Glucan Unmasking Identifies Regulators of Temperature-Induced Translatome Reprogramming in *C. neoformans*

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ABSTRACT  The cell walls of fungi are critical for cellular structure and rigidity but also serve as a major communicator to alert the cell to the changing environment. In response to stresses encountered in human hosts, pathogenic fungi remodel their cell walls. Masking the β-1,3-glucan component of the cell wall is critical to escape detection by innate immune cells. We previously demonstrated that β-1,3-glucan is unmasked in response to host temperature stress when translatome reprogramming is defective in *Cryptococcus neoformans*. Here, we used β-1,3-glucan unmasking as an output to identify signaling modules involved both in masking and in translatome reprogramming in response to host temperature stress. We reveal that the high-osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) pathway is involved in translatome reprogramming and that mutants in this pathway display moderate unmasking when grown at 37°C. Additionally, we show that mutants of the cell wall integrity (CWI)/Mpk1 MAPK pathway extensively unmask β-1,3-glucan. While the CWI pathway does not impact translatome reprogramming, our data suggest that it may play a role in the posttranslational regulation of transcription factors that govern masking.

IMPORTANCE  *Cryptococcus neoformans* is a fungal pathogen that causes devastating morbidity and mortality in immunocompromised individuals. It possesses several virulence factors that aid in its evasion from the host immune system, including a large polysaccharide capsule that cloaks the antigenic cell wall. Studies investigating how the cell wall is remodeled to keep this pathogen disguised in response to stress have been limited. We previously found that host temperature stress results in translatome reprogramming that is necessary for keeping the highly antigenic β-(1, 3)-glucan component masked. Our data reveal signaling modules that trigger these responses and suggest the points of regulation at which these pathways act in achieving masking. Understanding these mechanisms may allow for therapeutic manipulation that may promote the immune recognition and clearance of this fungal pathogen.

KEYWORDS  *Cryptococcus neoformans*, cell signaling, cell wall, glucans, host evasion, stress response

*Cryptococcus neoformans* is an environmental basidiomycete fungus that is able to enter the mammalian lung and persist. In cases of defective adaptive immunity, it can cause pulmonary infection or disseminate to the central nervous system, where it causes a deadly meningitis. The primary comorbidity associated with cryptococcosis is HIV coinfection; however, recent use of anti-tumor necrosis factor alpha (TNF-α) monoclonal antibodies in the context of rheumatoid arthritis is associated with an increased risk of cryptococcosis (1).

*C. neoformans* possesses multiple complex traits that promote its persistence in the lung and its virulence. One of these traits, thermotolerance, is required for mammalian...
infection. Previous work in our laboratory has implicated deadenylation-dependent mRNA decay mediated by Ccr4 in promoting adaptation to host temperature (2–4). This work suggests that reprogramming of the mRNAs associated with translating ribosomes, the translatome, is a prerequisite for temperature adaptation (3). Coupled to temperature adaptation is the maintenance of cell wall integrity (CWI) and the masking of the ubiquitous fungal pathogen-associated molecular pattern (PAMP) β-1,3-glucan.

Another environmental Cryptococcus species, C. amylolentus, is deficient in thermo-tolerance and, like our ccr4Δ mutant, also unmasks β-1,3-glucan at 37°C (3). In addition, C. amylolentus fails to repress abundant ribosomal protein (RP) transcripts, which is necessary for reprogramming the translatome in response to temperature stress. This led us to a model in which thermotolerance and β-1,3-glucan masking are linked, likely via changes that ensue from translatome reprogramming.

We have previously coined the term “adaptive agility,” defined as the extent and speed at which an organism can reshape its proteome to one that is suited for its new environment, and speculated that it is essential for and determines an organism’s pathogenic potential (5). Host temperature-induced mRNA decay and translatome reprogramming occur immediately in response to stress (3). Posttranslational modifications of proteins allow for rapid, efficient, and dynamic responses by altering the functions of proteins that are already present (6, 7). There have been several examples of how the translational landscape of fungi is regulated by kinases in response to different stressors (5).

The β-1,3-glucan component of the fungal cell wall is highly antigenic. The pattern recognition receptor Dectin-1 specifically recognizes β-1,3-glucan and promotes phagocytosis and inflammatory responses (8). In response to several stressors encountered in the human host, Candida albicans engages specific pathways that reduce the exposure of this PAMP. The CEK1-mediated mitogen-activated protein kinase (MAPK) pathway seems to generally regulate β-1,3-glucan masking (9), and signaling through the protein kinase A pathway is required for β-1,3-glucan masking in hypoxic and iron-limiting conditions (10, 11). Interestingly, the Gpr1 receptor, but not the canonical downstream cyclic AMP (cAMP) pathway, regulates lactate-induced masking in C. albicans (12). While the cell wall integrity MAPK pathway has been identified in C. neoformans (13), pathways that regulate glucan masking specifically in response to stressors have not been investigated.

In this report, we investigate the linkage between glucan masking, translatome reprogramming, and thermotolerance by using β-1,3-glucan unmasking as the phenotypic output of a screen of kinase deletion mutants. We identify two core MAP kinase modules that mediate glucan masking. Mechanistic investigation of the point of action suggests that the Hog1 pathway regulates unmasking via translatome reprogramming, whereas the cell integrity pathway likely regulates unmasking posttranslationally, perhaps through the activation of transcription factors.

RESULTS

A screen of kinase mutants for glucan unmasking implicates core MAP kinases. Reprogramming of the C. neoformans translatome through the action of the mRNA deadenylase Ccr4 is required for both temperature adaptation and the maintenance of masking of β-1,3-glucan at the host temperature (3). We reasoned that identification of regulators of glucan masking might also identify factors that control translatome reprogramming in C. neoformans. Using β-1,3-glucan unmasking as an output, we screened mutants from the C. neoformans kinase deletion collection that were predicted to be sensitive to either the host temperature or cell wall stress (14) and identified two core mitogen-activated protein kinase (MAPK) modules that unmask β-1,3-glucan at 37°C, as detected by flow cytometry. Null mutants for the three components of the Hog1 MAPK pathway, namely, MAP3K pbs2Δ, MAP2K ssk2Δ, and MAPK hog1Δ, exhibit a moderate level of unmasking, defined as 2- to 5-fold over that of the wild type (WT). In addition, all three components of the cell integrity MAPK signaling
pathway were found to unmask extensively, defined as unmasking at a level greater than 5-fold over the wild-type level. This is true for MAP3K bck1Δ, MAP2K, mkk2Δ, and MAPK mpk1Δ. We went on to validate the screen in larger-scale cultures and found that the identified mutants did indeed reproducibly unmask at 37°C (Fig. 1).

Comparison of glucan exposure by fluorescence microscopy to that of the WT, which completely masks β-glucan, and the ccr4Δ mutant, which exposes β-glucan, confirmed our findings by flow cytometry and revealed extensive unmasking in a speckled pattern around the periphery of a large portion of the cell wall integrity MAPK mutants and moderate unmasking in a crescent-like pattern around the periphery of a subpopulation of the Hog1 MAPK pathway mutants when grown at 37°C (Fig. 2).

**Unmasking kinase mutants exhibit differential sensitivity to temperature and cell wall perturbation.** Given that the identified kinase mutants exhibit unmasking at mammalian host temperature, we tested sensitivity to both host temperature stress and cell wall stressors by spot dilution assays (Fig. 3A). All of the mutants demon-

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**FIG 1** Validation of β-(1,3)-glucan unmasking in kinase mutants of the CWI MAPK and HOG1 MAPK pathways. Kinase mutants grown overnight in YPD at 37°C were stained with anti-β-(1,3)-glucan antibody and assessed for fluorescence by flow cytometry. Fluorescent populations of mutants were compared to the WT to calculate fold change in β-(1,3)-glucan exposure. Statistical analysis was performed with the Kruskal-Wallis test. Error bars depict standard errors of the means (SEM) (n = 3 or 4). *, P < 0.05; **, P < 0.01.

**FIG 2** Mutant strains null for components in the CWI MAPK and HOG MAPK pathways unmask β-(1,3)-glucan at 37°C. Cells grown overnight at 37°C were stained with an anti-β-(1,3)-glucan antibody and imaged by fluorescence microscopy. Insets show a portion of the larger image at ×4 magnification. DIC, differential interference contrast.
strated a strong sensitivity to SDS and moderate sensitivity to caffeine. All of the cell integrity MAPK pathway mutants exhibited pronounced sensitivity to Congo red, a dye that interacts with β-1,3-glucans, and a modest sensitivity to calcofluor white. None of the mutants exhibited growth defects when they were spotted onto yeast extract-peptone-dextrose (YPD) agar plates and incubated at 37°C, nor were the cell wall stress sensitivities exacerbated at elevated temperatures.

To further examine the effect of host temperature, we also determined growth curves for the mutants in liquid YPD media at 37°C. We have observed differences in growth between liquid and solid media, and it has recently been shown that humidity plays a role in viability and transmission (15). While the Hog1 MAPK mutants demonstrated WT-like growth, all of the CWI MAPK mutants demonstrated slower growth in liquid media despite exhibiting comparable growth by the spot dilution method (Fig. 3B).

Hog1, but not Mpk1, controls unmasking at the level of translatome reprogramming. Our previous data demonstrate that deadenylation-dependent mRNA decay promotes the reprogramming of the translatome to allow for expression of genes required for temperature adaptation and glucan masking. We next set out to investigate if Hog1 and Mpk1 act at the level of translatome reprogramming. The first step in translatome reprogramming is the rapid, but transient, removal of highly abundant and efficiently translated mRNAs from the translating pool (16). In the wild type, nearly every ribosomal protein mRNA is removed from the translating pool by 1 h at 37°C (3). Removal of ribosomal protein mRNAs permits the association of transcription factor mRNAs and stress response effector mRNAs with ribosomes. We asked if transient repression of RPL2, a representative ribosomal protein mRNA, was intact in the identified unmasking mutants of each pathway. Analysis of RPL2 repression by Northern blotting revealed a decreased rate of repression in the pbs2Δ, ssk2Δ, and
mutants compared to that in the wild type (Fig. 4). RPL2 repression in the bck1Δ, ssk2Δ, and mpk1Δ mutants exhibited the same repression kinetics as the wild type, with a rapid decline in levels during the first hour, followed by a gradual increase toward prestress levels. These data suggest that the Hog1 pathway, but not the Mpk1 cell integrity pathway, regulate the first step in translatome reprogramming.

Temperature adaptation in C. neoformans is accompanied by a moderate translational repression consistent with the removal of a large group of highly abundant and highly translated mRNAs from the translating pool. We reasoned that if RP transcripts were delayed in their removal from the translating pools in the hog1Δ mutant, then we might observe a defect in host temperature-associated changes in the translational landscape. We used polysome profiling to compare the translatonal states of the wild type, the mpk1Δ mutant, and the hog1Δ mutant at 30 min after a shift to 37°C (Fig. 5). Like the WT, the mpk1Δ mutant exhibited a decrease in the number of low-molecular-weight polysomes and an increase in the number of high-molecular-weight polysomes in response to host temperature. The hog1Δ mutant demonstrated a defect in translational regulation, with very little change in the polysomes and a large increase the 60S subunit. This is consistent with the Hog1 pathway regulating glucan masking through translatome reprogramming.

**Hog1 and Mpk1 are activated during host temperature adaptation.** We next sought to establish that these phenotypes in the hog1Δ and mpk1Δ mutants are due to direct participation of these kinase modules during host temperature adaptation. In order to evaluate this, we measured the levels of active phosphorylated Hog1 and Mpk1 by Western blotting in WT cells during growth at 37°C (Fig. 6, left). We found that shifting to 37°C activated both kinases in the WT, with pronounced activation occurring after 1 h.

The observed delay in RP transcript decay in the hog1Δ mutant suggested that
Hog1 activity influences removal of RP transcripts from actively translating ribosomes. We wondered then if Hog1 activation would be reduced in the absence of RP transcript decay. To test this, we also examined Hog1 and Mpk1 activation in a ccr4Δ mutant, which is severely deficient in RP decay (3) (Fig. 6, right). We found that temperature-induced Hog1 activation was delayed in the ccr4Δ mutant, beginning after 2 h, and was not as robust as in the wild type. We also observed a small but reproducible acceleration in Mpk1 activation in the ccr4Δ mutant. Together, these results indicate that both the Hog1 and Mpk1 kinase modules participate in adaptation to growth at 37°C and that, while Ccr4-mediated mRNA decay is not a prerequisite for Mpk1 activation, Ccr4 either directly or indirectly modulates Hog1 activation during host temperature adaptation.

Ccr4, Hog1, and Mpk1 cooperatively regulate the host temperature transcriptome. In the absence of deadenylation-dependent mRNA decay, many transcription factors fail to enter the translatome, leading to gross defects in transcriptome reprogramming (3). In order to evaluate potential genes involved in the glucan-masking phenotype, we compared the existing mpk1Δ transcriptome sequencing (RNA-seq) data set (17; GEO accession no. GSE57217) to our own data set from the ccr4Δ mutant (3; accession no. GSE121183) (Fig. 7A). We filtered the resulting overlap of 232 genes for those that would be cell surface associated or involved in cell wall remodeling, and among these genes were two chitin synthases, CHS4 and CHS5, as well as an uncharacterized exoglucanase, EXG1, which were predicted to be downregulated in both sets. We hypothesized that if Hog1 influences removal of RP mRNAs and, thus, translatome reprogramming and if translatome reprogramming precedes transcriptome remodeling as we predict (Fig. 8), then these genes would also be affected in the hog1Δ mutant. We reported previously that CHS6 was downregulated in the ccr4Δ mutant and included that in our analysis. We compared the levels of expression of these four genes by reverse transcription-quantit-
Gene expression was evaluated in the WT and the mpk1Δ, ccr4Δ, and hog1Δ mutants, following a shift to 37°C for 1 h (Fig. 7B). We observed a statistically significant downregulation of all four cell wall genes in each of the mutant strains. This finding indicates that Hog1, Mpk1, and Ccr4 all coordinate the expression of cell wall genes at 37°C and suggests that these proteins cooperatively enforce the transcriptional regulon involved in glucan masking.

**FIG 7** Genes involved in cell wall remodeling are downregulated in unmasking mutants at 37°C. (A) Differentially expressed genes (DEGs) in the mpk1Δ and ccr4Δ strains were compared to identify genes regulated by both the CWI pathway and Ccr4-mediated mRNA decay. (B) The WT, hog1Δ, mpk1Δ, and ccr4Δ strains were grown to mid-log phase in YPD at 30°C and then shifted to 37°C for 1 h. qPCR was performed on cDNA from these cells to evaluate the expression of CHS4, CHS5, CHS6, and EXG1. The ΔΔCt method was used to calculate expression normalized to that of mitofusin. Statistical analysis was performed using one-way ANOVA with Dunnett's post hoc test by comparing the normalized expression of each gene in the mutants to their expression in the WT. Error bars depict SEM (n = 3). *, P < 0.05; **, P < 0.01; ***, P < 0.001.

The model illustrated in **FIG 8** depicts the role of the CWI MPK and HOG MPK pathways in host temperature-induced β-(1,3)-glucan unmasking. Our data suggest that the HOG MAPK pathway acts at the level of translatome reprogramming. The decay of RP transcripts may impact the activation of Hog1, perhaps via a complex feedback loop. The CWI MAPK pathway does not affect translatome reprogramming but may act downstream on newly synthesized transcription factors (TF) that govern glucan masking.
DISCUSSION

The fungal cell wall is essential and not only impacts the structure and rigidity of the cell but also mediates all interactions that the cell encounters with its environment. It is logical then that this structure is dynamic and displays plasticity. β-1,3-Glucan is an important component of fungal cell walls, serving to anchor other components by covalent attachment (18). It is also highly antigenic in the context of a host, and exposure of this glucan results in rapid detection by Dectin-1, resulting in protective immune responses (19). Pathogenic fungi utilize mechanisms of glucan masking to help evade the host immune system. While C. albicans hides its glucan layer under a blanket of mannoproteins (20), Aspergillus species mask β-1,3-glucan with the hydrophobic surface protein RodA on conidia (21) and a polysaccharide cloak of galactosaminogalactan on hyphae (22), and C. neoformans is protected by a polysaccharide capsule (23). The continual changes that a pathogen encounters in a host must elicit responses that include cell wall remodeling to keep it cloaked from attack.

Unlike in other pathogenic fungi, β-1,3-glucan is not the most abundant cell wall carbohydrate in C. neoformans; however, it is essential, as deletion of the sole β-1,3-glucan synthase, FKS1, is lethal (24, 25). Studies investigating β-1,3-glucan masking have been lacking for this organism. We previously reported that Ccr4-mediated mRNA decay and translome reprogramming in response to host temperature was required for keeping β-1,3-glucan masked. Here, we identified additional signaling modules that mediate this outcome.

The Pkc1 CWI MAPK pathway is the major pathway that regulates cell wall integrity in C. neoformans (13); thus, we were not surprised to find that mutants with mutations in this pathway unmasked β-1,3-glucan. Like the Cek1 MAPK pathway in C. albicans (9), this pathway may control masking in a general manner by regulating genes that are involved in cell wall structure even under no stress, as these mutants are inherently sensitive to cell wall stressors. In fact, mutation of the most upstream kinase of this pathway, Pck1, is viable only in the presence of an osmostabilant (26). Host temperature may increase reliance on this pathway to help in combating stress-induced insult to the cell wall. The terminal kinase Mpk1 was previously reported to be activated in response to oxidative and nitrosative stresses (26); we show here that it is activated in response to 37°C and may explain both the growth defect in liquid culture at 37°C and the extensive β-(1, 3)-glucan unmasking seen in strains with null mutations in the kinase components of this pathway (Fig. 1 and 3B). We showed that Mpk1 activation in response to temperature stress was not hindered when translome reprogramming was inhibited by defective clearance of ribosomes in the ccr4D mutant (Fig. 6). Further, RP transcript repression (Fig. 4) and polysome profiles (Fig. 5) were unaffected in the mpk1D mutant, suggesting that the CWI MAPK pathway does not impact unmasking at the level of translome reprogramming. Given that our previous data suggest that translome reprogramming impacts transcription factor production and precedes transcriptional remodeling, we speculate that CWI MPK regulation of masking in response to host temperature acts in parallel with or after translome reprogramming, perhaps through posttranslational modification of newly translated transcription factors, thereby affecting their activity or localization (Fig. 8). Indeed, our previous work identified several genes that encode transcription factors that are downregulated in the ccr4Δ mutant that may be influenced by Mpk1, which was supported by the overlap of differentially expressed genes in these two strains (Fig. 6A). Importantly, we note that the RNA-seq data for the mpk1Δ strain was acquired from cells grown in YPD supplemented with the osmostabilant sorbitol due to the original inclusion of the pck1Δ mutant in those studies (17), while our data were acquired from cells that were stressed at mammalian host temperature for 1 h in YPD. Differential gene expression may be even greater in the mpk1Δ mutant in medium that does not aid in stabilizing the cell membrane. Regardless, the data suggest that Ccr4-mediated changes in gene expression in response to host temperature include components involved in regulation
of the CWI MAPK pathway or targets of the CWI-MAPK pathway that promote glucan masking.

The *C. neoformans* HOG MPK pathway has been studied largely in regard to osmoregulation and drug resistance (27–29). Interestingly, Hog1 negatively regulates capsule production, and in response to osmotic stress, the constitutive activation of Hog1 in *C. neoformans* is downregulated (27, 28). We show here that Hog1 activation is heightened in response to host temperature, and this activation is dependent, at least in part, on Ccr4-mediated clearance of actively translating ribosomes (Fig. 6). Intriguingly, the absence of *HOG1* resulted in significantly delayed repression of the abundant RP transcripts following a shift to 37°C. These mRNAs need to be cleared from ribosomes for translatome reprogramming to ensue. Delayed RP repression was supported by the lack of change in the translational landscape in response to host temperature in the *hog1Δ* mutant. If Hog1 activation were a prerequisite for Ccr4-mediated decay, then we would have expected Hog1 phosphorylation to occur in the *ccr4Δ* mutant similarly to in the WT, but this was not the case. The relationship between Hog1 activation and translatome reprogramming is therefore complex, and the following begs to be answered: at which time point in the temperature response does Hog1 act? In *Saccharomyces cerevisiae*, Hog1 has been indirectly linked to translational regulation via activation of its downstream target Rck2, which targets the translation elongation factor eEF2 (30). We are currently investigating the events that occur at the ribosome in response to stress to identify the order of operations that result in translatome reprogramming. Our work suggests that Hog1 is required for the immediate repression of RP transcripts and their exit from the translating pool to allow for translation of newly synthesized genes, such as those involved in masking (Fig. 8). The fact that the *hog1Δ* mutant grows as well as the WT at 37°C suggests that translatome reprogramming eventually occurs; however, glucan masking is not completely achieved. Future assessment of phagocytosis will determine if Hog1-mediated β-(1, 3)-glucan masking contributes to host evasion and if the loss of Hog1-mediated β-(1, 3)-glucan masking alters the immune landscape in the lung.

Our work here is the first to examine signaling modules that specifically play a role in the masking of the antigenic cell wall component β-(1, 3)-glucan in *C. neoformans*. Given that innate immunity is equipped to specifically recognize this pattern-associated molecular pattern and elicit protective immune responses, an understanding of the pathogen’s ability to cloak itself may be essential in future therapeutic endeavors, and given that protein translation has proven to be targetable, the role of factors that specifically manipulate the translational machinery to promote pathogen adaptation may be instrumental.

### Materials and Methods

#### Strains used in this study.
For all experiments, the *C. neoformans* serotype A var. *grubii* strain H99 was used as the WT. Kinase mutants were created by the Y. S. Bahn laboratory (14) and obtained from the fungal genetics stock center. The *ccr4Δ* mutant was created by biolistic transformation of the H99 strain using a PCR-amplified DNA construct in which the nourseothricin resistance cassette was flanked by sequences upstream and downstream of the *CCR4* gene. The URA resistance cassette was dropped from our previously constructed plasmid (31) and replaced with NAT. Primers for cloning and amplification are listed in Table 1. The knockout clone was verified by PCR and Southern and Northern blotting.

#### β-1,3-Glucan staining and detection.
For screening of the kinase mutant library, cells were grown overnight in 100 μl of YPD in a 96-well plate at 37°C. Cells were pelleted, washed twice in phosphate-buffered saline (PBS), and then stained in a 96-well plate according to the staining protocol below. For verification of identified mutants from the screen, cells were grown in 3 ml of YPD overnight at 37°C, with shaking at 250 rpm. Cells were washed once in PBS and resuspended in PBS to an optical density at 600 nm (OD600) of 1.0. For each strain, 100 μl of cells was aliquoted to 2 microcentrifuge tubes and pelleted. For staining, cells were resuspended in 100 μl of PBS containing 15 μg/ml anti-β-(1, 3)-glucan antibody (Biosupplies Australia). For no primary controls, cells were resuspended in 100 μl of PBS. All tubes were incubated at 37°C with gentle rotation for 1 h. Cells were pelleted and washed 3 times with PBS and resuspended in PBS containing 10 μg/ml Alexa Fluor 488-goat anti-mouse secondary antibody.

Cells were incubated at 37°C with gentle rotation for 40 min while being protected from light. Cells were pelleted and washed three times in PBS and then fixed in 3.7% formaldehyde at room temperature for 20 min while being protected from light. Finally, cells were washed and resuspended in PBS. For microscopy, cells were visualized and images captured using a Leica SP8 TCS confocal microscope. For
TABLE 1 Oligonucleotide sequences used in this study

| Primer        | Sequence (5’–3’) |
|---------------|-----------------|
| F-CCR4up      | AGTGAACAGGATTTGCGC |
| R-CCR4down    | ATCGTGCTCTACCTGGC |
| F-NAT-BglIII  | TAATAGATCGAGGATGCTG |
| R-NAT-Munl    | TAATACACGCTATAGAAGATG |
| CHS4 F        | CGGTCCTAGCATGGATT |
| CHS4 R        | TTCCGAGTGAATGATCTG |
| CHS5 F        | GCCGGATATCTCTATATCG |
| CHS5 R        | TACCTTCCATCATGGATGAC |
| CHS6 F        | TTGACCCCGACCTATCT |
| CHS6 R        | GTTGGAAAATGATCTG |
| EXG1 F        | ACCCAGATGATGAGC |
| EXG1 R        | GCCAGAACACTACATT |
| mitofusin F   | CTTGGGTTCTTCCACCG |
| mitofusin R   | CAGGTGCAACTGAGAGC |

CHS4 and CHS6 primers were previously published (32).

Primers for CHS5 were previously published (33).

flow cytometry, fluorescence was measured using a BD LSRFortessa flow cytometer. Statistical analysis for flow cytometry was done using GraphPad Prism (version 8.4.3).

Growth curves. All strains were grown overnight in 3 ml of YPD at 30°C, with shaking at 250 rpm. Overnight cultures were used to seed fresh YPD cultures to an OD600 of 0.15 and were grown at 30°C for 5 h. These mid-logarithmic cultures were then diluted to an OD600 of 0.1 in a 100-μl final volume in a 96-well plate. Growth was assessed by measuring the OD600 every 10 min for 20 h using a Biotek Synergy hybrid plate reader.

Spot plate analyses. For spot plate analyses, all strains were grown in 3 ml of YPD overnight at 30°C. Cells were washed twice with sterile deionized water (SDW) and resuspended in SDW at an OD600 of 1.0. Five 10-fold serial dilutions were prepared in SDW, and 5 μl of each dilution was spotted onto YPD agar plates supplemented with cell wall inhibitors (0.5 mg/ml caffeine, 1 mg/ml calcofluor white, 5 mg/ml Congo red, 0.03% SDS). Plates were incubated at 30°C or 37°C for 3 days and photographed.

Polysome profiling. Polysome profiling was done as previously published (3), with minimal differences. Briefly, cells were grown to mid-log phase in YPD, and half of the culture was pelleted, resuspended in prewarmed 37°C YPD, and incubated at 37°C for 30 min. Cycloheximide (0.1 mg/ml) was added immediately, and cells were pelleted by centrifugation at 4,000 rpm and 4°C for 3 min. Pellets were flash frozen in liquid nitrogen and stored at −80°C until lysis. Pellets were washed in polysome lysis buffer (20 mM Tris HCl, pH 8.0, 140 mM KCl, 5 mM MgCl2, 1% Triton X-100, 25 mg/ml heparin sodium sulfate, 0.1 mg/ml cycloheximide) and pelleted. Cells were resuspended in residual buffer, transferred to a microcentrifuge tube, and pelleted at 13,000 rpm for 30 s. The supernatant was removed, and pellets were resuspended in 100 μl of cold lysis buffer and transferred to an Eppendorf tube containing glass beads. Cells were lysed mechanically in a Bullet Blender for 5 min, followed by the addition of 300 μl cold lysis buffer. The supernatant was transferred to a cold microcentrifuge tube and centrifuged for 5 min at 14,000 rpm and 4°C. Cleared lysates were quantitated for RNA, and 250 μg was loaded on top of 10% to 50% sucrose gradients. Gradients were subjected to ultracentrifugation for 2 h at 39,000 rpm and 4°C. Sucrose gradients were then pushed through a flow cell, and RNA was detected by UV-visible light to determine the A260.

Northern blot assessment of RPL2 mRNA repression. For all strains, cells were grown in 3 ml of YPD at 30°C and 250 rpm overnight. Overnight cultures were used to seed 40 ml of YPD at an OD600 of 0.2. Cells were grown at 30°C, with shaking at 250 rpm, until the OD600 reached 0.6. Cells were pelleted and resuspended in prewarmed 37°C YPD. Aliquots of 5 ml were pelleted every 30 min for 2 h and flash frozen in liquid nitrogen. Pellets were resuspended in 50 μl of buffer RLT (Qiagen RNeasy kit) plus 10 μl/ ml β-mercaptoethanol (β-ME), and cells were lysed by mechanical disruption with glass beads using a Bullet Blender. Lysates were resuspended in 450 μl of RLT plus β-ME, and RNA was extracted using the Qiagen RNeasy kit. For Northern blot analysis of RPL2, 3 μg of RNA per sample was electrophoretically separated in a 1% agarose-formaldehyde gel. The rRNA was detected and quantified by ImageLab software. RNA was transferred to a nylon membrane, hybridized with a P32-labeled DNA probe, and the signal was quantitated by ImageLab software. Western blot detection of phospho-Hog1 and phospho-Mpk1. The C. neoformans ccr4Δ strain (H99) were used to inoculate 5 ml overnight YPD cultures, shaken at 250 rpm at 30°C. Overnight cultures were used the following day to inoculate fresh YPD (at an OD600 of 0.2), followed by 5 h of shaking in baffled flasks in a 30°C incubator until mid-log phase (OD600 = 0.65). Mid-log-phase cells were pelleted, resuspended in prewarmed YPD, and shaken in a 37°C incubator at 250 rpm, with images recorded every half hour. At each time point, cells were pelleted, the supernatant was discarded, and the pellets were rapidly frozen in liquid nitrogen and stored at −80°C until lysis.

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Thawed pellets were rinsed in 1 ml SDW and then resuspended in whole-cell lysis buffer (15 mM HEPES [pH 7.4], 10 mM KCl, 5 mM MgCl2, 10 μg/ml Halt protease and phosphatase inhibitor, 1 mM dithiothreitol [DTT]). Cells were lysed by mechanical disruption using glass beads in a Bullet Blender, and the resulting lysate was clarified by centrifugation. Samples were reduced and denatured by boiling them in Laemmli buffer with β-ME, and 30 μg of protein per sample was electrophoretically separated through a Bio-Rad stain-free Tris-glycine gel. Prior to the transference of protein to nitrocellulose, the gel was imaged and total protein was quantified from the gel using ImageLab software. The blot was probed using a rabbit anti-phospho-p38 MAPK (Cell Signaling Technologies), followed by an anti-rabbit HRP secondary antibody, and visualized by chemiluminescence using a Bio-Rad ChemiDoc Imager. It was found that the phospho-p38 primary antibody, in addition to detecting phosphorylated Hog1, was cross-reactive with phosphorylated Hpk1. The specificity of this cross-reactive band was verified using a mouse anti-phospho-p42/44 MAPK antibody (Cell Signaling Technologies), which demonstrated the loss of the corresponding band in the mpk1Δ mutant and reliably showed the same pattern of Hpk1 phosphorylation as the phospho-p38 antibody across all biological replicates. The phospho-p38 signal was selected to show the phosphorylation of both Hog1 and Hpk1 due to its higher signal-to-noise ratio.

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We declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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