The Link between Morphotype Transition and Virulence in *Cryptococcus neoformans*

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Abstract

*Cryptococcus neoformans* is a ubiquitous human fungal pathogen. This pathogen can undergo morphotype transition between the yeast and the filamentous form and such morphological transition has been implicated in virulence for decades. Morphotype transition is typically observed during mating, which is governed by pheromone signaling. Paradoxically, components specific to the pheromone signaling pathways play no or minimal direct roles in virulence. Thus, the link between morphotype transition and virulence and the underlying molecular mechanism remain elusive. Here, we demonstrate that filamentation can occur independent of pheromone signaling and mating, and both mating-dependent and mating-independent morphotype transition require the transcription factor Znf2. High expression of Znf2 is necessary and sufficient to initiate and maintain sex-independent filamentous growth under host-relevant conditions in vitro and during infection. Importantly, ZNF2 overexpression abolishes fungal virulence in murine models of cryptococcosis. Thus, Znf2 bridges the sex-independent morphotype transition and fungal pathogenicity. The impacts of Znf2 on morphological switch and pathogenicity are at least partly mediated through its effects on cell adhesion property. Cfl1, a Znf2 downstream factor, regulates morphogenesis, cell adhesion, biofilm formation, and virulence. Cfl1 is the first adhesin discovered in the phylum Basidiomycota of the Kingdom Fungi. Together with previous findings in other eukaryotic pathogens, our findings support a convergent evolution of plasticity in morphology and its impact on cell adhesion as a critical adaptive trait for pathogenesis.

Introduction

Adaptation to the host environment by many eukaryotic pathogens is often accompanied by transition in cellular morphology [1,2,3,4,5,6,7,8,9]. The ubiquitous fungal pathogen *Cryptococcus neoformans* causes more than half a million deaths each year [10]. It can grow in the yeast form as well as the filamentous form. Earlier pre-genetic studies indicate an inverse relationship between filamentation and virulence [11,12,13,14,15,16,17]. These studies also point to the potential of filament-specific antigens as vaccines against *Cryptococcus* infections [18,19,20].

Because *Cryptococcus* typically grows in the yeast form and the morphological transition from the yeast form to the filamentous form appears to be coupled with mating, signaling pathways that lead to bisexual mating (α-α mating) and unisexual mating (mostly α-α mating) have been intensively investigated [21,22,23,24]. The roles of these signaling components in fungal pathogenicity are also scrutinized in animal models. However, accumulating evidence indicates that key signaling components that specifically lead to mating, such as those in the pheromone sensing pathway, have no or minimal direct effect on virulence [25,26,27,28]. Furthermore, conditions relevant to host physiology (e.g. aqueous environment, high temperatures, and high levels of CO₂) are mating-suppressive, suggesting sex-independent mechanisms in orchestrating morphotype and virulence in *Cryptococcus* [29].

Therefore, the existence and the nature of the link between morphological transition and virulence in *Cryptococcus* remain enigmatic.

Results

Activation of Pheromone Signaling Is Insufficient to Drive Filamentation under Mating-Suppressing Conditions

Although *Cryptococcus* morphological transition from the yeast form to the filamentous form is historically associated with mating, the observations that filamentation can be achieved in strains in the absence of key pheromone signaling components or meiotic genes [30,31,32,33], lead us to hypothesize that pheromone signaling pathways are not essential or sufficient for filamentation per se, but they are critical in stimulating filamentation in response to mating cues. To test this hypothesis, we decided to examine the effect of constitutive activation of the pheromone signaling circuit on morphogenesis under mating-inducing and mating-suppressing conditions.

It is known that the expression of genes in the pheromone signaling pathway, such as those encoding the pheromone Mis7, the pheromone receptor Ste3, the pheromone transporter Ste6, and the key pheromone response regulator Mat2 (Figure 1A), is low under mating-suppressing conditions but is dramatically higher during α-α bisexual mating (Figure 1B and data not
Author Summary

Although morphogenesis and virulence are commonly associated in many eukaryotic pathogens, the nature of such association is often unknown. For example, Cryptococcus neoformans, a fungal pathogen that causes cryptococcal meningitis, typically undergoes morphological transition between the yeast and the filamentous form during mating. However, molecules that are critical for mating do not directly impact fungal virulence. Thus, the nature of the long observed association between morphotype and virulence in this microbe remains elusive despite decades of effort. Here we demonstrate that constitutively activated pheromone signaling is insufficient to drive morphological transition under mating-suppressing conditions, including those relevant to host physiology. Rather, we demonstrate that sex-independent morphological switching is driven by the transcription factor Znf2 and this regulator controls the ability of this fungus to cause disease. Znf2 governs Cryptococcus morphotype and virulence potential at least partly through its effects on cell surface proteins. One novel adhesin Cfl1 functions downstream of Znf2 and it orchestrates morphological switch, cell adhesion, biofilm formation, and pathogenicity. Thus, cell adhesion at least partly underlies the link between morphological transition and pathogenicity in C. neoformans. Our findings provide a platform for further elucidation of the impact of morphotype on virulence in this ubiquitous pathogen. The discovery of Cfl1 and other novel adhesins in Cryptococcus could lay a foundation for the development of vaccines or alternative therapeutics to combat the fatal diseases caused by this fungus.

Filamentation Can Be Independent of Sex and Is Controlled by the Transcription Factor Znf2

We previously showed that the deletion of ZNF2, which encodes a zinc-finger transcription factor, locked cells in the yeast form during mating without impairing pheromone signaling [28]. This suggests that Zfn2 is not essential for mating signal relay; rather, it is crucial for filamentation. Although Znf2 functions downstream of Mat2 during mating [28] and its gene expression was significantly induced by MAT2 overexpression under the mating-inducing condition (Figure 2C and 2D), activation of the pheromone signaling pathway was unable to induce ZNF2 expression in the absence of mating stimuli. This was evidenced by the low expression level of ZNF2 in the P<sub>GPD1</sub>-MAT2 strain under mating-suppressing conditions (Figure 2C and 2D). The ability of Cryptococcus to undergo filamentation correlates with the expression level of ZNF2, but not that of MAT2 (Figure 1C, Figure 2A, 2C and 2D). Thus, Znf2 could be a master regulator that dictates Cryptococcus morphotype irrespective of environmental stimuli or mating type.

To test this hypothesis, we constructed the P<sub>GPD1</sub>-ZNF2 strains. Indeed, the P<sub>GPD1</sub>-ZNF2 triggered filamentation in Cryptococcus strains of either mating types a or a in both serotype A and serotype D backgrounds under all tested conditions, including those that are inducing or suppressive to mating (Figure 3A and Figure S1). In contrast to the P<sub>GPD1</sub>-MAT2 strain, filaments produced by the P<sub>GPD1</sub>-ZNF2 strain under mating-inducing condition maintained the filamentous morphology after being transferred to mating-suppressive conditions (Figure S2). However, it is notable that the P<sub>GPD1</sub>-ZNF2 strain produces more robust hyphae under mating-inducing condition, suggesting that other factors induced under mating-inducing condition could further activate Znf2.

The P<sub>GPD1</sub>-ZNF2 also conferred filamentation to mutants that harbor deletions in the key mating components under various conditions tested (MIF1-3, Mat2, or Ste12 functioning in a branching pathway in pheromone signaling) (Figure 3B). To confirm that filamentation conferred by Znf2 activation is not due to some cryptic restoration of mating ability, we measured the efficiency of cell fusion of the wildtype, the mat2a mutant, and the mat2Δ+P<sub>GPD1</sub>-ZNF2 strain during bisexual a-a mating. Indeed, overexpression of ZNF2 did not rescue the cell fusion defects of the mat2Δ mutant (Figure 3C). Consistently, gene ontology analyses of our previous transcription data indicate that Znf2, unlike Mat2, does not regulate genes involved in the cell fusion event critical for mating (Figure S3) [28]. Taken together, the results indicate that filamentation can be independent of mating and Znf2 is one key determinant of this sex-independent morphogenesis.
Expression Level of Znf2 Mediates Bi-directional Morphological Transition

To verify the correlation of Znf2 expression and Cryptococcus morphology, we constructed the Znf2 gene driven by two inducible promoters: the galactose-inducible GAL10 promoter (data not shown) [35] or the copper transporter CTR4 promoter (Figure 3D) (copper deprivation–on; copper repletion–off) [36]. Transformation of the P<sub>GAL10</sub>-Znf2 or the P<sub>CTR4</sub>-Znf2 construct into wildtype either the serotype D reference strain JEC21 or the serotype A reference strain H99 conferred filamentous growth under promoter-inducing conditions. These strains grew as yeasts under promoter-repressive conditions (YPD and Serum). Because unilateral mating is not observed in the wildtype strain, the induction of pheromone was evaluated during bisexual mating with the cotulure of H99α and its congenic partner KN99α incubated on different media for 72 hr. The expression level of MF1α during bisexual mating on V8 medium was arbitrarily set as 1 for comparison. (C, D, E and F) In order to analyze the effect of the deletion or the overexpression of Znf2 on the key elements of the pheromone pathway and to avoid potential complication due to higher expression of these elements in the presence of a compatible mating partner under mating-inducing conditions, only α strains alone in the H99 background were used in the these assays. Overexpression of Znf2 constitutively activated pheromone signaling in single strain cultures under all the conditions tested. The expression patterns of MAT2 (C), MF1α (D), STE3 (pheromone receptor gene) (E), and STE6 (pheromone transporter gene) (F) in wildtype (H99), and its derived mat2Δ mutant and the P<sub>GPD1</sub>-MAT2 strain were shown. Gene expression levels in the wildtype H99 grown on V8 medium were arbitrarily set as 1 for comparison. Cells were cultured on different media for 72 hr.

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To examine the effect of Znf2 on the dynamic morphological transition, we incubated the P<sub>CTR4</sub>-Znf2 strain in H99 background in liquid YPD medium containing 200 μM BCS (inducer) and examined cell morphology over time. Morphological transition from the yeast form to the filamentous form completed by 60 hours (Figure 4). At this time, the hyphae were transferred to YPD medium containing copper sulfate (inhibitor). Cryptococcus cells then switched from the filamentous form to the yeast form over time (Figure 4). The control of bi-directional morphological transition by Znf2 is also observed when cells were cultured in serum (data not shown), indicating that this control is independent of environmental cues. These results demonstrate that (i) the expression level of Znf2 determines Cryptococcus cell morphology: high expression level of Znf2 drives the cells to the filamentous form and low expression level of Znf2 renders cells unicellular yeast; (ii) Znf2 is necessary and sufficient to initiate morphological transition; (iii) High Znf2 activity is required to maintain cells in the filamentous morphotype.

Figure 1. Overexpression of MAT2 causes constitutive activation of pheromone signaling. (A) The C. neoformans pheromone signaling pathway. Pheromone signaling is triggered by environmental cues (matting cues) and it turns on the master regulator Mat2, which in turn activates pheromone signaling, thereby constituting a self-reinforcing system. Activated pheromone signaling determines the output mating-relevant behaviors (e.g. formation of shmoo cells and mating projections, and initiation and cell contact and cell fusion). (B) MF1α (pheromone) and other mating signal genes (not shown here) were highly induced in α x α cotulures under the mating-inducing condition (V8), but not under mating-suppressing conditions (YPD and Serum). Because unilateral mating is not observed in the wildtype strain, the induction of pheromone was evaluated during bisexual mating with the cotulure of H99α and its congenic partner KN99α incubated on different media for 72 hr. The expression level of MF1α during bisexual mating on V8 medium was arbitrarily set as 1 for comparison. (C, D, E and F) In order to analyze the effect of the deletion or the overexpression of MAT2 on the key elements of the pheromone pathway and to avoid potential complication due to higher expression of these elements in the presence of a compatible mating partner under mating-inducing conditions, only α strains alone in the H99 background were used in the these assays. Overexpression of MAT2 constitutively activated pheromone signaling in single strain cultures under all the conditions tested. The expression patterns of MAT2 (C), MF1α (D), STE3 (pheromone receptor gene) (E), and STE6 (pheromone transporter gene) (F) in wildtype (H99), and its derived mat2Δ mutant and the P<sub>GPD1</sub>-MAT2 strain were shown. Gene expression levels in the wildtype H99 grown on V8 medium were arbitrarily set as 1 for comparison. Cells were cultured on different media for 72 hr.

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Znf2 Controls Fungal Ability to Cause Disease

The relationship between morphotype and pathogenicity is typically defined through studying morphological mutants that are otherwise isogenic to the wildtype strains and are able to maintain given morphotype under host relevant conditions, even though mutants with such extreme phenotypes are unlikely to be encountered clinically due to natural selections in the host [1,2,3,4]. For Cryptococcus, host physiological environment (e.g. high body temperature, aqueous environment, and high levels of CO2) is extremely inhibitory to mating. Consistently, constitutively activated mating signaling induced filamentation under mating-suppressing conditions irrespective of culture conditions. White arrows indicate shmoo-like cells and the red arrow indicates a hypha cell. (C and D) Activated pheromone signaling only induced the transcription of ZNF2 under mating-inducing condition either during unisexual mating (a cell culture alone) (C) or during bisexual mating (a-a cocultures) (D). Transcript levels of ZNF2 in the wildtype H99 grown on YPD agar were arbitrarily set as 1 for comparison. The cells were cultured on different media for 72 hr.

We tested the virulence of the wildtype H99 and the P_gpd1-ZNF2 strain in the murine inhalation model of cryptococcosis. The P_gpd1-ZNF2 strain exhibited heterogeneity in cell morphology and a mixture of cell types is always present in this strain. To obtain accurate inoculation and to avoid potential problems caused by differences in cell types at initial infection, only cells in the yeast form were used for animal inoculation. Remarkably, the P_gpd1-ZNF2 strain was completely avirulent (Figure 5A). By day 60 post infection (DPI 60) when the study was terminated, the P_gpd1-ZNF2 cells were either completely cleared from animal lungs or existed in very low numbers (1000 fold lower than the original inocula). We further examined the fungal burden in the lungs and the brain of animals infected with H99, the znf2D mutant, and the P_gpd1-ZNF2 strain at DPI 10 before any animal succumbed to cryptococcosis. Consistent with the animal survival rates, the lung fungal burden in animals infected with the znf2D mutant and the P_gpd1-ZNF2 strain was 236% and 0.6% respectively compared to those infected with the wildtype (Figure 5B). The brain fungal burden showed a similar
trend with larger variations due to individual differences in the timing of dissemination in this inhalation model (Figure S4), and no fungal cells were recovered from the brains of animals infected by the PGPD1-ZNF2 strain. To examine the effects of Znf2 on fungal morphology in vivo, we infected animals intranasally with H99, the znf2D mutant, and the PGPD1-ZNF2 strain and performed histological examination of lung tissues at DPI 1, 7, and 12. Remarkably, even though only yeast cells from the PGPD1-ZNF2 strain were used in the original inoculation into animals, lungs infected by the PGPD1-ZNF2 strain contained Cryptococcus cells of mixed morphology: yeast, pseudohyphae, and hyphae in all the time points examined (Figure 5C and Figure S5). This is consistent with the morphological heterogeneity of the PGPD1-ZNF2 strain in vivo (Figure S2). In comparison, only yeast cells were observed in the wildtype H99 or the znf2D mutant infected animals (Figure 5C and Figure S5). This histological examination indicates that activation of Znf2 can drive filamentation in vivo.

Znf2 Controls Cell Adhesion through Its Regulation of Adhesion Proteins

Tolerance of host temperatures is a pre-requisite of fungal virulence. In some fungal pathogens, morphological changes are
often a response to temperature and some morphological defective mutants lose the ability to cause diseases in mammalian hosts due to growth inhibition by high temperatures in vivo. To determine if alteration of virulence potential in the \textit{znf2} mutants are simply due to altered sensitivity to high temperature, we compared the growth of the wildtype H99, the \textit{znf2} mutant, and the P\textsubscript{CTR4-2-ZNF2} strain at 30\degree C and 37\degree C on a variety of media via the spot assay. No apparent growth defects were observed in the \textit{znf2} mutant or the \textit{ZNF2} overexpression strain when compared to the wildtype under the conditions tested (Figure S6). Furthermore, the observation that the \textit{ZNF2} overexpression strain was capable of amplification during early stages of infection based on the fungal burden time course experiment (Figure S7) also suggests that factors other than growth inhibition by high temperature are mainly responsible for the effects of \textit{Znf2} on virulence.

As morphological changes reflect changes in cell surface properties, we predict that \textit{Znf2} controls cell surface constitutes. One property likely regulated by \textit{Znf2} is cell adhesion, as supported by the following observations. First, increasing the \textit{ZNF2} expression led to increasingly wrinkled colony morphology and flocculation (Figure 3D, and Figure 6A, B and C). Both phenotypes are likely caused by increased expression of flocculins (adhesins or adhesion proteins), as previously shown in bacteria and in yeasts [37,38]. Second, aerial hyphae of the \textit{ZNF2} overexpression strains formed on solid media also tended to attach to each other, forming bundles (Figure 6D), as observed in flocculated strains of the filamentous fungus \textit{Ashbya gossypii} [39]. Third, deletion of \textit{ZNF2} impairs agar invasion whereas overexpression of \textit{ZNF2} remarkably promoted invasive growth (Figure 6E), and invasive growth reflects cell-substrate adhesion. The results suggest that \textit{Znf2} plays a pivotal role in morphogenesis-associated cell flocculation in \textit{Cryptococcus}.

Given that \textit{Cryptococcus} strains with increased flocculation are reduced in virulence [40,41], this transcription factor likely impacts pathogenicity at least partly through its effects on cell adhesion. Ontology analysis of our previous transcriptional profiling data [42] revealed that of those genes that are differentially expressed in the \textit{znf2} mutants, 23% encode secretory proteins based on WoLF PSORT prediction (http://wolfpsort.org/) (Figure 7A). We selected 9 such genes and examined their transcript level in a \textit{ZNF2} overexpression strain incubated in serum at 37\degree C in 5\% CO\textsubscript{2} by quantitative realtime PCR. All genes tested were also differentially expressed in the \textit{ZNF2} overexpression strain (Figure 7B).

We overexpressed these 9 genes using the constitutively active \textit{GPD1} promoter and examined if their overexpression could recapitulate some of the phenotypes caused by the \textit{ZNF2} overexpression (Figure 7C). Interestingly, strains with overexpression of CNAG\_00795 (designated as \textit{CFL1}; Cell FlOcculin 1) formed extremely wrinkled colonies, like \textit{ZNF2} overexpression strains (Figure 6A). Interestingly, the expression of \textit{CFL1} was also most dramatically induced by the \textit{ZNF2} overexpression
Figure 6. Znf2 regulates cell adhesion in *Cryptococcus*. (A) The P<sub>CTR4-2-ZNF2</sub> strain and a strain transformed with the empty vector (control) were incubated on YPD agar medium that contains either BCS (inducer) or CuSO<sub>4</sub> (inhibitor). Cells scraped from the colony were examined microscopically (images below). (B) Wildtype H99 and the P<sub>CTR4-2-ZNF2</sub> strain were pre-grown in YPD medium containing 25 μM CuSO<sub>4</sub> (inhibitor) for 12 hrs (no cell aggregation). The yeast cells were washed twice, inoculated into fresh YPD medium containing 200 μM BCS (inducer) and grown for additional 4 hrs with shaking before they were allowed to settle. Cell concentration of the upper stagnate culture (OD<sub>600</sub>) was measured every 30 min. (C) Cells from the bottom of the cultures were examined microscopically. (D) Overexpression of ZNF2 leads to the formation of hyphal bundles. Wildtype H99 and its derived P<sub>CTR4-2-ZNF2</sub> strain were grown on YPD BCS agar plate at 22°C for 5 days. Multiple hyphae were attached together forming bundles in the P<sub>CTR4-2-ZNF2</sub> strain (scale bar: 100 μm). (E) Znf2 controls invasive growth. Wildtype XL280 and its derived znf2<sup>Δ</sup> mutant and the P<sub>GPD1-ZNF2</sub> strain were grown on YPD agar medium at 22°C for 5 days. The left column shows the original colonies; the middle column shows invasive cells after surface cells were washed off; and the right column shows enlarged images of the remaining invasive cells.

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Figure 7. Znf2 regulates the expression of many extracellular Proteins. (A) Classification of genes differentially expressed in znf2<sup>Δ</sup> mutants compared with wildtype. (B) Selected genes predicted to encode extracellular proteins were also differentially expressed in the ZNF2 overexpression strains by qPCR. (C) Gene overexpression strains in the H99 background were grown on YPD medium at 22°C for 3 days. (D) H99, the P<sub>CTR4-2-ZNF2</sub> strain, and the P<sub>CTR4-2-CFL1</sub> strain were pre-grown for 12 hrs in YPD liquid medium containing CuSO<sub>4</sub> (inhibitor). The yeast cells were washed twice and then incubated on YPD agar medium containing BCS (inducer) for 3 days. Cells scraped from the colony above were examined microscopically (shown below).

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Because acapsular *Cryptococcus* mutants also form wrinkled colony, we examined capsule production in the *CFL1* overexpression strain and *cfl1Δ* mutants. No apparent defect in capsule production was detected based on microscopic examination (data not shown).

To confirm that cell adhesion is indeed caused by increased *CFL1* expression, we then constructed P<sub>CTR4-2</sub>*CFL1* strains. These strains grew as yeast cells in liquid cultures. A sharp increase in cell aggregation was observed when P<sub>CTR4-2</sub>*CFL1* cells were cultured under promoter-inducing conditions, a reminiscence of some of the phenotypes of the P<sub>CTR4-2</sub>*ZNF2* strains (Figure 7D).

To further confirm that *CFL1* is regulated by Znf2, we engineered a reporter strain where *ZNF2* expression is inducible by galactose and the fluorescent Cfl1 is driven by its native promoter. We grew the reporter strain under mating-suppressing conditions to avoid complication due to potential activation of mating signaling. Under such conditions, the colony formed by the reporter strain became fluorescent and wrinkled when the *ZNF2* expression was induced in the presence of galactose (Figure 8A and B), while the colony was non-fluorescent and smooth when the *ZNF2* expression was inhibited in the presence of glucose (Figure 8A and B). Thus the expression of the fluorescent Cfl1 is driven by Znf2. Taken together, Znf2 triggers morphological switch as well as flocculation (cell adhesion), and its downstream factor Cfl1 regulates cell adhesion.

**CFL1** Is Morphotype-specific and Its Secretion Is Required for Cell Adhesion

We examined the sub-localization of Cfl1 using a strain harboring the mCherry fused Cfl1 protein driven by its native promoter. Because Cfl1 is induced during mating and controlled by key components of mating signaling (Figure S8A and B), we examined microscopically the expression of *CFL1*-m-cherry during mating. Cfl1 was rarely detected in yeast cells (Figure 8C), but it was highly expressed in hyphae during both unisexual mating and bisexual mating (Figure 8D). The fluorescent Cfl1 delineated the periphery of hyphal cells, consistent with the function of adhesins on the cell surface and the prediction that Cfl1 is a secretory protein based on the presence of an N-terminal signal peptide for secretion.

Secretion is required for Cfl1’s function as an adhesin. This is supported by the observation that overexpression of the fluorescent Cfl1 that lacks the N-terminal signal peptide [Cfl1(sigPD)-mCherry] failed to confer wrinkled colony morphology or cell aggregation to *Cryptococcus* (Figure S9 and Figure 8E). This is not due to a failure of producing the mutant allele protein, as...
abundant Cfl1(sigPΔ)-mCherry protein was produced by the cells (Figure 8E). However, no fluorescence was detected from the culture supernatant (Figure 8F), indicating defects in secretion. A few other fungal adhesins are also reported to be associated with cell surface as well as being released into surrounding environment [43,44]. Such property may facilitate their roles in mediating both cell-cell adhesion and cell-substrate adhesion, and it may also help circumvent the blockage by other extracellular components. Consistent with its role as an adhesin, Cfl1 regulates a broad spectrum of cell adhesion-related biological processes, including complex colony morphology [45,46] and formation of different biofilms (Figure S10).

Remarkably, deletion of CFL1 dramatically reduced hyphal production during either bisexual or unisexual mating while overexpression of CFL1 enhanced the hyphal formation (Figure 9A and B). Thus, both the expression pattern of CFL1 and the observed effects of CFL1 deletion or overexpression on hyphal development indicate the importance of this adhesin in hyphal morphogenesis. Like the ζNF2 overexpression strain, hyphae formed by the P_GPD1-CFL1 strain on YPD medium (mating-suppressive) tended to attach to each other, forming bundles (Figure 6C and Figure 9A).

Cfl1 Affects Fungal Virulence

Previous studies implicate an inverse association between flocculation and virulence in Cryptococcus [40,41]. Consistently, we found that overexpression of CFL1 resulted in attenuation in virulence, indicating that Cfl1-mediated cell adhesion negatively modulates virulence (Figure 9C). Consistently, organ fungal burdens were maintained at low level in the P_GPD1-CFL1 and P_GPD1-ζNF2 infected animals at DPI 7, whereas the wildtype H99 strain proliferated significantly (Figure 9D). Unlike the P_GPD1-ζNF2 strain, the P_GPD1-CFL1 strain was not completely avirulent and the P_GPD1-CFL1 strain proliferated significantly when examined at DPI 12 (Figure S11). This is surprising but not unexpected as the impact of ζNF2 overexpression is likely the combinational effect of additional adhesion proteins and morphogenesis factors. As noted for znf2 mutations, deletion or overexpression of CFL1 did not cause any apparent change in growth compared to wildtype when cultured at 37°C with 5% CO2 (Figure S12A and B). Cells aggregated when CFL1 was overexpressed at both 30°C and 37°C as expected.

Discussion

C. neoformans is the major fungal pathogen from the phylum Basidiomycota in the Kingdom Fungi. Its morphological differentiation is typically heterogeneous and stochastic, and has been historically associated with mating. Pheromone signaling is the master regulation system in fungal mating, and it is required for early mating events such as cell recognition, mating projection formation, and initiation of cell contact and cell fusion [47,48,49]. However, increasing evidence implies that filamentation in Cryptococcus is a plastic process that is not limited to mating or the production of recombinant progeny: Filamentation is occasionally observed under mating-suppressing conditions, even in some attenuated strains isolated from infected host tissues [50,51,52,53]; Filamentation can occur in the absence of some key components of pheromone signaling or meiosis machinery [30,32,33,54]. Thus, filamentation could be used in behaviors unrelated with mating, such as foraging nutrients or defending predation. Such sex-independent cellular differentiation likely involves signaling pathways in response to cues other than the mating signal.

Here we show that sex-independent morphogenesis is linked with virulence in this fungus. We further demonstrate that the transcription factor Znf2 plays a pivotal role in cryptococcal morphological transition, and it is necessary and sufficient to drive...
filamentation irrespective of environmental cues, mating types, or pheromone signaling. Znf2 not only controls morphogenesis in vivo, but also the ability of this fungus to cause diseases. Thus Znf2 provides the key link between morphogenesis and virulence in Cryptococcus.

The exact mechanism by which Znf2 controls morphogenesis and links Cryptococcus pathogenicity is of great interest. Previous and this current in vitro studies indicate that Znf2 does not affect typical Cryptococcus virulence traits (e.g. melanization, capsule production, growth at high temperatures, growth in minimal media, and resistance to salt or H2O2 [42,55]). Although the P\textit{gpd}-ZnF2 strain is avirulent, this strain was capable of propagation during the first two weeks of infection (Figure S7). This is in contrast with other avirulent strains such as \textit{can1} or capsule mutants, which are less fit under various stress conditions and are rapidly cleared by the host [56,57]. These lines of evidence point to new traits regulated by Znf2 that influence pathogenicity.

Our observation that genes encoding secretory proteins are enriched within the regulon of Znf2 emphasizes the importance of changes in cell surface during morphogenesis. Given that Cryptococcus strains with increased flocculation have been noted to be reduced in virulence [40,41], Znf2 likely impacts pathogenicity at least partly through its effects on cell adhesion (flocculation). Cell adhesion mediated by microbial pathogens usually involves a repertoire of extracellular adhesion proteins. One of Znf2’s downstream factors, CFL1, is a prominent adhesion protein which orchestrates filamentation, cell adhesion, and virulence. To our knowledge, CFL1 is the first Cryptococcus adhesin discovered. Interestingly, CFL1 does not resemble any known adhesins characterized in ascomycetous fungi in terms of primary sequences and functional domains based on Pfam prediction (http://pfam.sanger.ac.uk/). There are four other homologues of CFL1 in the genome of Cryptococcus and in some other species in the phylum of Basidiomycota (Figure S15), in which no adhesin has been identified so far. This suggests that CFL1 and its homologues represent a novel adhesion family specific to Basidiomycota.

Unlike Znf2, overexpression of CFL1 attenuates but does not abolish Cryptococcus virulence in the murine model of cryptococcosis. This is not unexpected as studies show that microbes are typically endowed with multiple adhesins. The master regulator Znf2 likely controls additional adhesins and other morphogenesis factors, and it is the orchestrated effects of its downstream targets that give rise to its overall impact on morphogenesis and virulence. Further characterization of CFL1, other adhesins, and morphogens downstream of Znf2 can help parse out the effects of cell morphotype and other cell properties (e.g. changes in cell surface proteins like adhesins) on Cryptococcus virulence. Such investigation may lay a foundation for future endeavors to develop vaccines or alternative therapies against cryptococcosis.

**Materials and Methods**

**Ethics Statement**

This study was performed according to the guidelines of NIH and Texas A&M University Institutional Animal Care and Use Committee (IACUC). The animal models and procedures used have been approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University (protocol number: 2011-22).

**Strains, Mating, and in vitro Phenotypic Assays**

Strains used in this study are listed in Table S1. For mating assays, parental strains (\textit{a} and \textit{d}) with equal number of cells were cocultured together on V8 medium in the dark at 22°C, and mating was examined microscopically for formation of mating hyphae and spores [30]. For cell fusion assays, the coculture of marked parental strains were removed after 48 hours of incubation on V8 medium, washed, and plated on selective media to select fusion products at 37°C as described previously [28,33,59]. For self-filamentation assays, cells were patched on V8 medium alone and hypha formation was examined microscopically. Phenotypical assays in vitro were performed as previously described [59]. The serotype A strain H99 is highly virulent and has been widely used in pathogenesis studies. Thus strains generated in this genetic background were used in the animal experiments and many of the in vitro characterization experiments. However, because wildtype H99 has not been observed to undergo unisexual mating and its bisexual mating is rather weak compared to the well-characterized but less virulent serotype D strains such as JEC21 and XL290, strains generated in these genetic backgrounds were used in some of the morphogenesis and mating assays.

**Construction of Gene Deletion and Gene Overexpression Strains**

Plasmids and primers used in this study are listed in Table S2 and S3. For gene deletion, overlap PCR products with an appropriate selection marker connected with the 5’ and 3’ flanking regions of gene of interests were introduced into Cryptococcus strains by biolistic transformation and transformants with homologous replacement were selected as described previously [60]. For overexpression, genes were amplified by PCR and the amplified fragments were digested and inserted into pXL1 after the \textit{GPD1} promoter [61]. The \textit{P\textit{gpd}} of the resulting plasmids was replaced with either the \textit{P\textit{ctr4-2}} or the \textit{P\textit{gal10}} to generate the copper or the galactose inducible system. The \textit{P\textit{ctr4-2}} and \textit{P\textit{gal10}} were amplified from the plasmid pNAT/\textit{CTR4-2} and H99 genomic DNA respectively [35,62].

**Construction of Fluorescent Proteins and Microscopic Examination**

Because CFL1 contains a predicted secretory signal peptide at its N-terminus, the mCherry [63] was fused to the C-terminus. The fragment including CFL1 coding region and 1 kb upstream sequences (NCFL1) was pieced together with the mCherry by an overlap PCR. The resulting products were introduced into plasmid pXL1 to generate pXL1-NCFL1-mCherry (for the serotype \textit{A} H99 allele) and pXL1-NCFL1-mCherryD (for the serotype \textit{D} JEC21 allele). The CFL1-mCherry without the CFL1 promoter was amplified and introduced into pXL1 after the \textit{GPD1} promoter [61]. The \textit{P\textit{gpd}} of the resulting plasmids was replaced with either the \textit{P\textit{ctr4-2}} or the \textit{P\textit{gal10}} to generate the copper or the galactose inducible system. The \textit{P\textit{ctr4-2}} and \textit{P\textit{gal10}} were amplified from the plasmid pNAT/\textit{CTR4-2} and H99 genomic DNA respectively [35,62].

**RNA Purification and qPCR Analyses**

Total RNA was purified using the purelink RNA purification kit (Invitrogen) and was used as the template for the first strand cDNA synthesis using the Superscript III cDNA synthesis kit (Invitrogen). Relative expression level of selected genes was measured by real
time PCR using power SYBR qPCR premix reagents (Invitrogen) in a Realplex system (Eppendorf). Primer efficiency was determined by serially diluting the cDNA and monitoring DNA amplification by real-time PCR. Primers for qPCR used in this study are listed in Table S3. Gene-expression levels were normalized using the endogenous control gene TEF1. The relative transcript levels were determined using the comparative CT method as described previously [64].

Northern Blots
RNA was separated on agarose gels blotted to nylon membrane. Redi-Prime II kit (Amersham) was used to generate probes. The C. neoformans actin gene transcript served as a control. mRNA purification was performed using the PolyATtract mRNA Isolation System III (Fisher) according to the manufacturer’s instruction.

Measurement of Cryptococcal Biofilms
The cells were cultured in 96-well microtitre plates under a variety of growth conditions. The air-liquid interface biofilm was observed only in CFL1 overexpression strains. The strains were grown in YPD liquid medium for 8 days. Crystal violet method was used for the quantitative assessment of the ability of Cryptococcus strains to form biofilm as previously described [65].

Murine Models of Cryptococcosis
Animals were infected essentially as previously described [59,66]. Groups of 6- to 8-week-old female A/ mice (Jackson Labs) were infected intranasally with 1x10^5 Cryptococcus cells in 50 μl PBS. For the P_gpd1-ZNF2 strain, the culture of cells with mixed morphotype was centrifuged briefly at a low speed to allow the enrichment of yeast cells on the top. The top culture was then centrifuged again and only yeast cells were collected for infection. Ten mice per group were used for survival studies, and four or five were used for organ fungal burden studies and histological examinations. For organ fungal burden studies, fungal CFUs from lungs, kidneys, spleen, and the brains of sacrificed mice at each time point were measured as described previously [59,67]. Dunnett’s two-tailed t test was used to test statistical differences (P≤0.05). For histological examinations, organs from the sacrificed animals were fixed in 10% formalin, embedded in paraffin, sectioned at 5 μm in thickness, and stained with hematoxylin and eosin (H&E) and Gomori methenamine silver (GMS) as previously described [56,68]. For mortality studies, the infected animals were monitored until all mice were sacrificed due to sickness or up to DPI 60 when the experiment was terminated. If the experiment was terminated, surviving animals were examined for the presence of Cryptococcus cells. Statistical significance (P≤0.05) of the survival data between different groups was assessed by the Mantel-Cox log-rank test [69].

Accession Numbers for Genes and Proteins Mentioned in this Study
C. neoformans var. grubii (H99): ZNF2 (CNAG_00366); MAT2 (CNAG_06203); STE3x (CNAG_06800); STE6 (CNAG_03600); MF1x (CNAG_07407); CPL1 (CNAG_00795) and other secretory protein encoding genes controlled by Znf2 (CNAG_00596, CNAG_00925, CNAG_01211, CNAG_05778, CNAG_07422, CNAG_06239, CNAG_06411, CNAG_05729); KEL1 (CNAG_01149); CDC10 (CNAG_01373); CDC12 (CNAG_01740); cnCDC11 (CNAG_02196); cnMUC1 (CNAG_03234); cnCDC24 (CNAG_04243); cnCDC3 (CNAG_05925).

C. neoformans var. neoformans [JEC21]: ZNF2 (CNAG_02160); MAT2 (CNMD02020); STE12a (CNDO5010); CFL1 (CNAG07720). Gene ID numbers were obtained from either NCBI (Entrez or the Cryptococcus genome website at the Broad Institute http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/MultiHome.html)

Supporting Information

Figure S1 Znf2 governs filamentation in Cryptococcus neoformans. Wildtype XL280 (serotype D, y) and its derived znf2Δ mutant and the P_gpd1-ZNF2 strain were grown on V8 juice agar medium at 22°C (mating-inducing condition) (scale bar: 500 μm) or in YPD liquid medium at 30°C (mating-suppressing condition) (scale bar: 25 μm) for 5 days. (TIF)

Figure S2 Constitutively activated pheromone signaling is insufficient to maintain hyphal growth under a host-relevant condition. Wildtype H99 and its derived P_gpd1-MAT2 and P_gpd1-ZNF2 strains were grown on V8 agar medium at 22°C (mating-inducing condition). At the 5th day, cells were collected, washed, and transferred to serum at 37°C with 5% CO2 (host-relevant condition) and incubated for additional 5 days (scale bar: 40 μm). Only cells of the P_gpd1-ZNF2 strain remained in the hyphal form under such conditions. (TIF)

Figure S3 Znf2 does not control the expression of genes involved in the early events of mating. Comparative profiling of gene expression in the wildtype, the mat2Δ mutant, the ste7Δ mutant, and the znf2Δ mutant [28] revealed that S. cerevisiae homologues known to be involved in early events of mating (e.g. mating projection formation and cell fusion) were regulated by Mat2 and Ste7, but not by Znf2. CDC3 and CDC12 were also experimentally shown in Cryptococcus to be required for full mating efficiency [63]. The transcript level change (fold) is represented by a color code. The homologues of C. neoformans genes in S. cerevisiae were identified based on HUWU-BLASTUH program (http://amigo.geneontology.org); “Sce gene” shows the corresponding S. cerevisiae gene name of the C. neoformans homologue. (TIF)

Figure S4 Znf2 controls the level of fungal burden in the brain of infected mice. Mice were infected intranasally with wildtype H99, the znf2Δ mutant, and a P_gpd1-ZNF2 strain. Fungal burden in the brains at DPI 10 was determined. Differences among the groups are statistically significant (p<0.05). n. d.: Not detectable. CFU: colony forming unit. (TIF)

Figure S5 The P_gpd1-ZNF2 strain produces cells of the filamentous form during infection. Lung tissues from mice infected with Cryptococcus strains (H99, the znf2Δ mutant and the P_gpd1-ZNF2 strain) were fixed, sectioned, and stained with Grocott–Gomori methenamine silver to visualize fungal cells. Scale bar: 10 μm. (TIF)

Figure S6 The znf2 mutations do not cause any apparent growth defects at high temperature. (A) Diagram of the P_ctr1-x-ZNF2 inducible system. (B) Cells of C. neoformans strains (H99, znf2Δ mutant and P_ctr1-x-ZNF2) were cultured on YPD medium containing CuSO4 overnight and all strains were in the yeast form under such condition. The cells then were quantified by measuring the optical density at 600 nm.
Three-microliters of the cell suspensions with 10^3 serial dilutions were spotted onto media. Growth of cells on YPD, DME, and RPMI media containing either BCS or CuSO_4 at 30°C in the ambient air for 3 days were compared to those at 37°C under 5% CO_2. Cells grown on DME medium or RPMI medium at 37°C for 3 days appeared more mucoid due to enhanced capsule production. Capsule production was confirmed with India ink staining (data not shown).

(TIF)

**Figure S7** The ZNF2 overexpression strain proliferated in vivo. Mice were infected with the P_CFL1-ZNF2 cells in the yeast form intranasally. Fungal burden in the lungs was determined at DPI 1, 7, 12, and 16. The graph shows the changes in fungal burden over time. The average CFU at DPI 1 was 0.66×10^5.

(TIF)

**Figure S8** The CFL1 expression is dependent on Znf2 during bisexual mating. (A) CFL1 was highly expressed in a x α cocultures under the mating-inducing condition (V8) but not under mating-suppressing conditions (YPD and Serum). H99α and its congenic partner KN99a were cocultured on different media for 72 hr. The expression level of CFL1 during bisexual mating on V8 medium was arbitrarily set as 1 for comparison. (B) The expression pattern of CFL1 during bilateral matings of the cocultures (a X α, a znf2a X α znf2a, a mat2a X α mat2a and a ste7Δa X α ste7Δa in JEC21 background) on V8 medium (pH = 7.0) for 24 hr.

(TIF)

**Figure S9** Diagram of the m-Cherry labeled wildtype CFL1 allele and the mutant CFL1 allele that lacks the secretion signal. Both CFL1 alleles are constructed under the control of P_CFL1 to so that the transcriptional levels of CFL1-mCherry hybrid alleles can be readily manipulated by external addition of inducer (BCS) or inhibitor (CuSO_4). The arrow points to the 54-bp DNA region predicted to code the secretory signal peptide.

(TIF)

**Figure S10** Overexpression of CFL1 results in complex colony morphology and formation of different biofilms. (A) The CFL1 overexpression strain and wildtype H99 were grown on YPD agar medium for 4 days. The CFL1 overexpression strain showed an elaborate pattern of complex multicellular growth. This complex colony morphology resembles the mat biofilm formation reported in *Scecnanomyces cerevisiae* [45]. (B and C) The overexpression of CFL1 greatly enhances the ability of Cryptococcus to form different biofilms. CFL1 overexpression induced by inducer (BCS) triggers the formation of air-liquid interface biofilm (B) and it increases the formation of plastic surface-anchored biofilm (C).

(TIF)

**Figure S11** Overexpression of CFL1 results in reduced lung fungal burden. Mice were infected intranasally with 1×10^7 cells of either wildtype H99 or the P_CFL1-CFL1 strain. Fungal burden in the lungs at DPI 10 was measured. Differences among the groups are statistically significant (p<0.05).

(TIF)

**Figure S12** The cfl1 mutations do not cause any apparent growth defects at high temperature. (A) Cells of *C. neoformans* strains (H99, the cfl1A mutant, and the P_CFL1-CFL1 strain) were cultured on YPD medium containing CuSO_4 overnight and all strains were in the yeast form under such condition. The cells then were quantified by determining the optical density at 600 nm. Three-microliters of the cell suspensions with 10^3 serial dilutions were spotted onto media. Growth of cells on YPD, DME, and RPMI media containing either BCS or CuSO_4 at 30°C for 3 days in the ambient air were compared to those at 37°C under 5% CO_2. Notably, cells grown on DME or RPMI medium at 37°C under 5% CO_2 appeared more mucoid due to enhanced capsule production. Capsule production was confirmed with India ink staining (data not shown). (B) CFL1 overexpression leads to cell aggregation on RPMI agar.

(TIF)

**Figure S13** Phylogenetic tree of Cfl1 homologs. Protein sequences were aligned using the neighbor-joining method with MEGA v5.04 program [http://www.megasoftware.net/mega4/mega.html]. Cfl1 and its paralogues (Cfl2, Cfl3, Cfl4 and Cfl5) from *Cryptococcus neoformans* are indicated by red dots. Organisms whose genomes contain *CFL1* homologues all belong to the phylum Basidiomycota.

(TIF)

**Table S1** Strains used in this study.

**Table S2** Plasmids used in this study.

**Table S3** Primers used in this study.

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**Author Contributions**

Conceived and designed the experiments: LW BZ XL. Performed the experiments: LW BZ XL. Analyzed the data: LW BZ XL. Contributed reagents/materials/analysis tools: XL. Wrote the paper: LW XL.

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