

Autophagic Fungal Cell Death Is Necessary for Infection by the Rice Blast Fungus

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Rice blast is caused by the fungus *Magnaporthe grisea*, which elaborates specialized infection cells called appressoria to penetrate the tough outer cuticle of the rice plant *Oryza sativa*. We found that the formation of an appressorium required, sequentially, the completion of mitosis, nuclear migration, and death of the conidium (fungal spore) from which the infection originated. Genetic intervention during mitosis prevented both appressorium development and conidium death. Impairment of autophagy, by the targeted mutation of the *MgATG8* gene, arrested conidial cell death but rendered the fungus nonpathogenic. Thus, the initiation of rice blast requires autophagic cell death of the conidium.

Rice blast disease is one of the most serious and recurrent problems affecting rice production worldwide (1–4). The disease is caused by *Magnaporthe grisea* (Hebert) Barr (anamorph *Pyricularia grisea* Sacc), an ascomycete fungus that produces dome-shaped melanin-pigmented appressoria

that penetrate the leaves and stems of rice plants (*Oryza sativa*). Rice blast disease starts when three-celled spores land on the leaf surface, to which they stick tightly by means of an adhesive carried in the spore apex (5, 6). Within an hour after bonding to the leaf surface, each spore germinates and

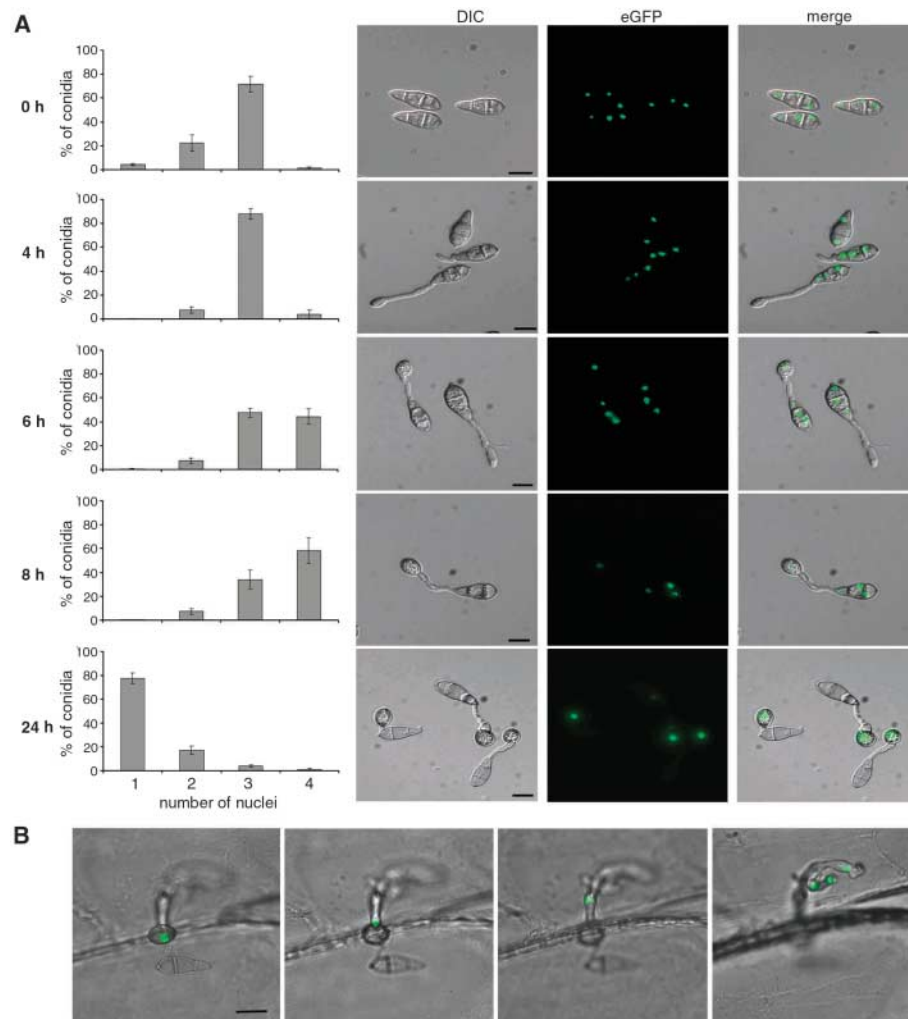
produces a polarized germ tube, which typically emerges from the apical cell of the conidium ($77.7 \pm 5.8\%$ of cases). Thereafter, the germ tube quickly differentiates at its tip to form an appressorium. The *M. grisea* appressorium generates up to 8 MPa of turgor to provide the motive force necessary to breach the tough cuticle of the rice plant via a narrow penetration peg (7, 8). After storage products are transported from the spore to the developing appressorium, the conidium and germ tube then collapse and die (movie S1).

To investigate the genetic control of appressorium morphogenesis and to observe the pattern of nuclear division during appressorium development in *M. grisea*, we used a strain of the fungus [expressing a histone H3–enhanced green fluorescent protein (eGFP) fusion protein (9)] in which individual nuclei were visible by epifluorescence microscopy (Fig. 1). During spore germination on an in-

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Fig. 1. (A) Bar charts showing the number of *M. grisea* germlings containing 0 to 4 nuclei (mean \pm SD; $n = 200$; three experiments). Conidia expressing *H3:eGFP* were examined by epifluorescence microscopy at 0, 2, 4, 6, 8, and 24 hpi. Representative bright-field [differential interference contrast (DIC)], fluorescence, and merged images at each time point are shown. (B) Merged bright-field and epifluorescence images from a Z-stack experiment using sterile onion epidermal strips inoculated with conidia expressing *H3:eGFP* and observed at 30 hpi. Similar observations were made using rice leaves. Scale bars, 10 μ m.



ductive hydrophobic surface, a fungal nucleus migrated into the germ tube, where mitosis occurred between 4 and 6 hours after inoculation (hpi, hours post inoculation) (Fig. 1A). After 8 hpi, one of the daughter nuclei migrated into the incipient appressorium, and the other nucleus returned to the conidium. After 12 to 15 hpi, the three nuclei that remained in the conidium broke down and could no longer be seen (Fig. 1A). By 24 hpi, the only surviving nucleus was contained within the mature appressorium. To determine whether the pattern of nuclear division and migration was associated specifically with appressorium morphogenesis, we conducted two experiments. First, we incubated conidia in the presence of exogenous nutrients, which inhibits appressorium formation in *M. grisea* (4) and leads to the germination of nondifferentiated fungal hyphae. Under these conditions, mitosis occurred later (6 to 8 hours) and nuclei in the conidium did not degenerate (fig. S1, A and B). Second, we observed mitosis in a *M. grisea* $\Delta pmk1$ mitogen-activated protein kinase mutant which is unable to form appressoria (10). The $\Delta pmk1$ mutant underwent successive nuclear divisions within the germ tube as it extended, but no break down of nuclei in the spore took place (fig. S1C).

Thus, in *M. grisea*, (i) mitosis consistently occurred within the germ tube before appressorium differentiation, and (ii) breakdown of nuclei within the conidium was correlated with appressorium development. After plant infection, the appressorium nucleus migrated to the penetration hypha and underwent mitosis before the development of the invasive hypha (Fig. 1B).

Next, we tested whether the formation of the appressorium was a prerequisite for infection-related development in *M. grisea*. We used two drugs, hydroxyurea (HU) and benomyl, to block cell-cycle progression at the G_1/S or G_2/M transitions, respectively (11). When HU (50 mM) or benomyl (20 $\mu\text{g ml}^{-1}$) was applied to conidia between 0 and 4 hpi, inhibition of mitosis was observed in the germ tube (fig. S2, A and B); at 24 hpi, appressorium development had declined significantly ($P < 0.05$) (fig. S2C). However, when HU or benomyl was applied later (at 6 or 8 hpi), mitosis had already taken place and the rate of appressorium differentiation was not significantly reduced ($P > 0.05$). Initiation of appressorium development, characterized by hooking of the apex of the germ tube, still occurred after benomyl treatment, but cells failed to mature (fig. S2D).

To investigate the importance of mitotic control to appressorium development in *M. grisea*, we generated a mutant that was unable to enter mitosis. In *Aspergillus nidulans*, the *NimA* gene (*never entry into mitosis*) encodes a protein kinase that is necessary for mitosis, in addition to the $p34^{cdc2}$ /cyclin B-dependent kinase (12, 13). We identified an equivalent gene in *M. grisea*, *MgNIMA* (fig. S3A), which was able to complement a *nimA5* mutant of *A. nidulans* (fig. S3D). We then generated a thermosensitive *MgNIMA*^{E37G} mutant allele (14) and introduced this into the *M. grisea* *H3:eGFP* strain by targeted gene replacement (fig. S3, B and C). *MgNIMA*^{E37G} mutants grew normally at 26°C but showed a reversible growth defect at 32°C (Fig. 2A). To investigate how the *MgNIMA*^{E37G} mutation affects mitosis in *M. grisea*, we counted the number of nuclei contained in conidia incubated at various temperatures (from 26° to 32°C) during a time course of appressorium development. When incubated at 26°C, conidia from the *H3:eGFP* strain and the *MgNIMA*^{E37G} mutant followed the same progression through mitosis, which had normally occurred by 6 hpi (Fig. 2, B to D). Mitosis was not affected when conidia of the *H3:eGFP* *M. grisea* strain were incubated at 29°C (Fig. 2, B to D). In contrast, when conidia of the *MgNIMA*^{E37G} strain were shifted to 29°C, they most commonly displayed

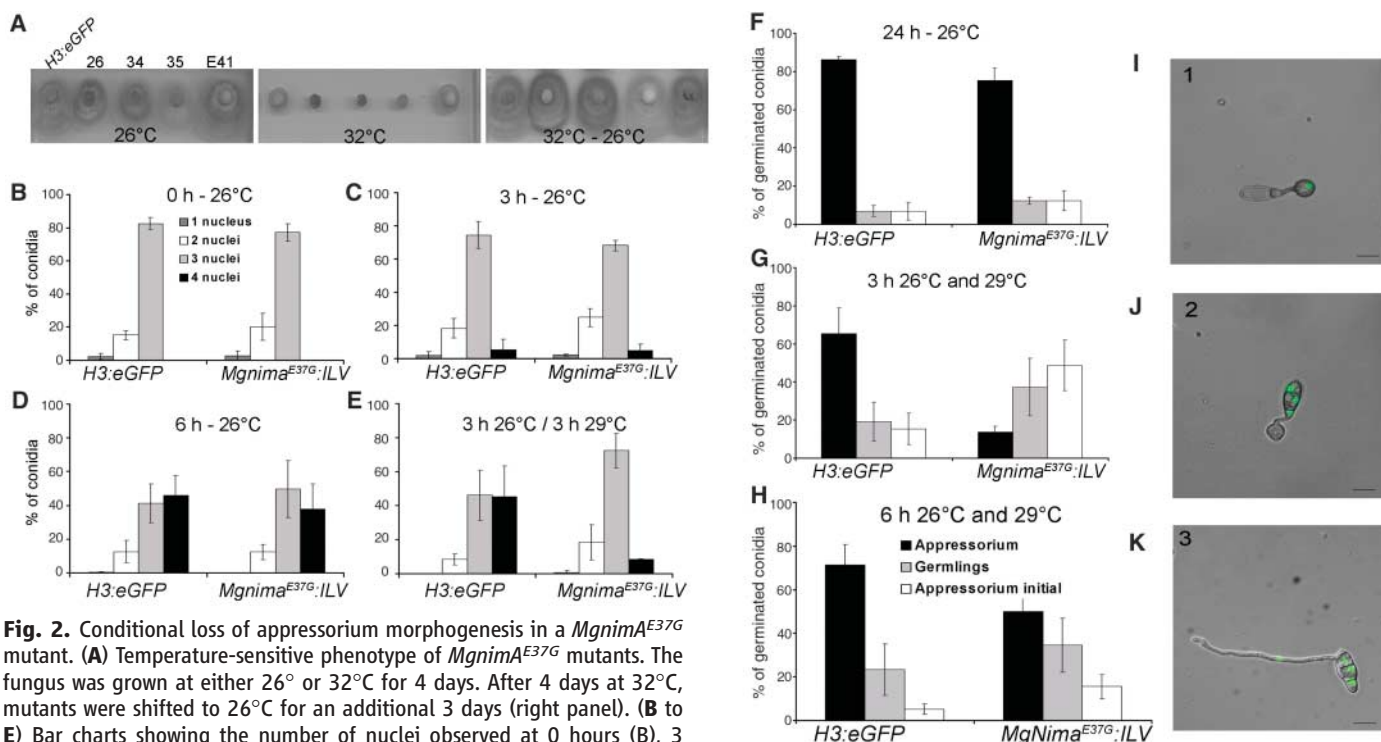


Fig. 2. Conditional loss of appressorium morphogenesis in a *MgNIMA*^{E37G} mutant. (A) Temperature-sensitive phenotype of *MgNIMA*^{E37G} mutants. The fungus was grown at either 26° or 32°C for 4 days. After 4 days at 32°C, mutants were shifted to 26°C for an additional 3 days (right panel). (B to E) Bar charts showing the number of nuclei observed at 0 hours (B), 3 hours (C), and 6 hours (D) after spore germination. Conidia from the *M. grisea* *H3:eGFP* strain and *MgNIMA*^{E37G} mutant were allowed to germinate and form appressoria at 26°C. After 3 hours at 26°C, slides were transferred to 29°C, a semi-permissive temperature that does not interfere with appressorium development (E), and the number of nuclei was recorded 3 hours later (mean \pm SD, $n = 200$, three experiments). (F to H) Bar charts showing frequency of appressorium formation. Conidia from the *H3:eGFP*

strain and the *MgNIMA*^{E37G} mutant were allowed to form appressoria at 26°C (F). After 3 hours (G) or 6 hours (H) at 26°C, slides were transferred to 29°C. Appressorium differentiation was recorded after 24 hours (mean \pm SD, $n = 200$). (I to K) Micrographs showing nucleated mature appressorium (I), appressorial initial (J), and undifferentiated germling (K). Scale bars, 10 μm .

Fig. 3. *MgATG8* is necessary for autophagy in *M. grisea*. (A) Micrographs showing hyphae of *M. grisea* *H3:eGFP* and Δ *Mgatg8::ILV* strains subjected to nitrogen starvation, in the presence or absence of 4 mM PMSF. DIC and epifluorescence images of MDC staining (10) are shown. The granular appearance of vacuoles corresponds to the accumulation of autophagic bodies in the hyphae of the *H3:eGFP* strain. Scale bars, 10 μ m. (B to E) Representative electron micrographs of vacuoles (v) in hyphae of a *M. grisea* wild-type Guy11 strain [(B) and (C)] or a Δ *Mgatg8* mutant [(D) and (E)] subjected to nitrogen starvation in the presence of PMSF. Arrows indicate autophagy-associated structures. Scale bars, 500 nm.

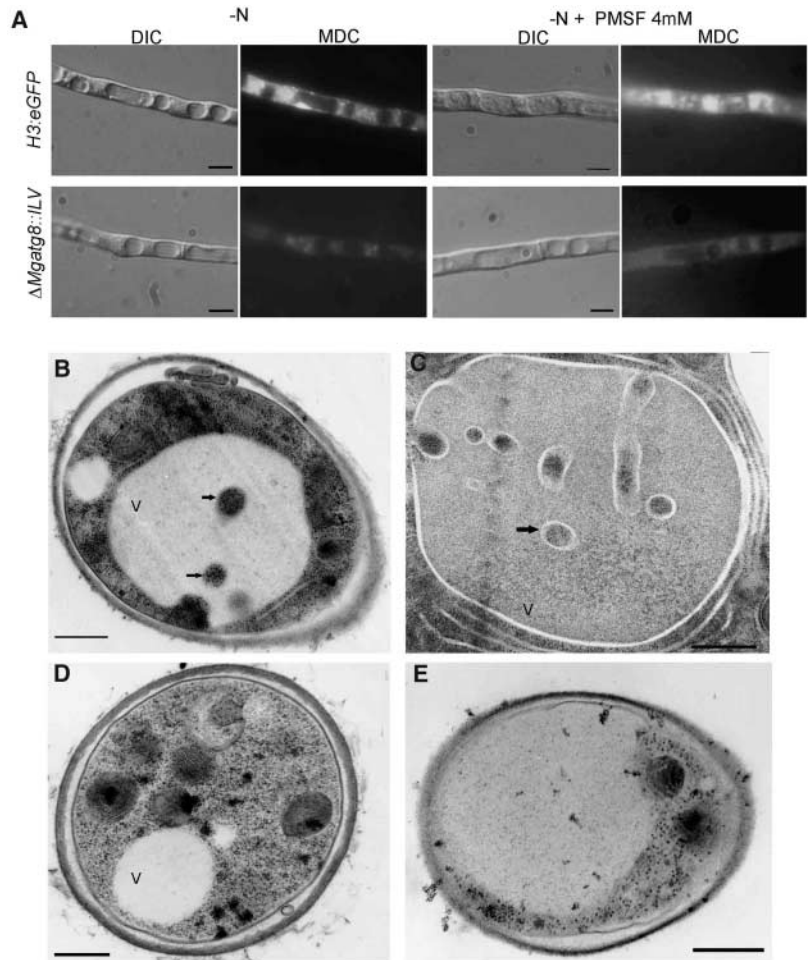
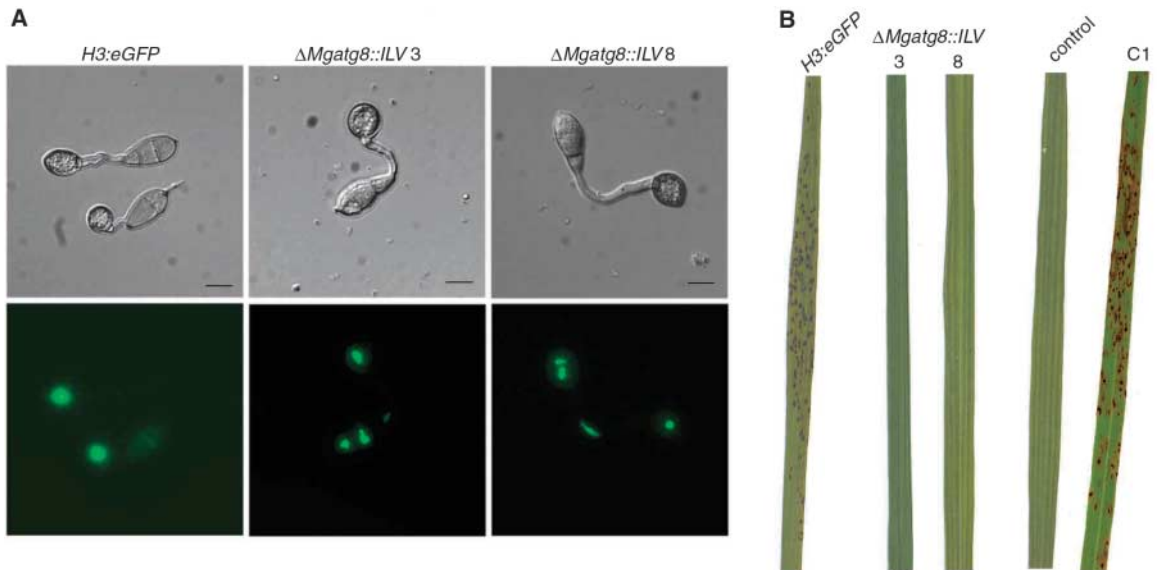


Fig. 4. Disruption of autophagy in *M. grisea* prevents rice blast disease. (A) Micrographs showing appressorium development and nuclei in *H3:eGFP* and two Δ *Mgatg8::ILV* mutants. Scale bars, 10 μ m. (B) Δ *Mgatg8::ILV* mutants are unable to cause rice blast disease. Seedlings of rice cultivar CO-39 were inoculated with uniform conidial suspensions of *H3:eGFP* or Δ *Mgatg8::ILV* mutants 3 and 8. Seedlings were incubated for 5 days to allow development of disease symptoms. Reintroduction of the *MgATG8* gene restored the ability to cause rice blast disease to a Δ *Mgatg8* mutant (C1). "Control" represents a mock inoculation with 0.2% gelatin.



three nuclei, demonstrating that mitosis had not taken place (Fig. 2E). The small number of germlings with four nuclei had probably completed mitosis before the shift in temperature.

To determine the effects of arresting mitotic progression on the formation of appressoria in the *H3:eGFP* strain and *MgnimA*^{E37G} mutants, we incubated conidia at 26°C and then trans-

ferred them to 29°C after 3 hours (before mitosis) or 6 hours (after mitosis). Three types of development were distinguished: mature melanin-pigmented appressoria, undifferentiated

germlings, or appressorial initials showing swelling of the germ tube apex (Fig. 2, I to K). No significant differences ($P > 0.05$) were observed in conidia from the wild-type strain and the *MgnimA^{E37G}* mutant maintained at 26°C (Fig. 2F) or when conidia were shifted to 29°C after 6 hours (Fig. 2H). However, the rate of appressorium differentiation declined significantly ($P < 0.01$) between the *MgnimA^{E37G}* mutant and the wild-type strain when conidia were shifted to 29°C before mitosis had occurred (3 hpi) (Fig. 2G). Thus, pharmacological and genetic interventions in mitotic progression both prevent the formation of the infection structure in *M. grisea*. Furthermore, blocking mitosis and appressorium development also prevented the collapse and death of the conidium.

Why is appressorium morphogenesis in *M. grisea* always accompanied by collapse of the spore and nuclear degeneration (Fig. 1B)? These processes might be a consequence of autophagy occurring within the spore after mitosis and nuclear migration. To test this idea, we identified the *M. grisea* *MgATG8* gene, which is very similar to the *ATG8/AUT7* gene of *Saccharomyces cerevisiae* (15), and were able to restore autophagy when the gene was expressed in a Δ *Mgatg8* mutant of *S. cerevisiae* (fig. S4). We generated Δ *Mgatg8* mutants in both Guy11 and *H3:eGFP* genetic backgrounds (11) and tested their ability to undergo autophagy in response to nitrogen starvation (Fig. 3A). When hyphae were starved in the presence of an inhibitor of vacuolar serine proteases [phenylmethylsulfonyl fluoride (PMSF) (16)], autophagic bodies accumulated in the vacuoles of the *H3:eGFP M. grisea* strain, leading to a granular appearance of the vacuole (Fig. 3A). In contrast, vacuoles of the Δ *Mgatg8::ILV* strains displayed normal morphology without granulation, indicating that autophagy did not occur in the mutant. We also used mono-dansyl cadaverine (MDC) as an indicator of autophagic activity (16, 17), which displayed strong fluorescence in the cytoplasm and vacuoles of the *H3:eGFP* strain, but little fluorescence in the starved mycelium of the Δ *Mgatg8::ILV* mutant (Fig. 3A). Electron microscopy showed autophagic bodies (15) inside the vacuoles of the wild-type strain (Fig. 3, B and C), which were absent in a Δ *Mgatg8::ILV* mutant (Fig. 3, D and E). *MgATG8* is therefore necessary for autophagy in *M. grisea*.

To investigate the effects of inhibiting autophagy during the development of appressoria in *M. grisea*, we incubated conidia on a hard hydrophobic surface to develop appressoria. In the wild-type strain, appressoria formed after 24 hours and the collapse of the conidium was accompanied by nuclear degeneration (Fig. 4A). Conversely,

conidia of the Δ *Mgatg8::ILV* mutants still formed appressoria, but three misshapen and elongated nuclei were present in spores at 24 hpi and conidia from the Δ *Mgatg8::ILV* mutants were still intact (Fig. 4A). However, the Δ *Mgatg8* mutants were unable to carry out plant infection (Fig. 4B) or form penetration hyphae efficiently even after 48 hpi (table S1). We verified that the different mutant phenotypes attributed to the *MgATG8* deletion, including an effect on the efficiency of conidiogenesis (table S1), were complemented by transforming the Δ *Mgatg8::hph* strain with a wild-type copy of *MgATG8* under the control of its native promoter (11) (Fig. 4A).

We have demonstrated a correlation among mitotic control, autophagic cell death, and infection-related morphogenesis of a pathogenic fungus. Mitosis is completed before the morphogenesis of the appressorium, suggesting that either a G₂/M or a postmitotic checkpoint may regulate appressorium formation in *M. grisea* (18). Autophagic cell death of the fungal spore is also coupled to mitotic completion, is a prerequisite for infection, and perhaps constitutes a further developmental checkpoint for the establishment of rice blast disease in *O. sativa*.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/312/5773/580/DC1

Materials and Methods

Figs. S1 to S4

Table S1

References

Movie S1

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Global Control of Dimorphism and Virulence in Fungi

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Microbial pathogens that normally inhabit our environment can adapt to thrive inside mammalian hosts. There are six dimorphic fungi that cause disease worldwide, which switch from nonpathogenic molds in soil to pathogenic yeast after spores are inhaled and exposed to elevated temperature. Mechanisms that regulate this switch remain obscure. We show that a hybrid histidine kinase senses host signals and triggers the transition from mold to yeast. The kinase also regulates cell-wall integrity, sporulation, and expression of virulence genes in vivo. This global regulator shapes how dimorphic fungal pathogens adapt to the mammalian host, which has broad implications for treating and preventing systemic fungal disease.

Microbial pathogens that inhabit our environment must undergo a radical change to survive inside a mammalian host. Among the more than 100,000 different species of environmental fungi are six phylogenetically related ascomycetes called the dimorphic fungi: *Blastomyces dermatitidis*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Sporothrix schenckii*, and *Penicillium marneffei*. These fungi

change morphology once spores are inhaled into the lungs of a mammalian host from hyphal molds in the environment to pathogenic yeast

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