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Molecules and Cells

The Iron-Responsive, GATA-Type Transcription Factor Cir1 Influences Mating in *Cryptococcus neoformans*

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Mating and sexual development have been associated with virulence in various fungal pathogens including *Cryptococcus neoformans.* This fungus is a significant pathogen of humans because it causes life-threatening cryptococcal meningitis in immunocompromised people such as AIDS patients. The virulence of *C. neoformans* is known to be associated with the mating type of the cells (α or a), with the α mating type being predominant among clinical isolates. However, the mechanisms by which mating and sexual development are controlled by environmental conditions and their relationship with virulence require further investigation. Cir1 is a GATA-type transcription factor that regulates the expression of genes required for utilization of essential metals such as iron and copper, and also genes required for major virulence factors including the polysaccharide capsule and melanin. Here we investigated the role of Cir1 in the mating of *C. neoformans*. Our results demonstrate that mutants lacking *CIR1* are defective in mating, and that Cir1 contributes to copper mediated enhancement of sexual filamentation. Furthermore, we found that Cir1 influences the expression of mating pheromone genes suggesting that this protein plays a role in the early phase of sexual development on V8 mating medium.

INTRODUCTION

The process of sexual development has been well characterized in many fungi including the model yeast *Saccharomyces cerevisiae,* several pathogens of plants and animals and commonly studied filamentous fungi such as *Neurospora crassa* (Lee et al., 2010). In the fungal pathogens, sexual development is often associated with virulence by mechanisms that are poorly understood (Hsueh and Heitman, 2008). The plant pathogens, *Ustilago maydis* and *U. hordei,* are exceptions because the formation of a filamentous, dikaryotic cell type by fusion of mating partners in these fungi is essential for host infection and subsequent completion of the coincident infection and sexual cycle (Hsueh and Heitman, 2008). *Ustilago* mutants that are incapable of mating and sexual development are known to be avirulent (Feldbrugge et al., 2004). Human fungal pathogens, such as *Cryptococcus neoformans*, *Candida albicans* and *Aspergillus fumigatus,* also possess the machinery for sexual development, but the role of mating in pathogenesis has yet to be clarified.

C. neoformans is a basidiomycete fungal pathogen that causes life-threatening cryptococcal meningitis in immunocompromised people such as AIDS patients (Bicanic and Harrison, 2004). The fungus is yeast-like and generally haploid, and it possesses a bipolar mating system in which a single *MAT* locus determines the mating type of cells (*MAT*α or *MAT***a**). Previous studies suggested that mating-type and sexual development may be associated with virulence in *C. neoformans*. For example, it has been found that strains of the *MAT*α mating type are predominant in the environment, and that a *MAT*α strain was more virulent than a *MAT***a** strain in a congenic mating pair of capsular serotype D strains (Kwon-Chung et al., 1992). The *MAT* locus in *C. neoformans* is somewhat unusual because it spans a region of more than 100 kb and contains ~20 genes encoding pheromones (*MF*α or *MFa*), pheromone receptors (*STE3*α or *STE3a*), components of the MAP kinase signaling pathway for pheromone perception, a homeodomain transcription factor (*SXI1*α or *SXI2a*), and proteins not clearly related to mating (Hsueh and Heitman, 2008; Hull et al., 2005). The mating pheromones of *C. neoformans* are short peptides that mediate initial signaling events via MAP kinase pathway components including the pheromone receptor Ste3, a heterotrimeric G protein β subunit Gpb1, a MAPKK kinase Ste11, a MAPK kinase Ste7, a MAP kinase Cpk1 and a transcription factor Ste12 (Lengeler et al., 2000; Wang and Heitman, 1999). In addition to the MAP kinase pathway, the cAMP/protein kinase A pathway also regulates sexual development in *C. neoformans.* The Gpa1 protein is a key upstream component of the cAMP pathway that participates in the sensing of nutritional signals and that encodes a conserved G-protein α subunit. The *gpa1* mutant has been shown to be defective not only in the expression of virulence factors (melanin formation and capsule induction), but also in mating; these results support a role in sexual development and further link this process with virulence (Alspaugh et al., 1997).

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In the laboratory, sexual development in *C. neoformans* can be induced by co-culturing both *MAT*α and *MAT***a** cells on V8 agar, a solid medium containing 5% V8 juice. A recent study by Kent et al. (2008) revealed that no single factor in V8 medium induces sexual development, but it appears, instead, that multiple factors coordinately contribute to the process. Moreover, it was discovered that one of the essential metals, copper, plays an important role in the induction of sexual development. It was also shown that copper increases the transcript levels for the pheromone genes (*MF*α or *MFa*), but the underlying mechanism for this influence remains to be determined.

Previously, we identified the GATA-type transcription factor Cir1 as a major regulator of the expression of the iron regulon and all of the known major virulence factors. These include the polysaccharide capsule, melanin deposition in the cell wall and the ability to grow at mammalian body temperature. Global transcriptome analysis using microarrays identified downstream target genes of Cir1 and revealed that Cir1 also regulates genes associated with the *MAT* locus (e.g., *STE11*α and *MYO2*). For example, *STE11*α was 2.28-fold and 2.42-fold upregulated in the *cir1* mutant in low-iron and high-iron medium, respectively (Jung et al., 2006). Moreover, genes in the signaling path-ways that influence sexual development in *C. neoformans*, such as the cAMP pathway and the MAP kinase pathway, were also differentially expressed in the *cir1* mutants (Idnurm et al., 2005; Jung et al., 2006). Cir1 may also regulate copper uptake and homeostasis because the *cir1* mutant showed differential expression of the copper exporting ATPase Ccc2 compared to the wild-type strain, as well as differential expression of laccase, which requires copper and catalyzes melanin formation.

Overall, the discovery of connections between Cir1, signaling components and copper led us to investigate the phenotypic characteristics of the *cir1* mutants in relation to sexual development. In the present study, we constructed and employed *cir1* mutants of both mating types to investigate the influence of Cir1 on the initial stages of sexual development: fusion and filament formation. The results reveal that Cir1 plays a role in mating and filament formation on V8 medium and that this protein contributes to the influence of copper on mating.

MATERIALS AND METHODS

Strains, growth conditions and mating assays

All strains used in this study have the D capsular serotype background and their genotypes are listed in Table 1. Strains were maintained in yeast extract, bacto-peptone medium with 2.0% glucose (YPD, Difco). To evaluate the initial stages of sexual development, mating crosses involving mixtures of strains were conducted on solid V8 medium (Erke, 1976). Briefly, cells were grown in YPD at 30°C overnight and washed twice with phosphate buffered saline (PBS). Cell number was determined with a hemocytometer and 1×10^8 *MAT*α or *MAT****a*** cells were withdrawn from the cell suspensions. These cells were mixed by pipetting and 10 μl of each was spotted on V8 medium. Plates were incubated at room temperature in the dark for two to seven days, and the periphery of each mixed colony was observed microscopically.

RNA extraction and Northern blotting

Petri plates of solid V8 medium containing mating cultures or control cultures of single mating type cells were incubated at room temperature in the dark for 24 h. Cells were scraped off the plates and lyophilized for RNA extraction. Trizol (Invitrogen) was used for total RNA extraction following the manufacturer's recommendations. Northern blotting was performed as described by Sambrook et al. (1989) with five μg of total RNA from each strain. Hybridization probes were designed for genes from each mating type and were amplified separately by PCR with the primers listed in Table 2. A Rediprime II random prime labeling system (GE Healthcare) was used for probe labeling, and the membrane was exposed to a Phosphor Screen (GE Healthcare) for 16 h, followed by scanning using a Pharos FX™ Plus Molecular Imager (Bio-Rad).

Table 1. Strains used in this study

Name	Genotype	Reference
JEC21	*MAT*α	Kwon-Chung et al. (1992)
JEC21cir1Δ#46	*MAT*α, *cir1Δ::NAT*	Jung et al. (2006)
JEC21cir1Δ#57	*MAT*α, *cir1Δ::NAT*	Jung et al. (2006)
JEC20	*MATa*	Kwon-Chung et al. (1992)
JEC20cir1Δ#7	*MATa, cir1Δ::NAT*	This study
JEC20cir1Δ#37	*MATa, cir1Δ::NAT*	This study

Table 2. Primers used in this study

Name	Sequence
J2CIR-KO-F	GCGCAGTTCTGTCGATCGTCCCGAATTG
J2CIR-KO-R	CCGATTTTCGAACACTTCCAGTACATCC
MFα-F	TCACTGCCATCTTCACCACCTTCA
MFα-R	GATGACACAAAGGGTCATGCCACC
MFa-F	TCACTGCTACCTTCTCAACCCTTT
MFa-R	AACGCAAGAGTAAGTCGGGCCCT
J2GPD1-F	ACACATGGTCGCTTCAAGGGCTCCG
J2GPD1-R	ACATAGGAGCATCAGCAGAAGGAGCG

Mutant construction

A disruption cassette was employed to construct *cir1* mutants of the *MAT***a** strain. The cassette contained the nourseothricin acetyltransferase gene (*NAT*) and 5′ and 3′ flanking sequences of *CIR1* amplified by PCR using primers J2CIR-KO-F and J2CIR-KO-R (Table 2), and the plasmid pWH008 as a template (Jung et al., 2006). The disruption cassette was biolistically transformed into the JEC20 wild-type strain (Table 1) (Toffaletti et al., 1993). Positive transformants were identified by PCR and confirmed by Southern blot analysis (Fig. 1). Two independent mutants were selected and used throughout the study. Surface reductase activity of the mutant cells was evaluated by the 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) overlay method, as described previously (Hassett and Kosman, 1995; Ogur et al., 1957).

RESULTS AND DISCUSSION

Construction of a serotype D *MAT*a *cir1* mutant

We previously constructed the *cir1* mutation in *MAT*α cells to study the iron regulatory roles of Cir1 in the serotype D background of *C. neoformans* (Jung et al., 2006). In this paper, we designate this mutant as *cir1*$\alpha\Delta$ to indicate that the mutation is in the *MAT*α mating type. In the current study, we also deleted *CIR1* in a *MAT****a*** strain to investigate the influence of Cir1 on mating. Two independent *cir1* mutants were constructed in *MAT***a** cells (designated *cir1a*Δ) (Materials and Methods), and confirmed by Southern blot analysis (Fig. 1). These *cir1a*Δ mutants (JEC20cir1Δ#7 and JEC20cir1Δ#37) and two independ-

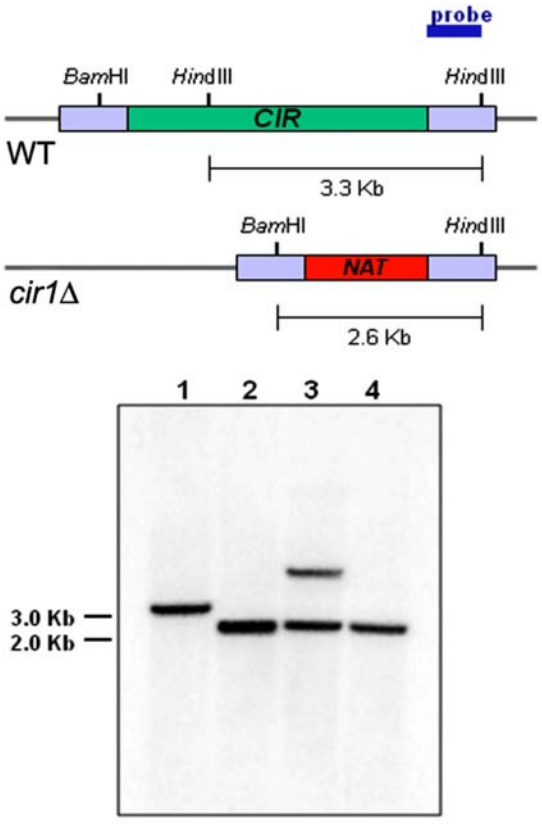

Fig. 1. Disruption of *CIR1* in the *MAT*a strain was confirmed by Southern blot analysis. Restriction maps of the genomic regions containing the wild-type or the disrupted *CIR1* allele are shown. Genomic DNA of the serotype D *MAT*a strain (JEC20) was digested with *Bam*HI/*Hin*dIII and hybridized with the probe indicated. Lane 1 contains DNA from the serotype D wild-type strain JEC20. Lanes 2 and 4 contain DNA from the *cir1* mutant strains and show that the disruption cassette integrated at the *CIR1* locus. The mutants represented by lanes 2 and 3 were designated as *cir1*Δ#7 and *cir1*Δ#37, respectively. Lane 3 contains DNA from a transformant that has an ectopic integration of the disruption cassette.

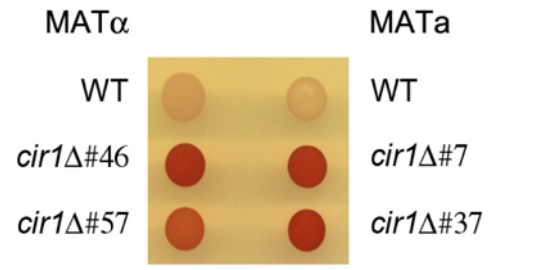

Fig. 2. Disruption of *CIR1* in the *MAT*a strain caused elevated cell surface reductase activity. Both *MAT*α *cir1* mutants and *MAT*a *cir1* mutants displayed increased cell surface reductase activity, as indicated by the red colony color in the presence of TTC (Materials and Methods). The two independent mutants in each mating type displayed identical phenotypes.

ent *cir1*$\alpha\Delta$ mutants (JEC21cir1Δ#46 and JEC21cir1Δ#57) from our previous study were used in all subsequent mating experiments (Jung et al., 2006). Initially, we confirmed that deletion of *CIR1* in *MAT***a** cells resulted in iron-related phenotypes by performing a 2, 3, 5-triphenyltetrazolium chloride (TTC) overlay assay to assess surface ferric reductase activity. We previously found that this activity is higher in the *cir1*$\alpha\Delta$ mutants compared to the wild-type strain (Jung et al., 2006). As with the *cir1*$\alpha\Delta$ mutants, we found elevated reductase activity for the *cir1a*Δ mutants, thus confirming loss of Cir1 function in *MAT***a** cells (Fig. 2).

Influence of Cir1 on mating in the presence and absence of copper

The ability of *cir1* mutants to mate was tested on V8 medium and we found that mating mixtures involving *cir1*$\alpha\Delta$ ($\alpha\Delta \times$ a, $\alpha\Delta \times a\Delta$) showed significantly reduced mating filamentation when compared with mixtures of wild-type strains (Figs. 3A and 3B). To rule out the possibility of a growth deficiency of the *cir1*$\alpha\Delta$ or *cir1a*Δ mutants on V8 medium, the same assay plates were incubated up to seven days and the mixtures were again observed for mating filaments. Prolonged incubation did not remedy the filamentation defect observed in mixtures containing the *cir1*$\alpha\Delta$ mutants ($\alpha\Delta \times$ a, $\alpha\Delta \times a\Delta$), suggesting that Cir1 indeed plays a role in mating in *MAT*α cells. The *cir1a*Δ mutants showed no distinguishable mating phenotype, in contrast with the *cir1*$\alpha\Delta$ mutants. That is, the mixtures containing the *cir1a*Δ mutants and the wild-type *MAT*α cells ($\alpha \times a\Delta$) showed wild-type levels of mating filamentation on V8 medium (Fig. 3A). Taken together, these results indicated that loss of Cir1 caused a unilateral mating defect in *MAT*α cells, but not in *MAT***a** cells. Unilateral mating responses and difference between cells of opposite mating type have been reported previously for *C. neoformans*. For example, overexpression of the G-protein β subunit Gpb1 triggers the formation of more mating conjugation tubes in *MAT*a cells than in *MAT*α cells (Wang et al., 2000). Additionally, Wickes et al. (1996) found that only *MAT*α strains display filamentous growth associated with monokaryotic fruiting. Therefore, we hypothesize that Cir1 is one of a number of regulatory functions that govern mating filamentation in a cell type-specific manner in *C. neoformans*.

Kent et al. (2008) previously developed a defined V8 medium to examine the role of different components, and they demonstrated that the inclusion of copper is important to achieve robust mating. We therefore tested the effect of copper on mating mixtures with the *cir1* mutants to investigate whether Cir1 mediates the response. We also tested the influence of iron because of the prominent role of Cir1 in iron homeostasis (Jung et al., 2006). Iron or copper was added to V8 medium and mating was assayed by observing filamentation. As expected from the work of Kent et al. (2008), we found an overall enhancement of mating filamentation by exogenously added copper, but there was no significant influence of iron (Fig. 3A). The level of filamentation for a unilateral mating mixture containing the *cir1*$\alpha\Delta$ mutants ($\alpha\Delta \times$ a) was also enhanced to a small extent by copper addition (Figs. 3A and 3B). In contrast, the mixture with a bilateral defect in Cir1 ($\alpha\Delta \times a\Delta$) was not responsive to copper addition, thus indicating a role for Cir1.

It is possible that differential expression of genes required for copper homeostasis in the *cir1* mutant background may explain the influence of Cir1 on copper stimulation of filamentation. Previous transcriptome analysis with the *cir1*$\alpha\Delta$ mutant demonstrated differential regulation of genes involved in copper uptake (Jung et al., 2006). For example, the transcript for the copper exporting ATPase Ccc2 was 8.82-fold up-regulated in the mutant compared to the wild-type in the low-iron condition. The transcript for the copper uptake transporter Ctr4 was 4.86-fold up-regulated under this condition. The V8 mating medium contains a relatively low level of iron (~1 μM) (Kent et al., 2008). It

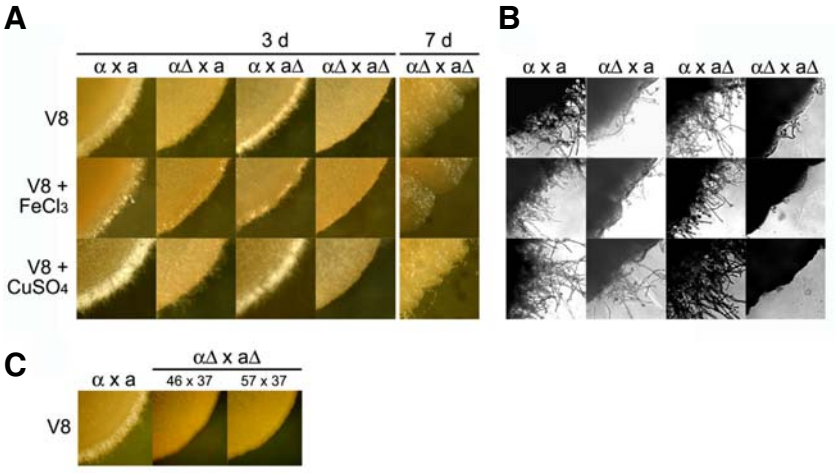

Fig. 3. Cir1 in the *MAT*α strain influences mating filament formation. Mixtures of cultures from the following strains were carried out on solid V8 medium, V8 medium containing 100 μM $FeCl_3$ or V8 medium containing 100 μM $CuSO_4$: *MAT*α wild-type and *MAT***a** wild-type (α × a), *MAT*α *cir1Δ*#46 and *MAT***a** wild-type (αΔ × a), *MAT*α wild-type and *MAT***a** *cir1Δ*#7 (α × aΔ), *MAT*α *cir1Δ*#46 and *MAT***a** *cir1Δ*#7 (αΔ × aΔ). (A) Each panel represents the periphery of a mixed colony. (B) Higher magnification (× 400) revealed significantly reduced levels of filamentation from a mixture containing *MAT*α *cir1Δ*. (C) The mixture containing independently generated *MAT*α *cir1* mutants (46 and 57) and *MAT***a** *cir1* mutants (37) displayed a lack of filamentation at the colony periphery.

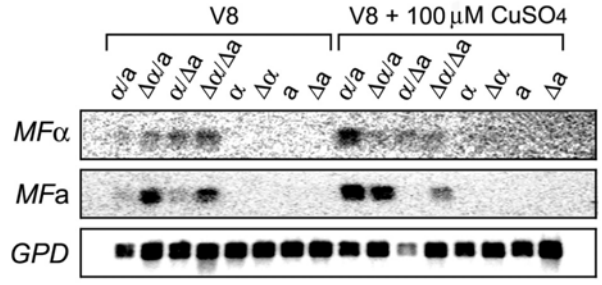

Fig. 4. Cir1 influences of the transcript levels for pheromone genes. RNA was extracted from either mixtures of cells undergoing mating or haploid cells on V8 medium or V8 medium containing 100 μM $CuSO_4$. Northern blot analysis was performed to assess the transcript levels of genes encoding the mating pheromones *MFα* and *MFa*.

is therefore possible that the regulatory influence of Cir1 on copper homeostasis functions reduces the intracellular accumulation that would otherwise stimulate mating. The absence of an influence of iron is consistent with the findings of Kent et al. (2008) that iron was not a key component of their defined V8 mating medium.

Cir1 regulates genes involved in mating and sexual development

To further investigate the role of Cir1 in the early stages of sexual development, we determined the expression of genes encoding the mating pheromones (*MFα* and *MFa*) in *cir1* mutants. Transcript levels of the genes in mating mixtures containing wild-type cells and/or *cir1* mutants were analyzed by Northern hybridization (Fig. 4). Our results showed that the transcript level of *MFα* was increased on V8 medium in mixtures of the *cir1Δα* mutant with the wild-type *MAT***a** strain or the *cir1Δa* mutant (αΔ × a and αΔ × aΔ), compared with cultures of single wild-type or mutant cells (Fig. 4). Interestingly, the mixture containing the *cir1Δa* mutant also displayed increased expression of *MFα* (α × aΔ). This result suggests that deletion of *CIR1* in the *MAT***a** cells may increase the expression of secreted pheromone (or another unidentified factor) to induce transcription of *MFα* in *MAT*α cells. The involvement of Cir1 in expression of *MFa* was also observed in that the mixtures containing the *cir1Δα* mutants showed increased transcript levels of *MFa* (αΔ × a and αΔ × aΔ). This result indicated that deletion of *CIR1* in the *MAT*α cells triggers increased transcription of *MFa* in *MAT***a** cells. We did note that, in contrast to the situation with *MFα* upon deletion of *CIR1* in *MAT*α cells, elevated *MFa* transcription was not observed in the mixture containing the *cir1Δa* mutant (α × aΔ). This result further reinforces the difference in the relative influence of the *cir1* mutation for *MAT*α and *MAT***a** cells, as observed for mating filament formation (Fig. 3).

As observed by Kent et al. (2008) our analysis also revealed that addition of copper significantly increased expression of *MFα* in mixtures containing wild-type cells (Fig. 4, α × a in V8 + $CuSO_4$ versus V8). However, the same was not true in mixtures containing *cir1Δα* and/or *cir1Δa* mutants (αΔ × a, α × aΔ or αΔ × aΔ) thus indicating that Cir1 is required for the elevated transcript level of *MFα* that results from copper addition. As mentioned above for the previous transcriptome analysis (Jung et al., 2006), this result could represent an indirect influence on the expression of genes required for copper homeostasis in the *cir1* mutants. Addition of copper increased the transcript level of *MFa* in mixtures with wild-type strains (α × a, in the presence of $CuSO_4$), as was found with *MFα*. However, the copper response was not observed for the *MFa* transcript in the *cir1Δa* mutant mixed with a wild-type partner (α × aΔ, in the presence of $CuSO_4$ versus V8 alone). This result suggested that the *cir1Δa* mutants may be deficient in copper uptake or perception. Taken together, our data indicated that Cir1 generally has a negative influence on the expression of the pheromone transcripts in both mating types in V8 medium. The situation appears to be more complex in V8+ $CuSO_4$ where Cir1 may make a positive contribution to the influence of copper. Overall, the reduction in mating filamentation in the *cir1* mutants suggested that Cir1 is required for the morphological transition during early sexual development in both *MAT*α and *MAT***a** cells. Our hypothesis is well supported by recent findings that suggest temporally distinct pathways during mating filamentation in *C. neoformans*. Specifically, Stanton et al. (2010) reported that pheromones and pheromone receptors play a major role in mating partner recognition during the initial period of sexual development, and that the transcription factor Sxi2a is required for subsequent filamentation and spore formation.

Our data are consistent with the view that Cir1 influences sexual development via regulation of mating pheromone expression, and it may also contribute to the morphological transition resulting in filamentation. Moreover, we found that Cir1 plays a role in the influence of copper on mating by a mecha-

nism that likely involves regulation of metal homeostasis. The influence of Cir1 appears to be more substantial in *MAT*α cells than in *MAT***a** cells, for unknown reasons. One possibility is that Cir1 may differentially influence the expression or the activity of the **a**- and α-specific homeodomain transcription factors (Sxi2a and Sxi1α) that negatively regulate the pheromone genes (Hull et al., 2005). As suggested by our previous study, differential expression of various components in the cAMP pathway and the MAP kinase pathway may also contribute to the deficiency of the *cir1* mutant in sexual development. It is possible that the influence of signaling components, copper, and Cir1 on mating are interconnected. Overall, these studies provide new insights into environmental factors (such as copper) that influence mating in *C. neoformans* by revealing coordination with the extensive Cir1 regulatory network for metal homeostasis and virulence.

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