *MAT2*^{oe} strain in the *sxi2***a**Δ mutant background. Consistent with our hypothesis, the mtDNA inheritance pattern in the cross D(α) × A(**a**) *sxi2***a**Δ *MAT2*^{oe} is similar to the cross D(α) *sxi1*$\alpha\Delta$ × A(**a**) *MAT2*^{oe} (Table 2, crosses 8 and 10; see also Fig. S2B in the supplemental material), where a modestly predominant proportion of the progeny (84% and 74%, respectively) inherited mtDNA from the **a** parent where *MAT2* is overexpressed. In contrast, in the control crosses D(α) × A(**a**) *sxi2***a**Δ and D(α) *sxi1*$\alpha\Delta$ × A(**a**), only 24.5% and 25% of the progeny inherited mtDNA from the **a** parent, respectively. This again supports our hypothesis that the parental origin of the *MAT2* overexpression determines the type of mitochondria to be preserved in the progeny, and a tighter uniparental mtDNA inheritance pattern can be achieved with the cooperation between Mat2 and Sxi1α/Sxi2**a**.

DISCUSSION

Previous studies have demonstrated the predominantly uniparental **a** mtDNA inheritance pattern in α-**a** crosses, and such an inheritance pattern is suggested to be established during the early stages of sexual development (11, 12). In this study, we investigated the roles of the postzygotic factors Znf2 and Sxi1α/Sxi2**a** and the prezygotic factor Mat2 in mtDNA inheritance in this fungus. Our findings indicate that mtDNA inheritance is established in the original zygote, before the formation of dikaryotic hyphae or subsequent developmental stages that lead to the production of meiotic spores (Fig. 1). In addition, we have shown the existence of a prezygotic control by Mat2. Although Mat2 is known to be highly upregulated during mating in both partners (26, 27), we posit that the timing and the level of Mat2 activation in the parental strains are critical in determining the origin of mitochondria to be inherited. It was shown previously that wild-type **a** cells express pheromone even in the absence of the α partner while α cells produce pheromone as a response to the pheromone produced by **a** cells (8). Therefore, under natural **a**-α matings, **a** cells likely initiate the mating process and their mitochondria are preserved to be inherited in the progeny. When the *MAT2* gene is constitutively expressed using the *GPD1* promoter, cells with *MAT2*^{oe} act, in effect, as the parent initiating the mating process and their mitochondria are preferentially inherited in the progeny.

To confirm that Mat2 is differentially regulated in **a** and α cells when they are cultured alone and when they are cocultured together during mating, we measured the expression level of the *MAT2* gene and the pheromone genes controlled by Mat2 (*MF***a** and *MF*α) at multiple time points during the process. The pheromone genes were included as they are highly sensitive to the change of the *MAT2* expression level (27) and thus can robustly reflect the activity of Mat2 even when the change in the *MAT2* expression level itself is subtle. As shown in Fig. S4A in the supplemental material, the expression level of *MF***a** increased more than 70-fold by 4 h in the culture of **a** cells alone. In contrast, the expression level of *MF*α increased less than 3-fold by 4 h in the culture of α cells alone and the level gradually increased to about 18-fold by 10 h. The patterns of *MF***a** and *MF*α expression levels are largely correlated with the *MAT2* expression level in these cultures (see Fig. S4B). During **a**-α mating, the expression levels of both *MF***a** and *MF*α were drastically higher due to the positive feedback regulation of the pheromone sensing and response cascade in both α and **a** cells. Nonetheless, the expression level of *MF***a** again increased earlier and stronger than that of *MF*α during the mating process (see Fig. S4C). Thus, **a** cells initiate the mating process through activation of the pheromone pathway controlled by Mat2.

Based on these observations, we propose the following model to explain mtDNA inheritance in *Cryptococcus* (Fig. 5). During natural α-**a** matings, the activation of Mat2 in **a** cells leads to the marking of **a** mitochondria for preservation. In response to the pheromone produced by **a** cells, α cells also activate their Mat2 and send conjugation tubes toward **a** cells. After the α cell fuses with the **a** cell, the nuclei and mitochondria from both parental cells coexist in this newly formed zygote, which allows the formation of the Sxi1α/Sxi2**a** complex. This protein complex then mediates downstream factors to help eliminate α mitochondria before the zygote matures and sends out the dikaryotic hyphae. Thus, the cooperation between Mat2 and the Sxi1α/Sxi2**a** complex ensures stricter uniparental mtDNA inheritance from the **a** parent. Based on this model, one would predict that variations in the timing and the relative level of Mat2 activation between the mating pair, due to genetic differences or environmental factors, might cause variations in the tightness of the uniparental mtDNA inheritance. For instance, high temperatures inhibit the expression of *MAT2* in both α and **a** cells (27) and may diminish the differences in the initial Mat2 activity between the mating pair, which could lead to the leakage in the uniparental mtDNA inheritance observed previously (14). In contrast to the α-**a** bisexual mating, the unisexual α-α mating yields a biparental mtDNA inheritance pattern (9). This could result from similar regulations on Mat2 in the two α partners. Further investigation with a large number of natural isolates with different Mat2 expression levels or with strains where Mat2 expression level and timing can be precisely controlled is necessary to validate this model.

The cooperation between the prezygotic and postzygotic control to ensure a stricter uniparental mtDNA inheritance has been studied in other systems, although there are cases where either the prezygotic control or the postzygotic control prevails. In mammals, sperm mitochondria were tagged with ubiquitin during

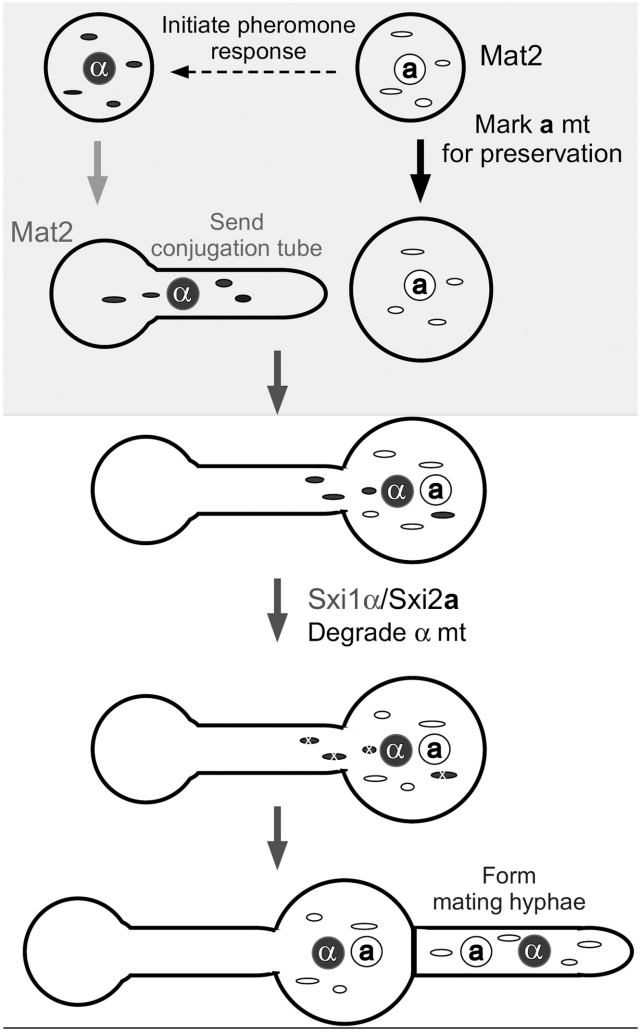

FIG 5 Model of mtDNA inheritance in *Cryptococcus*. Under mating-inducing conditions, the **a** cell activates Mat2 and produces high levels of the *MF***a** pheromone (8). In response to *MF***a**, the α cell activates Mat2 and produces a conjugation tube. The earlier induction of Mat2 at a higher level in the **a** cell marks its mitochondria for preservation. Immediately after cell fusion, the Sxi1α/Sxi2**a** complex forms, and it helps degrade the unprotected α mitochondria in the newly formed zygote. Thus, the **a** mitochondria dominate in the mature zygote and cells subsequently generated from that zygote (e.g., hyphae and spores).

spermatogenesis, and they rapidly disappeared during zygote development (30). In the fish *Oryzias latipes*, the abundance of sperm mtDNA decreases 5-fold during spermatogenesis and the rapid elimination of remaining paternal mtDNA takes place within 2 h after fertilization (31). A 10-fold reduction in paternal mtDNA occurs during mouse spermatogenesis (32). The size difference between sperms and oocytes in these systems results in unequal contributions of paternal and maternal mtDNA in the zygote that facilitate a highly stringent uniparental mtDNA inheritance pattern (1, 30, 33). In the fruit fly *Drosophila melanogaster*, paternal mtDNA is completely eliminated during sperm development (34), and thus, the prezygotic control is sufficient to guarantee a uniparental mtDNA inheritance pattern.

Our study reinforces the concept that there are intricate connections between factors controlling sexual development and organellar inheritance. The high-mobility group (HMG) domain transcription factors are critical regulators controlling sexual development in fungi (Mat2 in *Cryptococcus* and *sexP*/*sexM* in *Phycomyces*) (35), and they are critical factors controlling mtDNA inheritance during sexual reproduction (this study and personal communication with Alexander Idnurm). Sex is proposed to enhance species fitness in changing and stressful environments. Uniparental mitochondrial inheritance is proposed to enhance the coadaptation of mitochondrial and nuclear genomes and therefore to improve species fitness more rapidly (36). Thus, intricate connections between the regulation of sexual development and mtDNA inheritance might have coevolved during the development of different eukaryotes. The highly virulent *Cryptococcus gattii* strains responsible for the cryptococcosis outbreak in British Columbia and the northwestern United States might have increased their fitness (virulence) in the temperate climate due to changes in their mitochondrial and nuclear genomes (37, 38). Given the robust genetic and molecular tools available to *Cryptococcus* and the vast ecological and epidemiological knowledge regarding its natural distribution and genotypes, *Cryptococcus* could serve as an excellent model to study the evolution of uniparental mitochondrial inheritance, the ecological and medical importance of this phenomenon, and the underlying molecular mechanisms.

MATERIALS AND METHODS

Strains and growth conditions. Strains used in this study are listed in Table S1 along with their genotypes. Strains were maintained on YPD medium (2% Bacto peptone, 1% yeast extract, 2% dextrose, 2% Bacto agar) at 30°C before mating. Mating was carried out on V8 juice medium (5% V8 juice, 0.5 g/liter KH_2PO_4, 4% Bacto agar) at 22°C in the dark as described previously (26).

Generation of *MAT2* overexpression strains. The *MAT2*^{oe} in the mating-type **a** strain was obtained by crossing LW80 (*MAT*α, P_{GPD1}-*MAT2*-NEO) (27) and JF99 (MAT**a**, *ura5*). Mating was carried out for 14 days on V8 juice agar medium (pH 5). The mating type of the spores generated was confirmed by crossing the derived colonies with reference strain JEC20**a** or JEC21α. Colonies that mated with JEC21α but not with JEC20**a** and were resistant to G418 were selected. Auxotrophic strains were confirmed by the absence of growth on SC-URA medium. The *MAT2* overexpression in the *sxi2***a**Δ strain was obtained by introducing the construct P_{GPD1}-*MAT2*-NEO (27) into the *sxi2***a**Δ mutant as described previously (39). Stable transformants were selected, and the ability of these strains to produce filamentation on their own and the enhanced mating filaments were confirmed on V8 juice medium as described previously (27).

Generation of *ura5* auxotrophic strains. To generate *ura5* auxotrophic strains, freshly grown strains on YPD were collected and washed twice with water. Strains were then plated on 5-fluoroorotic acid (5-FOA) plates (6.7 g/liter or 0.67% yeast nitrogen base without amino acids, 50 mg/liter uracil, 2% Bacto agar, 2% dextrose, 1 g/liter 5-fluoroorotic acid, SC-URA medium). Strains that grew on 5-FOA plates but failed to grow on SC-URA plates were selected as *ura5* auxotrophic strains as described previously (40). Only strains showing a stable auxotrophic phenotype after several passages were used in this study.

Mating assays. Parental serotype D and serotype A strains of different mating types were grown on YPD agar at 30°C. Mating was performed on V8 juice medium (pH 7) by mixing equal numbers of cells of opposite mating types. The coculture was incubated at 22°C in the dark for 2 to 3 days. Mating mixtures were then scraped off the V8 agar plates, washed with water, and plated on selective medium. To select products of the cell fusion events between two auxotrophic strains, cells were plated on min-

imal YNB medium (6.7 g/liter or 0.67% yeast nitrogen base with no amino acids, 2% Bacto agar, 2% dextrose) and grown for an additional 3 days at 37°C. To select products of the cell fusion events between two dominant marked strains, cells were plated on YPD medium supplemented with G418 and NAT (Nourseothricin or clonNAT) and incubated for an additional 3 days at 37°C. To select products of the cell fusion events between a *ura5* auxotrophic strain and a dominant marked strain (NAT or NEO/G418), cells were plated on proline plus NAT or proline plus G418 agar medium and incubated for an additional 3 days at 37°C as described previously (16).

Determining the mitochondrial genotype. The natural size polymorphism present in the cytochrome *b* subunit 1 gene (*COB1*) between serotype D and serotype A strains was used to determine the mtDNA genotype of the fusion product as described previously (17). In serotype A, *COB1* contains 1 intron, whereas in the case of serotype D, it carries 2 introns. Thus, PCR amplification of the *COB1* gene using the forward primer 5′-CCACAACCTATTAACATTAGCTACGC-3′ and the reverse primer 5′-CGTCTCCATCTACAAAGCCAGCAAAC-3′ yielded products of 610 bp in serotype A and 1,585 bp in serotype D (17). Total DNA extracted from the fusion products and their parental strains was treated with RNase A to get rid of RNA contamination. The treated DNA samples then served as the templates for PCRs. PCR amplicons were separated on agarose gels and stained with ethidium bromide for visualization under UV light.

Statistical tests. Statistical tests were performed using GraphPad Prism, version 5.04. Fisher's exact test was used to analyze if the inheritance pattern of mitochondrial DNA from the **a** and the α parent is statistically different between various crosses and the control crosses. A *P* value lower than 0.05 is considered statistically significant.

Phenotypic assays. Phenotypic assays were performed as previously described (29). Briefly, yeast cells of tested strains were grown on YPD medium overnight and washed three times with water. The optical density of the cultures was measured at 600 nm, and all cultures were adjusted to the same cell density. Cells were then serially diluted by 10-fold. To examine melanin production, cells were spotted on melanin-inducing medium containing L-dihydroxyphenylalanine (L-DOPA) (100 mg/liter) and incubated at 22°C and 37°C in the dark for 2 to 6 days. Melanization was visualized as the colonies developed a brown color. To analyze growth at different temperatures, cells were spotted on YPD medium and incubated at the indicated temperatures for 2 days.

Quantification of the ratio between mitochondrial DNA and nuclear DNA. Strains KN99α, XL1598 (Aα *mat2*Δ), LW80 (Aα *MAT2*^{oe}), KN99**a**, and RG40 (A**a** *MAT2*^{oe}) were collected after incubation in YPD or V8 medium for 24 h. The cell number of each strain was calculated based on hemocytometer counting, and total DNA was extracted from the same number of cells for each strain using the cetyltrimethylammonium bromide (CTAB) method as previously described (41). DNA samples were treated with RNase A, and then the RNA-free DNA samples served as the templates for real-time PCRs using Kapa SYBR Fast PCR mix. The quantification of relative levels of the mitochondrial genome-borne *COB1* (primers 5′ GCAAACACCACCTTCAATT 3′ and 5′ AGAAGGCAAATC GTGTTAGA 3′) and the nuclear genome-borne *TEF1* (Lin lab 329/330; 5′-CGTCACCACTGAAGTCAAGT-3′ and 5′-AGAAGCAGCCTCCATA GG-3′) was used to compare mutant strains with the wild-type strains cultured under the same conditions.

Microscopic examination of mitochondrial morphology. Cells were grown in V8 (pH 7) or YPD medium overnight, washed, and then suspended in phosphate-buffered saline (PBS). Mitotracker CMXRox stain was added to the cell suspension to a 100 nM final concentration as described previously (42). The cells were incubated with Mitotracker for 1 h at 30°C before they were washed three times with PBS. Washed cells were then visualized using a Zeiss Axioplan2 microscope.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00112-13/-/DCSupplemental.

Text S1, DOCX file, 0.1 MB.
Figure S1, TIF file, 0.6 MB.
Figure S2, TIF file, 4.6 MB.
Figure S3, TIF file, 1 MB.
Figure S4, TIF file, 7.3 MB.
Table S1, DOCX file, 0 MB.

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