Fungal adhesion protein guides community behaviors and autoinduction in a paracrine manner

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Microbes live mostly in a social community rather than in a planktonic state. Such communities have complex spatiotemporal patterns that require intercellular communication to coordinate gene expression. Here, we demonstrate that *Cryptococcus neoformans*, a model eukaryotic pathogen, responds to an extracellular signal in constructing its colony morphology. The signal that directs this community behavior is not a molecule of low molecular weight like pheromones or quorum-sensing molecules but a secreted protein. Znf2, a master regulator of morphogenesis in *Cryptococcus*, is necessary and sufficient for the production of this signal protein. Cfl1, a prominent Znf2-downstream adhesion protein (adhesin), was identified to be responsible for the paracrine communication. Consistent with its role in communication, Cfl1 is highly induced during mating colony differentiation, and some of the Cfl1 proteins undergo shedding and are released from the cell wall. The released Cfl1 is enriched in the extracellular matrix and acts as an autoinduction signal to stimulate neighboring cells to phenocopy Cfl1-expressing cells via the filamentation-signaling pathway. We further demonstrate the importance of an unannotated and yet conserved domain in Cfl1's signaling activity. Although adhesion proteins have long been considered to be mediators of microbial pathogenicity and the structural components of biofilms, our work presented here provides the direct evidence supporting the signaling activation by microbial adhesion/matrix proteins.

fungal community behavior | extracellular matrix signaling | fungal dimorphism | flocculin

Social behaviors in microbes, such as cooperation in foraging, differentiation, reproducing, and signaling, confer resistance to harsh environments and bestow competitiveness when resources are limited (1–3). Complex social behaviors in microbes are orchestrated by precise communication within or between communities and are best known to be initiated via secreted low-molecular-weight molecules (LMWMs) (1, 4). Besides LMWMs (e.g., pheromones/hormones), other intercellular communicatory mechanisms are established in higher multicellular eukaryotes. For example, matrix/adhesion proteins (adhesins) and some growth factors conduct intercellular communications that are critical for cell migration, proliferation, tissue patterning, and cellular differentiation (5–10). The adhesion/matrix protein-mediated signaling is well established in these higher eukaryotes with highly differential tissues and the requirement of cellular differentiation based on position cues (11). In "simple" microbes, matrix or adhesion proteins have long been considered to be mediators of pathogenicity and structural components of biofilms. Although some adhesion proteins are shown to undergo shedding (12, 13) and potential regulatory roles of adhesion/matrix proteins have been implicated in previous studies on a variety of microbes (14–19), their signaling function is unrecognized. Here, we present evidence of a microbial extracellular adhesion/matrix protein signal that controls the formation of complex communities and directs cellular differentiation in the model fungal pathogen *Cryptococcus neoformans*.

Results

A Secreted Signal Controlled by the Zinc Finger Transcription Factor Znf2 Regulates Colony Morphology in *C. neoformans*. *Cryptococcus* is a ubiquitous environmental pathogen, and this fungus can develop communities with complex colony morphology and yeast-to-hypha transition in response to environmental factors or mating cues (Fig. 1*A*) (19–22). We noticed that the wild-type XL280 strain aggregated and formed a complex colony morphology, generated hyphae and became fluffy, and grew more invasively when it was in close proximity to a culture that contained a mixture of cells of α and **a** opposite mating types on yeast extract peptone dextrose (YPD) medium (Fig. 1*B* and Fig. S1*A*). No such responses in this recipient were detected when α or **a** cells alone were the donor (Fig. S1*B*). This suggests that secreted signal(s) from the nearby α+**a** coculture elicited the responses. Disruption of Znf2, a master regulator of morphogenesis (19, 23), in the α+**a** donor cells severely reduced the ability of the α+**a** mixture to elicit the responses from the recipient strain (Fig. 1*B* and Fig. S1*A*). This suggests that Znf2 is a major regulator of the signaling. Consistent with this notion, overexpression of *ZNF2* in the α donor alone, in both a laboratory-generated strain and a clinical isolate, was sufficient to trigger similar responses from the nearby recipient (Fig. 1*D* and Fig. S1*B*).

The Paracrine Regulation of Colony Morphology Is Not Mediated by Pheromone or Other Secreted Low-Molecular-Weight Molecules. This paracrine communication regulated by Znf2 is unlikely attributable to sexual pheromones because the deletion of Znf2 does not impair pheromone sensing or production (19). Furthermore, a donor strain overexpressing Mat2 ($MAT2^{oe}$), a transcription factor that induces high levels of pheromone production but not *ZNF2* under this condition (19, 23), did not elicit the expected responses from adjacent cells (Fig. 1 *C* and *D*).

Given the importance of LMWMs in fungal intercellular communication (4, 24), we examined whether any LMWMs act as the signals controlled by Znf2. Surprisingly, the signaling molecules controlled by Znf2 are not LMWMs or gaseous messengers, because a membrane with a molecular-mass cutoff of 3 kDa blocked the communication when placed between the $ZNF2^{oe}$ donor strain and the wild-type recipient strain (Fig. 1*E*).

The Cryptococcal Adhesin Cfl1, a Downstream Target of Znf2, Plays an Important Role in Conducting the Paracrine Regulation of Colony Morphology. We previously found that ∼23% of the predicted Znf2 regulon are secreted proteins (19). In contrast, only 4.3% of the genome encodes secreted proteins, indicating that the Znf2 regulon is enriched with extracellular proteins (Fisher extract test, $P < 0.0001$). Many secreted proteins under the control of Znf2 do not contain any known domain associated with binding to the cell wall or the plasma membrane and, thus, could be released and act as signaling molecules. To test this hypothesis, we performed

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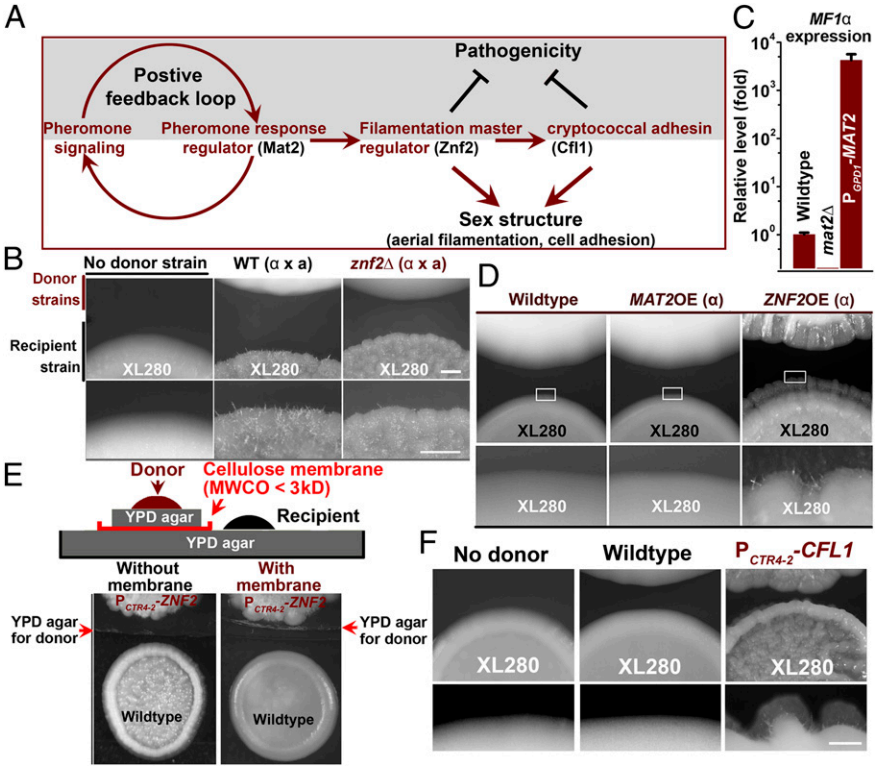

Fig. 1. Znf2 controls community behaviors in a paracrine manner. (*A*) Diagram depicting the pheromone and filamentation signaling pathways in *Cryptococcus*. The pheromone-sensing and response regulator Mat2 and the secreted pheromone signals form a positive-feedback loop. Under mating-inducing environmental conditions, Mat2 turns on Znf2, which orchestrates morphogenesis and virulence partially through its control of adhesin Cfl1 (19). (*B*) Znf2 is involved in the paracrine regulation of morphogenesis in the recipient elicited by the α+**a** coculture. All confrontation assays were performed on YPD media. The colony morphologies of the recipient strain were photographed at 60 h in confrontation with the donors. (Scale bars: 500 μm.) See Fig. S1 for the effect of Znf2 overexpression on the paracrine regulation of morphogenesis. (*C*) The pheromone gene *MF1*α is highly induced in response to the *MAT2* overexpression. (*D*) Overexpression of *ZNF2*, but not the pheromone (*MAT2*) in the donor, is sufficient to trigger paracrine responses in the recipient. (*E*) The signal molecule regulated by Znf2 is likely large. The *ZNF2* overexpression strain and the wild-type recipient were grown on YPD medium. A membrane with a molecular-mass cutoff of 3 kDa was placed between the two strains. (*F*) The overexpression of *CFL1* is sufficient to elicit paracrine responses in the recipient. (Scale bars: 200 μm.)

confrontation assays with strains overexpressing nine of these secretory genes as the donors (Table S1). All of these nine genes were previously shown to exhibit significant increase in expression in the *ZNF2* overexpression strain (19). The wild-type recipient strain formed a biofilm colony and aerial hyphae only in response to the donor strain overexpressing *CFL1* ($CFL1^{oe}$) (Fig. 1*F*). Cfl1 is an adhesion protein that promotes flocculation and biofilm formation (19). Intriguingly, *CFL1* is one of the most induced genes during sexual development in wild-type *Cryptococcus* (Fig. 2 *A* and *B*). Our transcriptional results indicate that the induction of *CFL1* is comparable to that of pheromone-producing gene (*MF1*α). Pheromone-producing genes are highly induced during mating and their products directly serve as an autoinducer during mating. This exceptionally high induction of *CFL1* further strengthens the possibility that released Cfl1 product may act as a signal to regulate cryptococcal community behaviors. Indeed, the deletion of *CFL1* in the α+**a** donor cells considerably reduced the responses in the nearby recipient strain, including adherence to agar and filamentation (Fig. 2 *C* and *D*). This suggests that Cfl1 is a major factor controlled by Znf2 in this paracrine regulation.

Cfl1-Expressing Donor Cells Elicit the Expression of Endogenous Cfl1 in Neighboring Cells. Recently, we demonstrated that the activation of Cfl1 or Znf2 promotes the formation of aerial hyphae and complex colonies (Fig. S2) (19). Because the responses elicited in the wild-type recipient strain resemble the phenotypes of the strain with *CFL1* overexpression (19), we postulated that the endogenous Cfl1 is induced in the recipient strain, ultimately stimulating the formation of wrinkled colony and aerial hyphae. To test this hypothesis, we used a strain harboring the Cfl1 protein fused with mCherry under the control of the native *CFL1* promoter (P_{CFL1}-*CFL1-mCherry*), which does not fluoresce when grown in isolation (19) (Fig. 3*A*). Strikingly, bright fluorescence was detected in the P_{CFL1}-*CFL1-mCherry* strain when it was placed in close proximity to the $CFL1^{oe}$ donor strain (Fig. 3*A*). This indicates that the endogenous Cfl1 in the recipient is indeed highly induced. Consistent with this idea, when the P_{CFL1}-*CFL1-mCherry* strain was mixed with the nonfluorescent $CFL1^{oe}$ donor strain to form one single colony, the fluorescence emitted from the wild-type P_{CFL1}-*CFL1-mCherry* cells became stronger (Fig. S3). These findings indicate that Cfl1 can conduct autoinduction either inter- or intracolonially in a paracrine manner.

The magnitude of the endogenous Cfl1 production and filamentation in the recipient colony depends on the distance from the $CFL1^{oe}$ donor strain (Fig. 3*B*), likely reflecting the strength of the diffusible signals released from the donor strain. Interestingly, after longer incubation (>4 d) in confrontation, the filamentation on the distal side of the recipient colony became comparable or even better than that on the proximal side. We think this phenomenon is likely caused by greater extent of nutrient exhaust at the proximal regions between the two colonies and/or higher levels of accumulation of inhibitory factors/waste products generated by both colonies with prolonged incubation.

To test whether the endogenous Cfl1 elicited by the donor signal ultimately leads to the biofilm-like morphology and aerial hypha formation, the *cfl1*Δ mutant was used as the recipient in the

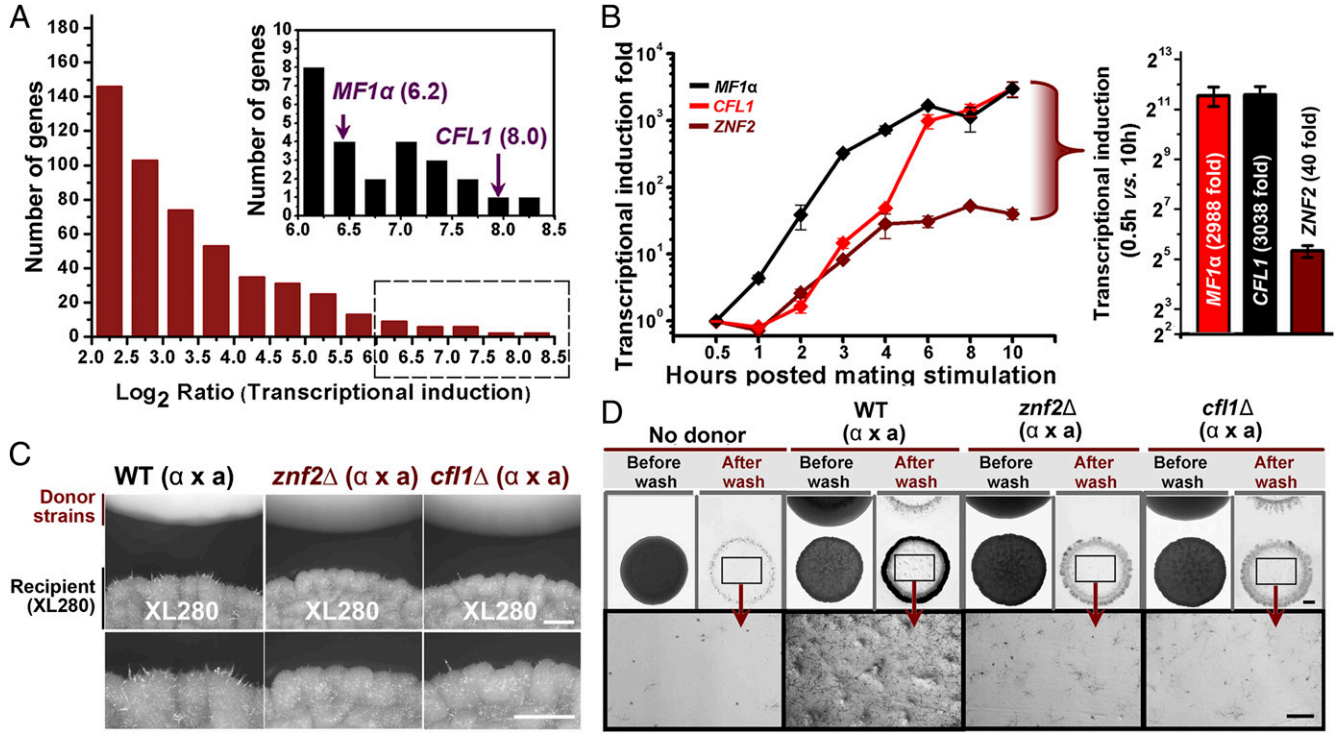

Fig. 2. Cfl1 is strongly induced during colony development and required for both paracrine signaling and adhesion to agar. (*A*) The histogram of the number of highly differentially expressed genes (≥fourfold) in the wild-type α+**a** (α x **a**) coculture at 72 h after mating induction in comparison with the initial state. *Inset* highlights the genes with ≥64-fold of induction in expression. The original transcriptome data were obtained from a previous study (41). [Bin width: Log_2 (fold change) =0.3.] (*B*) Transcriptional dynamics of *MF1α*, *ZNF2*, and *CFL1* during mating development. The α+**a** mixture was cultured on V8 juice agar medium at 22 °C in the dark for the indicated time periods and total RNA was extracted for the transcriptional analyses by real time PCR. Both *ZNF2* and its downstream target *CFL1* show a delayed transcriptional induction compared with the pheromone (*Left*). Such delay is expected given the role of pheromone in the initiation of mating (23) and the role of Znf2 in directing hypha generation at later stages of mating (Fig. 1*A*) (19, 23). The level of transcriptional induction of *CFL1* is similar to that of the α pheromone at 10 h postmating induction (*Right*). (*C*) Cfl1 is involved in the formation of biofilm colony and aerial hyphae in a paracrine manner. (Scale bars: *Upper*, 1 mm; *Lower*, 200 μm.) (*D*) Cfl1 is involved in controlling invasive growth in a paracrine manner. The α+**a** coculture colonies of the wild-type strains, the *znf2*Δ mutants, and the *cfl1*Δ mutants were grown on YPD agar as the donors. After confrontation with the recipient strain for 5 d, the plates were washed by cold water and the remaining invasive cells embedded in the agar were photographed. (Scale bar: 500 μm.)

confrontation assay (Fig. 3*C*). Deletion of *cfl1* in the recipient only reduced, but not did abolish, the responses stimulated by the donor signal, suggesting that production of Cfl1 in the recipient strain is not required for sensing or responding to the donor signal (Fig. 3*C*). By contrast, disruption of *ZNF2* in the recipient abolished the donor signal-stimulated morphogenesis and the induction of endogenous Cfl1 in the recipient strain (Fig. 3 *C* and *D*). This indicates that Znf2 is not only critical for producing the paracrine signal but it is also required for responding to that signal.

Cfl1 Can Undergo Processing, and the Shed Cfl1 Product Serves As a Signal to Direct Colony Morphology and Autoinduction. We demonstrated previously that Cfl1 is a cell wall constituent and a released factor (19). To understand the temporal and spatial pattern of Cfl1 expression and localization under natural conditions, we probed the wild-type P_{CFL1}-*CFL1-mCherry* strain during colony development under mating-inducing conditions that naturally induce the expression of this adhesin. Consistent with the role of Cfl1 in the regulation of filamentation, the fluorescence signal was tightly associated with the hyphal population (Fig. 4*A*) (19). At the early stage of filamentation, Cfl1 was localized in vesicles in the majority of Cfl1 expressing hyphal population (Fig. 4*A*). These vesicles are similar in appearance to known secretory vesicles described in other fungi (25, 26). During the late stage of colony development, Cfl1-mCherry could also be detected around the surface of some hyphal tips and basidia (19). Released Cfl1 from the intact wild-type colonies under mating-inducing conditions or the $CFL1^{oe}$ colonies was detected directly by colony immunoblot assays (Fig. 4*B*). Such released Cfl1 was not detected when its predicted signal peptide for secretion was deleted (Fig. 4*B*). Interestingly, when the $CFL1^{oe}$ strain was grown on solid media with a high surface tension, such as yeast nitrogen base (YNB) agar, the colony was visually encased by a mucous matrix layer where the released Cfl1 (designated as rCfl1) was highly enriched based on Western blot analyses (Fig. 4*C* and Fig. S4*A*). However, such a matrix layer was not observed when the secretion of Cfl1 was disrupted by removing the signal peptide, indicating that the formation of the matrix layer is associated with Cfl1 secretion (Fig. S4*B*). The released Cfl1 was a cleaved C terminus containing ~two-thirds of the full-length protein (Fig. 4*B* and Fig. S4*A*). The cell wall-associated form of Cfl1 was the mature full-length protein after the removal of the N-terminal signal peptide (Fig. 4*B* and Fig. S4*A*). This suggests that Cfl1 can undergo shedding and the predicted cleavage site is immediately after the EGF motif with a putative chitin-binding capability (Fig. S5*A*) (27). The ability of *C. neoformans* to shed Cfl1 appears to be highly efficient, because rCfl1 was readily detected in the extracellular matrix from Cfl1-expressing colonies (e.g., the wild-type α+**a** coculture and the $CFL1^{oe}$ strain) on the rich YPD medium (Fig. 4*B*). As expected, extracellular rCfl1 was below the detection level in the strain expressing the mutant Cfl1 allele without the signal peptide (Fig. 4*B*). The lack of signal was not attributable to a failure in producing the mutant allele protein, because abundant full-length Cfl1(sigPΔ)-mCherry was detected in the cell pellet (Fig. 4*B*). Similarly, extracellular Cfl1 was not attributable to increased cell

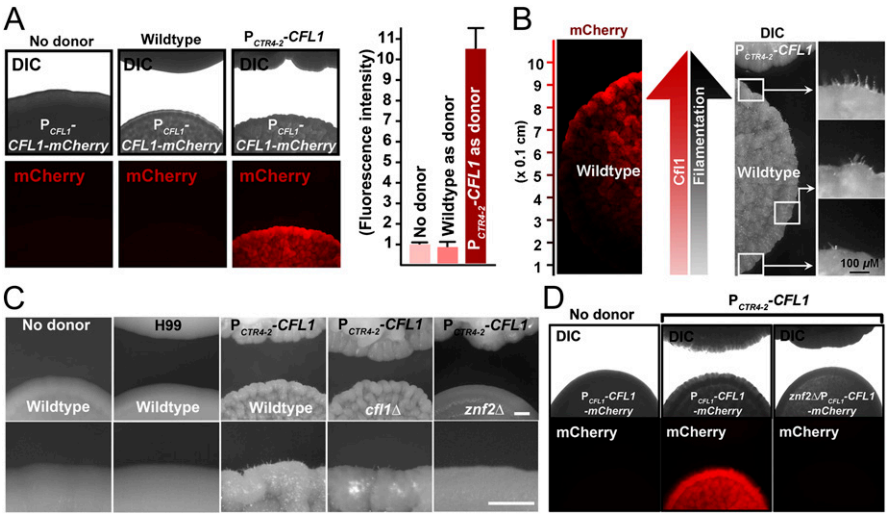

Fig. 3. The signal from the *CFL1* expressing donor cells induces the expression of endogenous Cfl1 in a paracrine manner. (*A*) The donor strain (*CFL1*^{oe}) activates the expression of endogenous Cfl1 in the recipient in a paracrine manner based on the images (*Left*) and the quantification of the fluorescence intensity (*Right*). (right graph). See Fig. S3 for the induction of fluorescent Cfl1 proteins in the wild-type subpopulation (P_{CFL1}-*CFL1-mCherry*) when mixed with the nonfluorescent donor cells (*CFL1*^{oe}) in a single community. (*B*) The signal from the *CFL1*^{oe} donor strain regulates the filamentation and endogenous Cfl1 expression in the recipient in a distance-dependent manner. The recipient cells closest to the *CFL1*^{oe} donor strain yielded the brightest fluorescence (mCherry) (*Left*) and produced the most robust filamentation (*Right*). (*C*) Znf2 is involved in the response of the recipient to the signal from the *CFL1*-expressing donor cells. The *CFL1*^{oe} strain (P_{CTR4-2}-*CFL1*) induced the adjacent wild-type strain, but not the *znf2*Δ mutant, to become wrinkled and to undergo filamentation. The *cfl1*Δ mutant as a recipient showed a reduced response compared with the wild-type recipient. (*D*) The deletion of *ZNF2* in the recipient strain abolishes its ability to respond to the donor signal and there is no induction of endogenous Cfl1. (Scale bar: 500 μm.)

lysis because cell viability was similar in *CFL1*^{oe}, wild-type, and *CFL(sigPΔ)*^{oe} colonies (75.3%, 65.7%, and 68.4%, respectively). This suggests that rCfl1 detected in the extracellular matrix was derived from secretion by viable cells and not from released intracellular proteins by dead cells.

Importantly, the secretion of rCfl1 is critical for the paracrine regulation of morphogenesis and autoregulation mediated by Cfl1 (Fig. 5*A* and Fig. S4*C*). The expression of Cfl1(sigPΔ)-mCherry by the donor strain was not able to stimulate the morphological responses or the expression of endogenous Cfl1 in the recipient strain. Collectively, the presented evidence raises the possibility that Cfl1 can directly serve as an extracellular signal to direct morphogenesis and autoinduction.

To verify this idea, we purified rCfl1 from the culture supernatant of *Cryptococcus* cells expressing *CFL1* (Fig. 5*B*) and assessed its paracrine function. The purified rCfl1 elicited expected responses from the recipient strain, including the expression of its endogenous Cfl1 (Fig. 5*C*), whereas BSA or digested rCfl1 fragments did not. This indicates that rCfl1 is a functional signaling molecule. The purified rCfl1 elicited weaker responses in the recipient than the *CFL1*^{oe} donor strain (Figs. 3*A* and 5*C* and Fig. S3), likely because of some reduction in protein activity during the purification process, the lack of a constant supply of fresh rCfl1 proteins, or the absence of augmentative cofactors in the purified rCfl1 proteins. The activity of rCfl1 with higher concentrations was not tested because of protein precipitation at those concentrations. Nonetheless, the evidence presented above demonstrates that the transcription factor Znf2 promotes the formation of complex colony morphology by activating the expression of its downstream targets, including adhesin Cfl1. Released Cfl1 acts as a signal to stimulate nearby *Cryptococcus* cells to induce the production of endogenous Cfl1, forming a reinforcing positive-feedback loop.

The domain prediction analyses based on Pfam and motif scan program (http://myhits.isb-sib.ch/cgi-bin/motif_scan) indicated that Cfl1 possesses an EGF motif with a putative chitin-binding capability and an amylogenic region with a predicted function in mediating cell–cell adhesion at its N terminus (Fig. S5*A*). However, the C-terminal region of the protein, corresponding to rCfl1, does not contain any annotated domain that is demonstrative of its signaling activity. A Position-Specific Iterative BLAST analysis with regions of low complexity masked revealed an 80-residue, cysteine-rich region at the C terminus that is highly conserved among Cfl1 homologs from different fungal species in the phylum Basidiomycota (28) (Fig. S5 *A* and *B*). Intriguingly, this putative domain resides exclusively at the C termini of these homologs, and all of these homologs are expected to function extracellularly based on their predicted subcellular location (Fig. S5*B*). We designated this domain signal C-terminal domain (SIGC), given its apparent association with secretion and its C-terminal location.

To further probe the possible common biological processes associated with proteins containing the SIGC domain, we searched the Pfam and InterPro (www.ebi.ac.uk/interpro) databases for other known functional domains that coexist with SIGC in these Cfl1 homologs. Ontology analyses indicate that 90% of the domains residing together with SIGC are associated with secretion, and 50% are responsible for extracellular ligand–receptor interaction (Fig. S5*C*). These results suggest that the SIGC domain may play a role in the extracellular factor-mediated signal transduction.

Because both the released and the cell wall-bound Cfl1 contain the SIGC domain, both forms of Cfl1 could be involved in guiding the paracrine signaling and/or cell–cell contact. The multifunctionalities of Cfl1 resemble those of matrix proteins found in higher multicellular eukaryotes. These matrix proteins regulate intercellular communication via a crosstalk through growth factors that bind to receptors with intrinsic tyrosine kinase activity, or through their direct interactions with some members of this receptors family (8, 29–32). The coexistence of SIGC and the receptor tyrosine kinase domain (Pfam accession no. PF07699.8) in some of the Cfl1 homologs (Fig. S5*C*) is intriguingly suggestive of coevolution of these two domains. It also suggests a general role of the SIGC domain in extracellular matrix/adhesion protein-initiated signal transduction.

Discussion

LMWMs are widely found in microbes as communicatory signals to regulate gene expression and community behaviors (1, 33). The

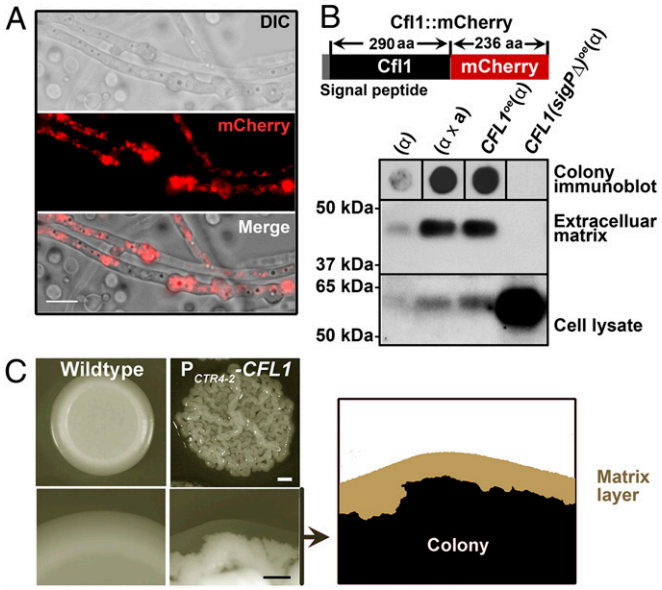

Fig. 4. Cfl1 undergoes shedding during colony differentiation, and shed Cfl1 is an important component of the extracellular matrix. (*A*) Cfl1-mCherry is observed in hyphal-secretion vesicles at 24 h postmating stimulation. (Scale bar: 20 μm.) See *Materials and Methods* for a detailed description of the experimental condition for the subcellular localization of Cfl1-mCherry. (*B*) Released Cfl1 is derived from protein shedding. Diagram of the mCherry-labeled Cfl1 detected by the antibody against mCherry. The gray bar indicates the predicted signal peptide for secretion. The overlay and immunoblot for Cfl1-mCherry are shown for the indicated cryptococcal colonies. Western blot analysis for the products of mCherry labeled wild-type *CFL1* allele and the mutant *CFL1(sigPΔ)* allele in different fungal colonies is shown below. "(α)" indicates the colony of XL280α harboring *CFL1-mCherry*–fused gene under the control of the *CFL1* native promoter (P_{CFL1}-*CFL1-mCherry*); "(α x **a**)" indicates the mating colony consisting of the equal number of cells of opposite mating types, both containing the construct of P_{CFL1}-*CFL1-mCherry*; and "*CFL1*^{oe} (α)" and "*CFL1(sigP)*^{oe} (α)" indicate the colonies containing mCherry labeled wild-type *CFL1* allele and the mutant *CFL1* allele that lacks the secretion signal, respectively. Both were constructed under the control of $P_{CTR4\text{-}2}$, so that the transcriptional levels of *CFL1-mCherry* could be highly induced by the addition of inducer (BCS) into the medium. All of the cryptococcal colonies were grown on the same YPD agar plate containing 200 μM BCS. (*C*) A matrix layer encases the *CFL1*^{oe} strain. Strains were grown on YNB agar medium for 5 d and photographed. The architecture of the colony edge is illustrated by a diagram (to the right). (Scale bar: 1 mm.)

strength of regulation mediated by LMWMs is dependent on the dynamics of molecular diffusion (34). Because of their small sizes, LMWMs are well equipped to synchronize gene regulation across the entire microbial community or even between communities. Our experiments presented here indicate that the exogenous Cfl1 signal exerts autoregulatory activity and that it guides community behaviors. Cfl1's regulation on community behaviors is also dynamic and depends on the distance of the recipient cells and the diffusion rate of Cfl1 proteins (Fig. 3*B*). Given the size of rCfl1 (over 20 kDa) and the fact that it is highly enriched in the extracellular matrix of the cryptococcal community (Fig. 4 *B* and *C* and Fig. S4*A*), Cfl1 likely exerts a greater impact on a matrix-connected subpopulation, rather than synchronizing the whole community. Because Cfl1 is drastically induced and actively secreted when cells begin to enter the filamentation stage (Figs. 2*B* and 4*A* and 4*B*), Cfl1 may serve as a matrix signal to regulate filamentation. In *C. neoformans*, filamentation often occurs in response to mating cues (35, 36), and it structurally and physiologically bridges early mating events (e.g., cell fusion) with late mating events (e.g., fruiting body formation) (35). During mating, the induction level of *CFL1* is comparable to that of the gene producing pheromone, an abundant extracellular signal controlling early mating events (Fig. 2*B*) that initiate subsequent mating hyphal production. However, the effect of the released Cfl1 on filamentation is unlikely mediated through enhancing the early cellular responses to mating input based on the following evidence. Firstly, *CFL1* exhibits a delayed transcriptional induction compared with the early mating gene *MF1*α (Fig. 2*B*). Secondly, Cfl1 is not required for cell fusion, and exogenous Cfl1 does not influence cell fusion (Fig. S6*A*). These results are consistent with the established essential role of its upstream regulator Znf2 in filamentation but not in cell fusion. Lastly, overexpression of Cfl1 is able to drive filamentation under the condition that suppresses cryptococcal mating behavior (Fig. S6*B*). Thus, the paracrine regulation by the rCfl1 signal is likely dedicated to filamentation, which occurs during the midstage differentiation of a mating community.

Although Cfl1 provides an example of regulatory adhesins in fungi, the regulatory role of adhesion proteins in modulating gene expression and controlling cell differentiation, in addition to their established structural role in cell–cell and cell–extracellular matrix contact, has long been established in higher eukaryotes (37, 38). The adhesive property of these structurally divergent molecules (39) allows these adhesion proteins to physically interact with their cognate partners presenting on the surface of cells or in the extracellular matrix (8, 37). Such interactions trigger appropriate

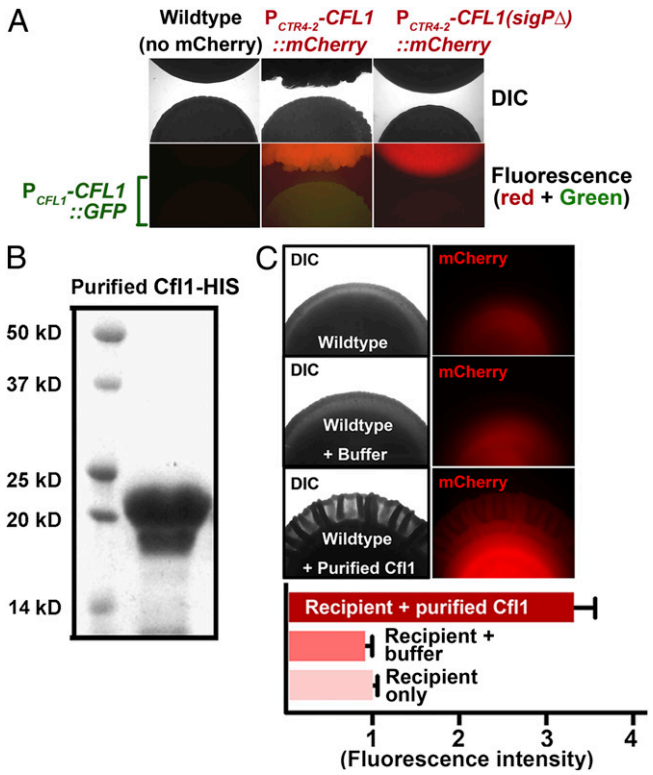

Fig. 5. Released Cfl1 serves as a signal to direct community behavior and autoinduction. (*A*) Secretion of Cfl1 is required for the paracrine regulation on the expression of endogenous Cfl1 in the recipient strain. The $P_{CTR4\text{-}2}$-*CFL1-mCherry* strain and the $P_{CTR4\text{-}2}$-*CFL1(sigPΔ)-mCherry* strain were used as the donor strains in the confrontation assay. Both strains showed high level of expression as indicated by strong cherry fluorescence under the inducing condition in the presence of BCS. Only the expression of the wild-type *CFL1* allele in the donor triggered the endogenous expression of P_{CFL1}-*CFL1-GFP* in the recipient strain. (*B*) SDS/PAGE gel of purified rCfl1 with the His tag. (*C*) Purified rCfl1 proteins drive morphogenesis and induce the expression of the endogenous *CFL1* gene in the wild-type strain.

corresponding signaling-transduction events to regulate cellular differentiation and growth. These lines of investigation in higher eukaryotes provide substantial evidence supporting the signaling and regulatory roles of matrix/adhesion proteins and underscore their importance in development (11, 37). In contrast, studies on adhesion or matrix proteins in both eukaryotic and prokaryotic microbes have primarily focused on their roles in microbial pathogenesis or in biofilms as structural proteins. The Cfl1 communication system used by *Cryptococcus* may represent direct evidence of adhesion/matrix protein-initiated signaling in microbes. However, given the pervasive existence of diverse matrix proteins in microbes across different domains, and the multicellular-like social behaviors of many microorganisms (e.g., fruiting body development) (40), it is plausible that adhesins also play important regulatory roles in microbes.

Materials and Methods

Strains, Mating, and Phenotypic Assays. Strains used in this study are listed in Table S1. Strains were maintained on YPD media unless indicated otherwise. See *SI Materials and Methods* for a detailed description of the phenotypic assays.

Microscopic Examination for the Subcellular Localization of Cfl1. To examine the subcellular localization of Cfl1::mCherry, the wild-type strain (XL280) harboring P_{CFL1}-*CFL1-mCherry* was grown on V8 or YNB (RPI corp) agar medium at 22 °C for 24 h before examination with a BX50 microscope. The fluorescence signal of Cfl1-mCherry–expressing colonies was quantified using the ImageJ analysis software.

RNA Purification and Quantitative PCR Analyses. The Purelink RNA kit (Invitrogen) was used for total RNA purification, and the SuperScript III cDNA synthesis kit (Invitrogen) was used for the first strand cDNA synthesis according to the manufacturer's instructions. See *SI Materials and Methods* for a detailed description of the quantitative PCR analyses.

rCfl1 Protein Purification from *Cryptococcus neoformans*. The cryptococcal strain expressing Cfl1-His_6 protein was used here for rCfl1 protein purification. See *SI Materials and Methods* for a detailed description of the construction of the plasmid for the expression of Cfl1-His_6 protein in *C. neoformans* and rCfl1 protein purification.

Colony Immunoblot for Secreted Cfl1. Wild-type α, α+**a** coculture, and overexpression strains harboring the *CFL1::mCherry*-fused gene were inoculated onto the same plate for the assay. See *SI Materials and Methods* for a detailed description of the colony immunoblot analyses.

Protein Extraction and Western Blot Analyses. Cryptococcal cells were incubated on YPD plates for three days at 30 °C. Cells were then collected and dispersed in the suspension buffer [100 mM Tris, 13% (vol/vol) glycerol] in the presence of protease inhibitor mixture (100 μL/10 mL lysis buffer) (Roche). See *SI Materials and Methods* for a detailed description of the protein extraction and Western blot analyses.

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1. Nadell CD, Xavier JB, Levin SA, Foster KR (2008) The evolution of quorum sensing in bacterial biofilms. *PLoS Biol* 6(1):e14.
2. St'ovíček V, Váchová L, Palková Z (2012) Yeast biofilm colony as an orchestrated multicellular organism. *Commun Integr Biol* 5(2):203–205.
3. Crespi BJ (2001) The evolution of social behavior in microorganisms. *Trends Ecol Evol* 16(4):178–183.
4. Jones SK, Jr., Bennett RJ (2011) Fungal mating pheromones: Choreographing the dating game. *Fungal Genet Biol* 48(7):668–676.
5. Bukalo O, Dityatev A (2012) Synaptic cell adhesion molecules. *Adv Exp Med Biol* 970(970):97–128.
6. Bukalo O (2008) Introduction: Cell adhesion and extracellular matrix molecules in synaptic plasticity. *Neuron Glia Biol* 4(3):165–167.
7. Guan JL, Chen HC (1996) Signal transduction in cell-matrix interactions. *Int Rev Cytol* 168(168):81–121.
8. Hynes RO (2009) The extracellular matrix: Not just pretty fibrils. *Science* 326(5957): 1216–1219.
9. Heldin CH (1992) Structural and functional studies on platelet-derived growth factor. *EMBO J* 11(12):4251–4259.
10. Logan CY, Nusse R (2004) The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* 20(20):781–810.
11. Nelson CM, Bissell MJ (2006) Of extracellular matrix, scaffolds, and signaling: Tissue architecture regulates development, homeostasis, and cancer. *Annu Rev Cell Dev Biol* 22(22):287–309.
12. Zhao X, et al. (2006) *Candida albicans* Als3p is required for wild-type biofilm formation on silicone elastomer surfaces. *Microbiology* 152(Pt 8):2287–2299.
13. Karunanithi S, et al. (2010) Shedding of the mucin-like flocculin Flo11p reveals a new aspect of fungal adhesion regulation. *Curr Biol* 20(15):1389–1395.
14. Fu Y, et al. (2002) *Candida albicans* Als1p: An adhesin that is a downstream effector of the *EFG1* filamentation pathway. *Mol Microbiol* 44(1):61–72.
15. Guo B, Styles CA, Feng Q, Fink GR (2000) A *Saccharomyces* gene family involved in invasive growth, cell-cell adhesion, and mating. *Proc Natl Acad Sci USA* 97(22): 12158–12163.
16. Halme A, Bumgarner S, Styles C, Fink GR (2004) Genetic and epigenetic regulation of the *FLO* gene family generates cell-surface variation in yeast. *Cell* 116(3):405–415.
17. Sharkey LL, McNemar MD, Saporito-Irwin SM, Sypherd PS, Fonzi WA (1999) *HWP1* functions in the morphological development of *Candida albicans* downstream of *EFG1*, *TUP1*, and *RBF1*. *J Bacteriol* 181(17):5273–5279.
18. Vlamakis H, Aguilar C, Losick R, Kolter R (2008) Control of cell fate by the formation of an architecturally complex bacterial community. *Genes Dev* 22(7):945–953.
19. Wang L, Zhai B, Lin X (2012) The link between morphotype transition and virulence in *Cryptococcus neoformans*. *PLoS Pathog* 8(6):e1002765.
20. Kozubowski L, Heitman J (2012) Profiling a killer, the development of *Cryptococcus neoformans*. *FEMS Microbiol Rev* 36(1):78–94.
21. McClelland CM, Chang YC, Varma A, Kwon-Chung KJ (2004) Uniqueness of the mating system in *Cryptococcus neoformans*. *Trends Microbiol* 12(5):208–212.
22. Kronstad JW, et al. (2011) Expanding fungal pathogenesis: *Cryptococcus* breaks out of the opportunistic box. *Nat Rev Microbiol* 9(3):193–203.
23. Lin X, Jackson JC, Feretzaki M, Xue C, Heitman J (2010) Transcription factors Mat2 and Znf2 operate cellular circuits orchestrating opposite- and same-sex mating in *Cryptococcus neoformans*. *PLoS Genet* 6(5):e1000953.
24. Albuquerque P, Casadevall A (2012) Quorum sensing in fungi—a review. *Med Mycol* 50(4):337–345.
25. Schuster M, et al. (2012) Myosin-5, kinesin-1 and myosin-17 cooperate in secretion of fungal chitin synthase. *EMBO J* 31(1):214–227.
26. Santiago-Tirado FH, Legesse-Miller A, Schott D, Bretscher A (2011) PI4P and Rab inputs collaborate in myosin-V-dependent transport of secretory compartments in yeast. *Dev Cell* 20(1):47–59.
27. Brandhorst T, Wüthrich M, Finkel-Jimenez B, Klein B (2003) A C-terminal EGF-like domain governs BAD1 localization to the yeast surface and fungal adherence to phagocytes, but is dispensable in immune modulation and pathogenicity of *Blastomyces dermatitidis*. *Mol Microbiol* 48(1):53–65.
28. Altschul SF, et al. (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res* 25(17):3389–3402.
29. Shrivastava A, et al. (1997) An orphan receptor tyrosine kinase family whose members serve as nonintegrin collagen receptors. *Mol Cell* 1(1):25–34.
30. Leitinger B, Hohenester E (2007) Mammalian collagen receptors. *Matrix Biol* 26(3): 146–155.
31. Soung YH, Clifford JL, Chung J (2010) Crosstalk between integrin and receptor tyrosine kinase signaling in breast carcinoma progression. *BMB Rep* 43(5):311–318.
32. Tran KT, Griffith L, Wells A (2004) Extracellular matrix signaling through growth factor receptors during wound healing. *Wound Repair Regen* 12(3):262–268.
33. Ng WL, Bassler BL (2009) Bacterial quorum-sensing network architectures. *Annu Rev Genet* 43(43):197–222.
34. Hense BA, et al. (2007) Does efficiency sensing unify diffusion and quorum sensing? *Nat Rev Microbiol* 5(3):230–239.
35. Lin X, Hull CM, Heitman J (2005) Sexual reproduction between partners of the same mating type in *Cryptococcus neoformans*. *Nature* 434(7036):1017–1021.
36. Shen WC, Davidson RC, Cox GM, Heitman J (2002) Pheromones stimulate mating and differentiation via paracrine and autocrine signaling in *Cryptococcus neoformans*. *Eukaryot Cell* 1(3):366–377.
37. Braga VM (2002) Cell-cell adhesion and signalling. *Curr Opin Cell Biol* 14(5):546–556.
38. Morton PE, Parsons M (2011) Dissecting cell adhesion architecture using advanced imaging techniques. *Cell Adhes Migr* 5(4):351–359.
39. Amack JD, Manning ML (2012) Knowing the boundaries: Extending the differential adhesion hypothesis in embryonic cell sorting. *Science* 338(6104):212–215.
40. Sutherland IW (2001) The biofilm matrix—an immobilized but dynamic microbial environment. *Trends Microbiol* 9(5):222–227.
41. Kruzel EK, Giles SS, Hull CM (2012) Analysis of *Cryptococcus neoformans* sexual development reveals rewiring of the pheromone-response network by a change in transcription factor identity. *Genetics* 191(2):435–449.