Identifying a novel connection between the fungal plasma membrane and pH-sensing

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Summary

The mechanisms by which micro-organisms sense and internalize extracellular pH signals are not completely understood. One example of a known external pH-sensing process is the fungal-specific Rim/Pal signal transduction pathway. Fungi, such as the opportunistic pathogen Cryptococcus neoformans, use Rim signaling to sense and respond to changes in environmental pH. Mutations in this pathway result in strains that are attenuated for survival at alkaline pH, and often for survival within the host. Here, we used an insertional mutagenesis screen to identify novel genes required for C. neoformans growth at host pH. We discovered altered alkaline pH growth in several strains with specific defects in plasma membrane composition and maintenance of phospholipid assembly. Among these, loss of function of the Cdc50 lipid flippase regulatory subunit affected the temporal dynamics of Rim pathway activation. We defined distinct and overlapping cellular processes regulated by Rim101 and Cdc50 through analysis of the transcriptome in these mutant strains. We further explored how pH-induced membrane changes affect membrane-bound pH-sensing proteins, specifically the C-terminal domain of the Rra1 protein, an upstream Rim pathway activator and pH sensor. These results suggest both broadly applicable and phylum-specific molecular interactions that drive microbial environmental sensing.

Introduction

A key virulence attribute of any microbial pathogen is the ability to rapidly adapt to the conditions of the infected host. One of these host-associated conditions is neutral-to-alkaline extracellular pH. Disease-causing micro-organisms likely sense pH as a host-specific inducing signal in order to effectively adapt to this novel environment. Fungal pathogens use the fungal-specific Rim/Pal signal transduction pathway to sense and respond to host pH through the activation of an alkaline responsive transcription factor: Rim101. In the human fungal pathogen Cryptococcus neoformans, Rim101 is required not only for the maintenance of the protective polysaccharide capsule, but it is also required for growth under stressful host conditions, such as elevated cation concentrations and alkaline pH (Chun and Madhani, 2010; O’Meara et al., 2010).

The fungal-specific Rim101 activation and signaling pathway has also been well described in the context of virulence in Candida albicans, another human fungal pathogen. In C. albicans, the proper activation of Rim101 is required for the transition from a yeast to hyphal form, which is necessary for infection and dissemination (Davis et al., 2000; Cornet et al., 2009; Cornet and Gaillardin, 2014). In another opportunistic fungal pathogen, Aspergillus fumigatus, the Rim101 ortholog (PacC) is required for effective infection in a murine model of aspergillosis, which is characterized by conidial germination and hyphal growth within the host bronchioles and surrounding lung tissue (Dagenais and Keller, 2009; Bertuzzi et al., 2014). The Rim pathway has also been well described in the model ascomycete Saccharomyces cerevisiae, especially for its role in surviving alkaline pH stress (Lamb et al., 2001; Mira et al., 2009; Selvig and Alspaugh, 2011).

In contrast, this signaling pathway is less well-defined in C. neoformans and other related basidiomycete fungi. Some components of the pathway are highly conserved between the two fungal phyla. Specifically, both basidiomycetes and ascomycetes internalize the alkaline pH signal using a pH sensor physically located at the plasma membrane. The alkaline pH signal is then propagated to the endosomal membrane complex, which involves...
the Endosomal Sorting Complex Required for Transport (ESCRT) pathway components. Once this complex forms, the Rim101 protein is cleaved by the Rim13 protease, subsequently translocating to the nucleus to direct the expression of various virulence genes (Antonio et al., 2010; Ost et al., 2015). However, much of the upstream portion of the pathway has yet to be identified in basidiomycetes, including the remaining components of the pH-sensing complex and how this complex might interact with the plasma membrane to internalize external cues (Ost et al., 2015).

Other investigators have suggested that the arrangement of phospholipids in the S. cerevisiae plasma membrane plays an important role in stress response and the propagation of the alkaline pH signal through the Rim21 pH-sensing protein (Nishino et al., 2015). In this model, alterations in extracellular pH change the charge of the inner leaflet of the fungal plasma membrane, altering the biochemical interaction of the S. cerevisiae Rim21 pH sensor with the membrane, potentially mediated by histidine residues on the C-terminus of this protein. Furthermore, there is a well-established connection between membrane phospholipid asymmetry and the transmembrane pH gradient (Hope et al., 1989; van Meer, 2011). In artificial model membranes, increases in pH alter the distribution of membrane phospholipids resulting in a more symmetric phospholipid distribution between inner and outer leaflets. These changes in membrane symmetry might in turn affect the conformation, localization and function of membrane proteins. Together these studies suggest that pH-induced alterations of the plasma membrane might contribute to the activation of the fungal pH sensors (Nishino et al., 2015; Lucena-Agell et al., 2016).

We therefore sought to further define the mechanisms of pH sensing in C. neoformans as a model pathogen to explore how this host-relevant signal might be internalized in the setting of infection. As a basidiomycete, C. neoformans also offers insight into environmentally sensing processes in a microorganism distantly related to more common models such as Saccharomyces and Aspergillus species. C. neoformans is one of the best characterized and genetically tractable organisms in the basidiomycete phylum and serves as an excellent model organism to elucidate environment-sensing mechanisms shared by the members of this large and diverse fungal phylum. Other fungal species, such as the human pathogen Cryptococcus gattii (Datta et al., 2009) and the maize pathogen Ustilago maydis (Banuett, 1995), are two additional examples of basidiomycete fungal pathogens that can be better understood as a result of these studies. We have previously demonstrated that the C. neoformans Rim pathway is activated in vivo during infections and required for effective pathogenesis. Specifically, our laboratory has shown that C. neoformans Rim101 is required for the attachment of protective capsule, for the efficient masking of pathogen-associated molecular patterns (PAMPs), and for appropriate cell wall organization that shields this fungus from immune recognition (O’Meara et al., 2013). The lack of Rim101 regulation of these important cellular processes leads to failed immune avoidance and a subsequent hyper-inflammatory response in a murine inhalation model of cryptococcosis (O’Meara et al., 2013). The basidiomycete-specific, transmembrane Rra1 protein appears to function as the pH sensor in C. neoformans, and it is the most upstream identified component of Rim pathway activation. This protein was originally identified as a basidiomycete-specific gene with a pH-sensitive mutant phenotype that is suppressed by the expression of the active form of Rim101 (Ost et al., 2015). Interestingly, Rra1 shares predicted structural features, but limited sequence identity, to known Rim pathway pH-sensing proteins in ascomycetes (Ost et al., 2015). This suggests that the ascomycete and basidiomycete phyla may have independently developed convergent ways of sensing and responding to changes in extracellular pH. This also suggests that there are additional basidiomycete-specific, upstream components of the pH-sensing complex in this pathway.

Here, we have utilized a genetic screen and transcriptomic data to identify novel processes of the fungal alkaline response pathway. In this study, we have identified a connection between C. neoformans plasma membrane dynamics, Rra1 C-terminal tail function, and pH sensing. Specifically, we discovered that mutants with altered plasma membrane composition and symmetry are unable to grow at alkaline pH, and a subset of these mutations affects the temporal activation of Rim pathway signaling. Furthermore, we identified that a relationship between the newly identified C. neoformans Cdc50 lipid flippase regulatory subunit influences the temporal dynamics of Rim pathway activation. Together, these data suggest a model in which plasma membrane bilayer asymmetry is a dynamic cellular trigger involved in the response to altered extracellular pH. These results have further elucidated the molecular interactions that drive environment-sensing in a large and biologically diverse group of fungi.

Results

Forward genetics screen to identify additional Rim pathway components

We performed a random insertional mutagenesis screen to identify fungal features required for Rim pathway-dependent growth at elevated pH (Ost et al., 2015). As previously described, we used Agrobacterium tumefaciens mediated transconjugation (AMT) to create C. neoformans insertionals mutants in a strain background.
expressing a galactose-regulatable, active form of the Rim101 transcription factor (pGAL7-GFP-Rim101T) (Ost et al., 2015). This approach allows us to screen for mutants with reduced growth at alkaline pH on glucose-containing medium (Rim101T-repressing conditions), but with restored growth on galactose-containing medium (Rim101T-inducing conditions). In this way, we could distinguish between pH-sensitivity due to Rim pathway dysfunction and that due to more general alkaline pH growth defects.

Our library of random insertional mutants was screened on YPD pH 8 medium containing either galactose or glucose. Each strain was pin-replicated in quadruplicate to each medium using a BM3 benchtop robot, and heatmap software was used to objectively compare growth at each condition. Thirty-six of these strains were chosen for whole genome sequencing and further analysis based on reproducible primary and secondary screening that revealed that the pH-sensitivity of these insertional mutants was suppressed by the expression of constitutively active Rim101. The AIM-Seq bioinformatics pipeline was used to identify the genomic location of the insertions in these mutants (Esher et al., 2015). This analysis revealed 27 unique sites of genomic integration, five of which were within three genes that encode for known Rim pathway components (RIM101 (x2), RIM13 (x2), and SNF7) validating the screening approach. We performed further phenotypic assessment of independently created mutants of the remaining 22 strains defective in growth at pH 8. These included 14 strains with enhanced growth on galactose at pH 8 (true positives), 5 with no pH sensitivity (false positives) and 3 strains with general growth defects at pH 8, such as the sre1Δ strain with a mutation in the Sre1 transcription factor that directs ergosterol homeostasis in fungal membranes (Bien and Espenshade, 2010) (Table 1).

The cdc50Δ strain is sensitive to alkaline pH and is partially rescued by constitutive activation of the Rim pathway

The cdc50Δ mutant displays an alkaline pH growth defect that was reproducibly suppressed by galactose-mediated activation of Rim pathway signaling. The CDC50 gene encodes the regulatory subunit of type IV P-type ATPases (lipid flippases) (Huang et al., 2016; Shor et al., 2016). In C. neoformans, the Cdc50 regulatory subunit works in complex with the aminophospholipid translocase (Apt1) to perform membrane lipid flipping functions, similar to the Drs2-Cdc50 and Dnf1/2-Lem3 lipid flipase complexes in S. cerevisiae (Saito et al., 2004; Hu and Kronstad, 2010; Hu et al., 2017). Other investigators recently reported a similar growth defect of the cdc50Δ mutant at alkaline pH (Hu et al., 2017). In order to further validate our result, we used targeted mutagenesis
to make an independent cdc50Δ mutant in both wildtype (WT) and pGAL7-Rim101T backgrounds (Ost et al., 2015). Similar to Rim pathway mutants, the cdc50Δ strain had an alkaline pH sensitivity that was partially suppressed by expressing the active form of Rim101 (Fig. 1A). However, the incomplete rescue by Rim101T expression of the cdc50Δ defect in growth at pH 8 suggests that this lipid flippase regulatory subunit may have an indirect effect on Rim pathway activation or perhaps Rim pathway-independent functions. Interestingly, mutation of the APT1 gene, encoding the catalytic subunit of the lipid flippase complex, also resulted in a growth defect at alkaline pH (Fig. 1B). These observations suggest that the maintenance of membrane asymmetry by lipid flippase activity is an important mediator of the cellular response to alkaline pH.

Additional membrane homeostasis proteins have roles in Rim pathway-independent pH sensitivity

Interestingly, other genes that direct membrane composition and homeostasis were also identified in our forward genetic screen and initial mutant pool. For example, one of the alkaline pH-sensitive mutants contained a mutation in the SRE1 gene. Sre1 is a transcription factor regulating membrane sterol content in response to various cell stresses, including hypoxia. To confirm the association of alkaline pH tolerance and intact Sre1 activity, we tested independently created strains with mutations in the SRE1, STP1 and ERG4 genes. Stp1 is a protease required for cleavage and activation of Sre1, and Erg4 catalyzes the terminal catalytic step in the biosynthesis of ergosterol (Baumann et al., 2005; Chang et al., 2007; Bien and Espenshade, 2010). In contrast to the congenic WT strain, the sre1Δ, stp1Δ, and erg4Δ mutants were all growth impaired at pH 8 (Fig. 1B). However, in contrast to Rim pathway mutants and the cdc50Δ strain, the alkaline growth defect of the sre1Δ, stp1Δ and erg4Δ mutants was not reproducibly suppressed by overexpression of Rim101T in validation experiments (Fig. 1A and data not shown). Moreover, the hypoxia sensitivity of the C. neoformans sre1Δ mutant was not shared with the rim101Δ or rra1Δ mutants (Chang et al., 2007; Chang et al., 2009 and data not shown). Therefore, although multiple cellular defects in membrane composition result in altered growth at elevated pH, not all result in defective Rim101 activation.

Cdc50 influences Rim pathway signaling

Shortly after exposure to an alkaline pH signal, the GFP-Rim101 transcription factor is proteolytically processed from a 140 kDa pre-processed form to a 100 kDa truncated protein that translocates from the cytoplasm to the nucleus (Ost et al., 2015). Recent work exploring the role of Cdc50 in iron regulation and virulence in C. neoformans demonstrated intact Rim101 nuclear localization in the cdc50Δ mutant strain when exposed to alkaline conditions, suggesting that Cdc50 is not involved in Rim pathway signaling (Hu et al., 2017). However, given our observation that constitutive Rim pathway activation could suppress the growth defect of the cdc50Δ strain, we performed a detailed time course by western analysis defining the temporal dynamics of this cleavage event in order to assess for a subtler relationship between Cdc50 function and Rim pathway activation. We pre-incubated WT and cdc50Δ strains in Rim pathway non-inducing conditions and then assessed Rim101 cleavage after transfer to pH 7 at 10, 20 and 30 min. Protein processing assessed by western analysis demonstrated that cleavage of the GFP-Rim101 fusion protein was temporally delayed in the cdc50Δ mutant strain compared to WT. By 10 min we observed partial cleavage of the GFP-Rim101 protein in the WT strain, with complete proteolytic processing by 20 min (Fig. 2A). In contrast, in the cdc50Δ strain there was little processed GFP-Rim101 protein detected at 10 min, and uncleaved protein was still observed after 30 min in Rim pathway activating conditions.

The delay in Rim101 proteolytic cleavage in the cdc50Δ mutant strain resulted in an associated delay in GFP-Rim101 nuclear localization (Fig. 2B and C). Strains pre-incubated in synthetic complete medium (SC) pH 4 were transferred to SC pH 7, and the cells were imaged at

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Fig. 1. Insertional mutagenesis screen to identify upstream activators of the Rim pathway. A. The rra1Δ and cdc50Δ strain pH sensitivities are partially rescued by the expression of the constitutively active form of Rim101. Strains were spotted in serial dilutions onto YPD. YPD 150 mM HEPES pH 8, YP-galactose and YP-galactose 150 mM HEPES pH 8 media.

B. Proteins involved in membrane homeostasis and biosynthesis have varying levels of pH sensitivity. Strains were spotted in serial dilutions onto YPD and YPD 150 mM HEPES pH 8 media. Growth was assessed after 48 h of incubation.
Fig. 2. Altered membrane asymmetry results in a delayed activation of the Rim pathway.
A. The eGFP-Rim101 fusion protein is proteolytically processed from 140 kDa to 100 kDa at pH7 (activating conditions). Time course western blot analysis assessed the cleavage of Rim101 over time in the indicated strains when incubated for 10, 20 and 30 min in activating conditions (pH 7). Protein processing was determined by western blotting using an α-GFP antibody.

B. eGFP-Rim101 nuclear localization increases in response to increasing pH. Cells were cultured in the same manner as (A). After transfer to pH 7, GFP signal was assessed at 5, 10, 20, 30 and 60 min by (B) epifluorescence microscopy (Zeiss Axio Imager A1) using the appropriate filter and by (C) quantification of fluorescence intensity (FI) across a representative cell (plotted along the white dotted line in (B)). FI values were measured using ImageJ Software (Fiji). White scale bars indicate 5 microns.

5, 10, 20, 30 and 60 min. As shown through representative images and corresponding plots of fluorescence intensity across the cell, there is a delay in nuclear localization of Rim101 in the cdc50Δ strain compared to wildtype (Fig. 2B and C). A fluorescent cellular signal of GFP-Rim101 nuclear localization was observed in WT strains as early as 5 min after transfer to higher pH, becoming more distinctly localized to the nucleus over a 60 min period of observation. In contrast, initial GFP-Rim101 nuclear localization was not evident until 30 min in the cdc50Δ mutant strain. For each strain, cellular fluorescence was quantified and plotted from the microscopic images, showing an increasingly distinct fluorescent signal in the nuclear compartment during the pathway activation compared to the
cytoplasm and cellular membrane after pathway activation (Fig. 2B and C). Representative images and cellular fluorescence intensity plots of NucBlue nuclear-stained cells at 60 min were included as controls for expected cellular patterns of nuclear localization. The eventual but delayed nuclear localization of Rim101 in the cdc50Δ

![Diagram](image_url)

**Fig. 3.** Transcriptomic analysis of the cdc50Δ and rim101Δ strains. RNA-Seq was used to define transcript abundance in the cdc50Δ and rim101Δ strains incubated in Rim pathway activating conditions for 1.5 h. RNA-seq was performed in biological triplicate (n = 3) for each genotype (rim101Δ, cdc50Δ and WT).

A. Venn’s diagram of the overlap of 253 differentially expressed (DE) transcripts that were DE in both the rim101Δ and the cdc50Δ strains.

B. Pie charts showing the distribution of membrane-associated transcripts DE in the cdc50Δ and rim101Δ strains. 675 total membrane-associated transcripts were identified using species-specific modified GO-term analysis. Significance was determined using Chi-Square analysis as compared to the expected number of DE transcripts by random chance (.05). p < .05 for both strains.

C. Venn’s diagram showing the overlap of membrane associated transcripts that are specific to the cdc50Δ or rim101Δ strain or shared between them. The two mutant strains share 35 membrane-associated DE transcripts.

D. Heatmap of the transcript abundance for the 34 membrane-associated DE transcripts shared between cdc50Δ and rim101Δ. Clusters were assigned based on log2 fold change similarities between the strains through a kmeans algorithm. Further grouping (groups 1–4) was determined based on patterns of increased and decreased transcript expression compared to WT across the mutant strains. Complete lists of the RNA-Seq datasets from both the cdc50Δ, rim101Δ and the 34 overlapping transcripts from 3d can be found in Table S1.
mutant is consistent with observations from other investigators (Hu et al., 2017), suggesting that Cdc50-mediated cellular processes contribute to the timing and intensity of Rim101 cleavage and localization, but that they are not absolutely required for eventual pathway activation.

The cdc50Δ and rim101Δ strains show a partial overlap in transcriptional changes in tissue-culture conditions

To identify cellular processes similarly regulated by both Rim101 and Cdc50, we compared the transcriptomes of the rim101Δ and cdc50Δ strains against WT. We incubated these strains in tissue culture medium for 1.5 hours to define transcript-level changes induced by more host-like environment. RNA-sequencing was performed in triplicate for each strain, and pairwise analyses of the rim101Δ mutant versus WT and the cdc50Δ mutant versus WT were assessed. Genes with an adjusted p-value < 0.05 were considered significantly differentially expressed (false discovery rate = 10%). Our analysis of the transcriptomes of these two mutant strains revealed that there are 2821 differentially expressed (DE) genes in the cdc50Δ background compared to WT, and there are 460 DE genes in the rim101Δ strain compared to WT (Table S1). Among these, 253 DE genes are shared between the datasets, which represents more than half of the Rim101-regulated genes (Fig. 3A, Table S1). Therefore, there is a partial overlap in the transcriptional perturbation shared by these mutant strains.

Due to the established role of Cdc50 in the maintenance of membranes (Huang et al., 2016; Hu et al., 2017) and the observed partial overlap between the Rim101- and Cdc50-regulated transcriptomes, we examined the two transcriptome datasets for an enrichment in membrane-associated genes. We queried the FungiDB fungal genome database for a species-specific gene ontology term analysis to assess for enrichment of membrane-associated transcripts in these datasets (Stajich et al., 2012). This analysis predicted that the C. neoformans genome contains 675 transcripts with membrane-related functions. These membrane-associated transcripts included 64 of the Rim101-dependent transcripts and 278 of the Cdc50-dependent transcripts (Fig. 3B). Compared to the C. neoformans genome in general, membrane-associated transcripts were enriched in a statistically significant manner (p < 0.01) for both the Rim101 and Cdc50 datasets. Therefore, Rim101 and Cdc50 are each required for normal transcriptional responses for a group of membrane-associated genes in tissue culture conditions.

We determined that 34 membrane-associated transcripts were shared between the two mutants (Fig. 3C). To visualize the levels of expression of these 34 transcripts, we used heatmap imaging to plot their log2-fold expression changes in the individual mutant strains compared to WT (Fig. 3D, Table S1). Hierarchical clustering revealed 4 groups of genes based on patterns in log2-fold change (Fig. 3D). Groups 1 and 4 represent membrane-associated transcripts that are differentially regulated in opposite directions in the two mutant strains. Specifically, Group 1 includes transcripts that are positively regulated by Rim101 and negatively regulated by Cdc50, whereas genes in Group 4 have the opposite regulation pattern. Notably, both Groups 1 and 4 contain sugar/glucose transporters, but these are regulated in opposite directions. Group 2 encompasses membrane-associated transcripts that are positively regulated by both Rim101 and Cdc50. Interestingly, this group contains the CFT1 iron permease gene (a known target of Rim pathway signaling) as well as a siderochrome-iron transporter. This group also contains two of the mitochondrial import inner membrane translocase genes (TIM8 and TIM13). Group 3 includes transcripts that are negatively regulated by both Rim101 and Cdc50. This group contains a plasma-membrane proton efflux P-type ATPase (CNAG_03565) whose ortholog has been predicted to maintain pH homeostasis in organisms such as C. albicans (Monk et al., 1991). Therefore, many membrane-associated proteins, including those involved in maintaining a pH and ion gradient, demonstrate similar transcript level changes in both direction and magnitude in both the cdc50Δ and rim101Δ mutant strains.

Assessing the effect of alkaline pH on membrane content and asymmetry in the WT strain

Due to the observed connection between susceptibility to alkaline pH and defects in membrane composition and organization, we analyzed the membrane lipid content of the WT strain. In order to see if environmental growth condition had a direct effect on lipid content, the WT strain was grown in either standard lab YPD (pH 5.5) conditions or Rim pathway-activating, tissue-culture conditions (pH 7.4) for 1.5 hours. Lipidomic analysis using liquid chromatography/mass spectrometry (LC/MS) revealed a similar major lipid profile of the WT strain grown in the two very different growth conditions (Figure S1). Therefore, major compositional changes in the plasma membrane are not required for Rim pathway activation.

Due to the similar major lipid profiles we observed using mass spectroscopy of cell extracts, we assessed the effects of pH on the asymmetry of the plasma membrane of the WT strain. Susceptibility to the antifungal agent cinnamycin, has been used as a surrogate marker for plasma membrane asymmetry, due to the propensity of this agent to preferentially bind exposed PE and affect transbilayer movement (Hu and Kronstad, 2010; Hu et al., 2017). Similar to prior reports, the cdc50Δ strain displayed enhanced susceptibility to cinnamycin (Hu et al., 2017). At pH 4, the WT strain was inhibited at 10 μM cinnamycin,
and the MIC for the $cdc50\Delta$ strain at this pH was 2.5 $\mu$M. In contrast, in multiple biological and technical replicates, the MIC for the WT at pH 8 was 20 $\mu$M; the MIC for the $cdc50\Delta$ mutant strain at pH 8 was uninterpretable due to its profound growth defect at alkaline pH. This reproducible, but subtle pH-dependent enhancement in cinnamycin susceptibility suggests that the expected pH-induced changes in bilayer asymmetry are likely transient. In contrast to artificial membranes or stable mutant strains that lack flippase activity, the WT strain likely rapidly restores membrane asymmetry after cellular pH stress.

**Identifying functional domains and pH-dependent localization of the putative Rra1 pH sensor**

In a prior genetic screen, we identified *C. neoformans* Rra1 as a basidiomycete-specific activator of Rim pathway signaling (Ost et al., 2015). Similar to ascomycete pH sensors, Rra1 contains seven putative transmembrane domains, and it is required for recruiting other Rim pathway proteins to the plasma membrane upon pathway activation (Nishino et al., 2015; Lucena-Agell et al., 2016). Since Rra1 is the most upstream, membrane-associated protein in the *C. neoformans* Rim pathway, we created a Rra1-GFP fusion protein to define its localization as a function of extracellular pH (Fig. 4A). This fusion protein was functional since it complemented the alkaline pH-associated growth defects of the $rra1\Delta$ mutant (Fig. 5C). At pH 4 and pH 8, the Rra1-GFP strain demonstrated a low intensity fluorescent signal in endomembranous structures such as the endoplasmic reticulum and the Golgi complex with more intensely fluorescent punctate structures localized at the cell surface (Fig. 4A). The surface localization of these puncta was confirmed using deconvolution of Z-stacked images to create a three-dimensional image of the cell (Fig. 4C). Although the Rra1-GFP containing puncta were present and visualized at both extremes of pH, fewer puncta were consistently observed

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**Fig. 4.** Localization of the Rra1 pH sensor is dependent on pH.

A. Schematic and pH-dependent localization of the Rra1 protein GFP fusion construct in response to pH 4 and 8 media (SC medium buffered to either pH 4 or pH 8 with Mcllvaine’s buffer) for 1.5 hours. GFP signal was assessed by epifluorescence microscopy (Zeiss Axio Imager A1) using the appropriate filter.

B. Quantification of Rra1-GFP localization. The mean values and standard errors of cells with plasma membrane puncta at pH 4 vs. pH 8 was quantified using ImageJ software (Fiji) (~600 cells/condition). Student’s $t$-test, $p = .0363$.

C. 3-D projected image of Rra1-GFP cells incubated in SC at pH 4 and 8. Images were taken using high resolution Deltavision microscopy with deconvolution and z-stacking capabilities. White scale bars indicate 5 microns.

D. 3-D projected image of Rra1-GFP after incubation in pH 4 + PBS and pH 4 + Filipin. Images were taken using high resolution Deltavision microscopy with deconvolution and z-stacking capabilities. White scale bars indicate 5 microns.

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Fig. 5. pH-dependent localization of Rra1 is dependent on its C-terminal tail.
A. Constructs of Rra1 truncations indicating the 7 Transmembrane domains (7 TM), the Highly Charged Region (HCR) with indicated Lysine and Arginine residues (****), and the C-terminal tail.
B. Localization of Rra1-296T-GFP and Rra1-273T-GFP at pH 4 and pH 8. GFP signal was assessed after an incubation period of 1.5 h and was assessed by epifluorescence microscopy with the appropriate filter. Cell surface puncta are indicated by red arrows and internal perinuclear/endosomal aggregates are indicated by red triangles.
C. Rescue of rra1Δ pH sensitivity by Rra1 truncation constructs. Strains were spotted in serial dilutions onto YPD and 150 mM HEPES pH 8 media.
D. Nuclear localization of eGFP-Rim101 in the WT and Rra1-296T backgrounds in Rim pathway inactivating and activating conditions (pH 4 and 8 respectively). Cells were incubated and imaged as in 4a.
E. To compare the relative localization of the Rra1 C-terminus in various fractions of the cell, total lysates (T) from the GFP-CtRra1 strain, incubated for 1.5 h in either pH 4 or pH 8 YPD buffered with 150 mM HEPES, were subjected to ultracentrifugation (60,000 xg) to separate the soluble (S) and insoluble pellet (P) fractions of the cell. Samples were assessed by western blotting using an α-GFP antibody. White scale bars indicate 5 microns.
at pH 8 compared to pH 4. This observation was confirmed through quantification of the number of cells with membrane-associated puncta in the two different conditions (Fig. 4B). The reduced number of puncta observed at pH 8 correlated with a corresponding increase in less defined intracellular fluorescence signal, possibly consistent with alkaline pH-induced endocytosis of Rra1, as has been observed in S. cerevisiae with its Rim21 pH sensor (Nishino et al., 2015).

We treated the Rra1-GFP strain with Filipin III, a dye that binds membrane sterols and disrupts lipid rafts. As described for other yeast-like fungi, Filipin III displays a diffuse cell surface incorporation in stained cells with enrichment at sites of budding and cell separation (Figure S2) (Nichols et al., 2004; Siafakas et al., 2006; Mollinedo, 2012; Curto et al., 2014). In contrast to untreated controls, the Filipin III-treated cells display a marked reduction in the number of Rra1-GFP puncta at the cell surface (Fig. 4D, S2). Three dimensional reconstruction of Z-stacked images of cells pre-incubated at pH 4 confirmed the altered localization of Rra1-GFP puncta after Filipin III treatment (Fig. 4D). The Filipin III-dependent changes in Rra1 localization suggests that this pH-sensing protein resides in lipid-rich microdomains on the cell surface.

**The Rra1 C-terminus is necessary for pH-dependent localization**

The Rra1 protein is predicted to contain an intracellular C-terminal domain enriched in arginine and lysine residues (Fig. 5A and E). We investigated the role of the Rra1 C-terminus in protein function by creating a series of truncated and GFP-tagged versions of this protein, and by analyzing the effects of extracellular pH on their localization. The Rra1-296T-GFP protein was truncated after residue 296, and it lacks the majority of the C-terminal tail while conserving the highly charged region (HCR) (GFP-Rra1Ct) (Fig. 5A and E). We then assessed its localization using subcellular fractionation/western blot analysis to define the relative protein abundance in the soluble (cytoplasmic) and insoluble (membrane-associated) fractions of the cell at different pH conditions (Fig. 5E). With increasing pH, we documented a shift in localization of the Rra1 C-terminus away from the membrane. The GFP-Rra1Ct localized primarily to the insoluble cellular fractions when the strain was incubated at pH 4. In contrast, the GFP-Rra1Ct was present in both the insoluble and soluble fractions when the strain was incubated at pH 8 (Fig. 5E). These data confirm that the Rra1 C-terminal is absolutely required for protein function, the region after residue 296 may contain auto-regulatory or inhibitory domains.

Due to the potential importance of the C-terminal tail for Rra1 function and localization, we created a fusion protein of GFP and the Rra1 C-terminus, including the HCR (GFP-Rra1Ct) (Fig. 5A and E). We then assessed its localization using subcellular fractionation/western blot analysis to define the relative protein abundance in the soluble (cytoplasmic) and insoluble (membrane-associated) fractions of the cell at different pH conditions (Fig. 5E). With increasing pH, we documented a shift in localization of the Rra1 C-terminus away from the membrane. The GFP-Rra1Ct localized primarily to the insoluble cellular fractions when the strain was incubated at pH 4. In contrast, the GFP-Rra1Ct was present in both the insoluble and soluble fractions when the strain was incubated at pH 8 (Fig. 5E). These data confirm that the Rra1 C-terminal tail displays enhanced association with membranes at lower pH.

In conclusion, pH-dependent changes in the plasma membrane direct the degree of membrane association of the Rra1 C-terminus. These interactions in turn likely orchestrate Rra1 conformational changes, endocytosis, and other determinants of protein function in a pH-dependent manner. Distinct cellular processes that control plasma membrane composition may also contribute to the cellular pH response by mediating Rim pathway—independent events.
**Discussion**

*Mechanisms of extracellular pH-sensing*

This study explored the function of the newly identified *C. neoformans* Rra1 protein and confirmed that it changes its membrane localization as a function of pH. We have shown that the C-terminal tail of Rra1, including a region enriched for potentially charged residues such as arginine and lysine, is required for plasma membrane association at lower pH. We also demonstrated that this physical association with membranes decreases as the external environment becomes more alkaline. No obvious consensus sequences for post translational modifications, such as prenylation or myristoylation, exist in the ‘charged region’ of the Rra1 C-terminus. However, we propose that charged residues within the C-terminal HCR might transiently interact with the negatively charged phospholipids that are present within the inner leaflet of the lipid bilayer, a well-described membrane change that occurs in response to increases in pH (Hope et al., 1989; van Meer, 2011).

The connection between membrane phospholipid asymmetry and a transmembrane pH gradient has been most extensively studied in the context of artificial model membranes (Hope et al., 1989; van Meer, 2011). In these systems, pH changes alter the distribution of outer and inner membrane phospholipids. In response to altered extracellular ion concentrations, charged phospholipids, such as PS and PE, are asymmetrically redistributed to the inner leaflet. Phospholipid asymmetry between inner and outer membrane leaflets contributes to the maintenance of microdomains within the plasma membrane, as well as the localization, conformation and function of membrane proteins (Mollinedo, 2012). Therefore, pH-induced membrane changes would be predicted to functionally alter lipid-rich regions of membranes, such as lipid rafts, as well as their associated proteins.

Our studies support an emerging model in which pH-induced alterations in the plasma membrane might serve as a central signaling event to trigger the cellular response to pH (Fig. 6). The normal asymmetry between leaflets maintains Rra1 protein localization in sterol-rich domains of the PM, likely through charged-base interactions with the HCR of its C-terminal tail. As extracellular pH increases, transient pH-mediated membrane changes render the leaflets more symmetric with a less negatively-charged inner leaflet, perhaps repelling regions of the Rra1 C-terminus. These Rra1 protein conformational changes may in turn result in altered Rra1-protein interactions, altered association with the plasma membrane, and

![Fig. 6. Model of Rim pathway activation in response to external pH. The Rra1 protein is embedded in the plasma membrane through its core seven transmembrane domains. At pH 4, the plasma membrane is composed of asymmetrically distributed phospholipids, and the Rra1 C-terminus associates closely with the inner leaflet of the plasma membrane. At pH 8, the Rra1 C-terminus disassociates from the membrane in response to a transient loss of bilayer asymmetry (more symmetrical membrane portrayed to the right of the Cdc50 protein). The normal membrane asymmetry is actively restored by the Cdc50/Apt1 lipid flippase complex (restored asymmetrical membrane portrayed to the left of the Cdc50 protein). The differential membrane association of the Rra1 C-terminal tail results in pH-dependent activation of the Rim pathway and potential subsequent endocytosis of the pH sensor.](image-url)
possible endocytosis of the surface pH sensor. Energy-dependent processes, such as lipid flippase activity, help to restore normal membrane asymmetry and to maintain proper conformation and function of membrane proteins such as Rra1 (Fig. 6).

Our experimental results in C. neoformans (Cn) complement recent studies in signal initiation for the Rim pathway in S. cerevisiae (Sc). Similar to the Cn Rra1 protein, the Sc Rim21 pH sensor is a membrane protein with seven transmembrane domains (Ost et al., 2015). Also, its C-terminal tail contains multiple histidine residues that likely alter their charged states in response to fluxes in pH. Accordingly, this region appears to change its relative plasma membrane association in a pH-dependent manner (Nishino et al., 2015). In this way, despite very dissimilar primary amino acid sequences, the C-terminal tails of both Sc Rim21 and Cn Rra1 may function in a similar fashion, acting as ‘antennae’ that flip in and out of the membrane in response to changes in membrane dynamics and pH (Ikeda et al., 2008; Nishino et al., 2015). This antenna-like function may in turn change the conformation of the pH-sensing proteins, or the association of their C-termini with interacting proteins, affecting the downstream propagation of the pH signal.

We observed that Rra1 localizes most intensely in punctate structures on the cell surface at lower pH, and this localization becomes more intracellular both in response to higher pH and to the disruption of lipid raft domains using Filipin III. With either intervention, the microscopic images indicate fewer numbers of intensely fluorescent Rra1-containing puncta on the cell surface, as well as a suggestion of increased intracellular Rra1 localization. This observation is consistent with a model in which the C. neoformans Rim pathway pH-sensing complex might undergo endocytosis and potential degradation in response to an increase in pH. A similar mechanism of signal regulation has been proposed for the A. nidulans PalH pH sensor which becomes ubiquitinated and phosphorylated at increased pH (Herranz et al., 2005; Hervás-Aguilar et al., 2010; Selvig and Alspaugh, 2011). It was initially suggested that PalH ubiquitination and subsequent endocytosis led to enhanced intracellular interaction between the pH-sensing protein and the ESCRT machinery. These intracellular protein interactions were suggested to be the key events transmitting the alkaline response to the downstream elements of the A. nidulans Pal pathway (Hervás-Aguilar et al., 2010; Cornet and Gaillardin, 2014). This model was later suggested to be incomplete when subsequent fluorescent colocalization and genetic epistasis studies showed that ESCRT-mediated propagation of the pH signal occurs at the plasma membrane in both A. nidulans (Peňalva et al., 2014; Lucena-Agell et al., 2016) and S. cerevisiae (Obara and Kihara, 2014). Therefore, the cycling of pH-sensing proteins between the cell surface and intracellular sites is a conserved process among the divergent membrane proteins in diverse fungal species. However, the relative role of protein cycling in the propagation versus the dampening of Rim pathway activity remains to be precisely defined.

Our studies demonstrated a reduction in Cn Rra1 membrane-associated puncta at low pH in response to treatment with Filipin III, which is known to disrupt the formation of lipid rafts in the plasma membrane through its binding to sterol-rich regions (Curto et al., 2014). This observation suggests that Rra1 membrane association is dependent not only on external pH, but also on the presence of regions in the plasma membrane that are rich in sphingolipids and ergosterol. It is also likely that the Rim pathway itself contributes to the maintenance and organization of membranes. In C. albicans, the Rim101 transcription factor regulates the expression of IPT1, a gene involved in sphingolipid biosynthesis and therefore for lipid raft formation (Garnaud et al., 2018). Interestingly, the C. neoformans genome does not contain a clear IPT1 ortholog. However, our transcriptional studies identified several Rim101-regulated genes involved in phospholipid synthesis, such as a phospholipid synthase (CNAG_05813), a predicted phospholipid transporter (CNAG_04098), and a fatty acid ligase (CNAG_02449). Our transcriptomic analysis also demonstrated that the Rim101 transcription factor controls the expression of phosphatidylserine decarboxylase (CNAG_00834) that catalyzes the interconversion of PS to PE (Table S1). This finding suggests that Rim101 helps to regulate the balance between these two charged lipids, which in turn may contribute to Rim pathway activation.

In addition to suggesting mechanistic studies in the regulation of Rra1 protein function, our expanded inserational mutagenesis screen emphasized the importance of proper lipid flippase activity on cellular pH-sensing and the activation of the Rim pathway. In these studies we confirmed a recent series of observations that Cn Cdc50 protein function plays a prominent role in the dynamic maintenance of membrane integrity and survival at alkaline pH (Huang et al., 2016; Hu et al., 2017). These previous investigations demonstrated that the cdc50Δ mutant has a striking growth defect at elevated pH. Interestingly, in this mutant strain, a Rim101-GFP protein localized appropriately to the nucleus in response to alkaline growth conditions, suggesting intact Rim pathway activation and function. However, our studies indicated that the cdc50Δ mutant alkaline growth defect could be suppressed by constitutive activation of the Rim pathway, suggesting incomplete Rim signaling in the absence of the Cdc50 protein. These studies presented here offer a synthesis of these two observations, confirming that Rim101 processing is eventually accomplished in the cdc50Δ mutant.
but that activation, cleavage and nuclear localization of Rim101 are delayed in the absence of Cdc50 activity.

Rim pathway signaling and lipid flippase activity control nonredundant, but overlapping cellular functions

By defining the transcriptional profile of the respective mutants, we discovered that Rim101- and Cdc50-dependent cellular processes share a partial overlap. Additionally, much of this overlap is comprised of genes with predicted membrane-associated functions, such as a plasma membrane proton pump that has been shown to regulate pH homeostasis in other fungi (CNAG_03565) (Monk et al., 1991), a hypothetical protein with predicted function in the transport of membrane component precursors to the cell surface (CNAG_01354) (Stajich et al., 2012), and a long-chain fatty acid importer (CNAG_00651). Notably this overlap failed to contain all of the known Rim pathway outputs that were observed in previous studies (O’Meara et al., 2013) and were present in the Rim101-associated transcriptome in this study, such as SIT1, CDA2 and CIG1 (Table S1), which may explain the disparate mutant phenotypes that distinguish Cdc50 functions from those of other proteins more directly participating in the Rim pathway.

Additionally, the distinct transcriptional changes that are differentially regulated by Cdc50 and Rim101 might also explain the difference between the respective mutant virulence-associated phenotypes. Both cdc50Δ and rim101Δ mutant strains have a profound defect in growth at host-relevant pH. However, the cdc50Δ mutant strain is profoundly attenuated for virulence (Huang et al., 2016), and the rim101Δ mutant displays an unexpected increase in animal death during models of inhalational cryptococcosis (O’Meara et al., 2013). The paradoxical increased virulence of the rim101Δ strain is a reflection of failed immune avoidance of an otherwise unfit strain due to altered cell wall epitope exposure. The virulence effect of this strain is not due to prolonged survival, but rather due to its induction of an excessive immune response to the infecting strain (O’Meara et al., 2013; Ost et al., 2017). In contrast, the cdc50Δ mutant strain displays many other mutant phenotypes due to the distinct Cdc50-regulated transcriptome, likely tipping the balance of virulence toward more rapid pathogen clearance and away from immune activation.

Membrane composition effects on fungal susceptibility to pH

In this study we identified a pH-sensitive phenotype for mutants in components of the C. neoformans sterol homeostasis pathway (sre1Δ, stp1Δ) and ergosterol biosynthesis pathway (erg4Δ). Extensive work has explored the roles of both Sre1 and Stp1 in the context of hypoxia response and drug sensitivity in C. neoformans, S. cerevisiae, and A. fumigatus (Baumann et al., 2005; Chun et al., 2007; Chang et al., 2009; Bien and Espenshade, 2010; Florio et al., 2011; Chung et al., 2014; Kong et al., 2017). However, this pathway has not been robustly associated with pH-related cellular processes. Furthermore, the lack of tight association between Rim pathway activation and Sre1/Stp1 functions suggests that many diverse membrane lipid changes might result in similar susceptibilities to alkaline pH.

In contrast to the stable alterations of membrane composition known to occur in the sre1Δ, stp1Δ, and erg4Δ mutant strains, it appears that pH-induced membrane changes are transient and likely rapidly restored by energy-dependent processes such as flippase activity. We measured total membrane lipid content in the WT strain incubated for 1.5 hours in very distinct growth conditions. Despite differences in temperature and pH, we did not detect stable alterations of plasma membrane lipids in strains grown in very different environments. Therefore, any membrane changes occurring in response to external pH are likely transient changes in phospholipid organization rather than major, stable changes in lipid content.

The precise delineation of the phospholipid content of individual membrane leaflets remains a technical challenge. The measurement of lipid bilayer asymmetry has been assessed best in isolated membrane systems rather than whole microbial cells. Additionally, our indirect analysis of membrane asymmetry (e.g., cinnamycin susceptibility) was best able to capture the stable and unrestored membrane leaflet changes of the cdc50Δ mutant strain, as opposed to the expected transient changes in leaflet asymmetry known to occur in response to elevated pH (Hope et al., 1989). However, we were able to demonstrate the effect of pH-induced membrane changes and the resulting altered membrane association of the functional C-terminal domain of the Rra1 pH sensor.

We therefore conclude that these data support models in which the basidiomycete and ascomycete fungal phyla have independently developed functionally similar ways of sensing and responding to changes in extracellular pH. This is evident in the phylum-specific pH sensors, represented by C. neoformans Rra1 and S. cerevisiae Rim21, that similarly interact with the membrane in response to fluxes in environmental pH. This phenomenon further suggests that there are additional basidiomycete-specific, upstream components of the pH-sensing complex in this pathway, and that the detailed mechanism of Rim-dependent pH-sensing in C. neoformans might thereby be distinct from that of model ascomycetes such as S. cerevisiae. Future studies will certainly define novel basidiomycete genes and cellular processes involved in environmental stress sensing and response in this large, diverse fungal phylum.
Experimental procedures

Strains, media, and growth conditions

Strains utilized throughout this study are shown in Table 2. Unless otherwise stated, each mutant and fluorescent strain was generated in the *C. neoformans* H99 MATα genetic background and incubated in YPD (1% yeast extract, 2% peptone and 2% dextrose). YP-Gal media contained 1% yeast extract, 2% peptone and 3% galactose. The pH 4 and pH 8 media was made by adding 150 mM HEPES buffer to YPD or YP-Gal liquid media, adjusting the pH with concentrated HCl (for pH 4) or NaOH (for pH 8.15), prior to autoclaving.

To generate the *sre1Δ* and eGFP-Rim101 + cdc50Δ deletion and tagged deletion constructs respectively, we performed the previously described double-joint PCR with split drug resistance marker method to rapidly make targeted gene deletions (Kim et al., 2009). In brief, we generated the following two PCR products: 1 kb of the 5′ flanking region of the target locus with a truncated drug resistance cassette and the remainder of the drug resistance cassette with 1 kb of the 3′ flanking region of the target locus. We then biolistically transformed these amplicons into either the wild-type *C. neoformans* strain (H99) or the *C. neoformans* strain that contains endogenously expressed GFP-Rim101 (Toffaletti et al., 1993). The primers used to generate each mutant strain and fluorescent strain are listed in Table 3. Transformants were selected on YPD medium containing nourseothricin (NAT).

To generate all strains containing the GFP-tagged Rra1 and Rra1 truncations, the *pKS50* (pHIS3-RRA1-GFP-NAT), *pHS3-RRA1-296T-GFP-NAT* and *pHS3-RRA1-273T-GFP-NAT-NAT* plasmids were biolistically transformed into the *rra1Δ::NEO* full knockout strain. To generate the GFP-CtRra1 tagged strain, the *pKS50* (pHIS3-RRA1-GFP-NAT) was biolistically transformed into the H99, WT strain. All plasmids, including the *pKP18* plasmid were made using In-Fusion cloning (Clontech) with the primers listed in Table 2.

Insertional mutagenesis and mutant assessment

In order to induce large scale random mutagenesis, an *Agrobacterium tumefaciens* strain that expresses the Neomycin (NEO) resistance marker was incubated along with a *C. neoformans* strain encoding the truncated and active form of the most downstream component of the Rim pathway (GFP-RIM101T) under the galactose-inducible

| Strain  | Genotype                                      | Source                  |
|---------|----------------------------------------------|-------------------------|
| H99     | MATα                                         | (Perfect et al., 1980)  |
| TOC2    | rim101Δ::NAT                                  | (O’Meara et al., 2010) |
| KS161   | H99 + pTO22 (Gal7-GFP-Rim101T NAT) MATα       | (Ost et al., 2015)      |
| KS310   | ma1Δ::NEO + pKS85 (pHIS3-RRA1-GFP-NAT) MATα    | This study              |
| KS338   | ma1Δ::NEO + pHIS3-RRA1-296T-GFP-NAT MATα       | This study              |
| KS340   | ma1Δ::NEO + pHIS3-RRA1-273T-GFP-NAT MATα       | This study              |
| KS342   | ma1Δ::NEO + pHIS3-RRA1-296T-4FLAG-NAT X KS208 MATα | This study              |
| KS234   | H99 + pKS50 (pHIS3-GFP-RRA1 C-terminus) MATα   | This study              |
| KS336   | ma1Δ::NEO MATα                                 | (Ost et al., 2015)      |
| CUX196  | cdc50Δ::NEO MATα                              | (Huang et al., 2016)   |
| HEB21   | cdc50Δ::NEO + pKP18 (pGal7-GFP-Rim101T NAT) MATα | This study              |
| HEB25   | sre1Δ::NEO MATα                                | (Liu et al., 2008)     |
| HEB15   | sre1Δ::NEO + pKP18 (pGal7-GFP-Rim10170T NAT) MATα | This study              |
| HM19-B12a | apt1Δ::NAT MATα                              | (Liu et al., 2008)     |
| HM1-G11a | sp1Δ::NAT MATα                                | (Liu et al., 2008)     |
| HM5-F6a | erg4Δ::NAT MATα                               | (Liu et al., 2008)     |
| TOC106  | eGFP-Rim101 MATα                               | (O’Meara et al., 2014) |
| KS208   | eGFP-Rim101 MATα                               | (Ost et al., 2015)     |
| HEB46   | eGFP-Rim101(TOC106) + cdc50Δ::NEO MATα         | This study              |
| KS351   | cdc50Δ::NEO + GFP-CtRra1                      | This study              |

*aStrains obtained from the 2016 Madhani plates. Designated HM.#-xx for plate number (#) and well (xx).
GAL7 promoter (Ost et al., 2015). Through A. tumefaciens insertional mutagenesis, we generated 10,000 random mutants. Insertional mutants were tested for rim101Δ-like phenotypes, including sensitivity to alkaline pH and 1.5 M NaCl in the presence of glucose-containing medium (repressing conditions for GFP-RIM101T expression) that are rescued in the presence of galactose-containing medium (inducing conditions for GFP-RIM101T expression), indicating that we were effectively identifying upstream components in the Rim pathway. We screened through these 10,000 mutants using a BM3-bench top robot to increase the throughput. The robot allowed us to cleanly pin 2,000 mutant strains in quadruplicate per day and take quality images that can be analyzed for growth following 3 days of incubation at 30°C. The robot pinned these strains to agar plates containing glucose (YPD), agar plates containing glucose at pH 8 (YPD pH 8), and agar plates containing galactose at pH 8 (YPGal pH 8). Mutants were selected for further prioritization based on reduced growth on YPD pH 8 and rescued growth on YPGal pH 8 as compared to a negative control (WT H99) and a positive control (Rim pathway mutant strain with a constitutively active Rim101T under the GAL7 promoter).

This first pass screening technique identified 94 mutant strains generated from the random insertional mutagenesis that had reduced growth at alkaline pH that was rescued by the expression of the active, truncated form of Rim101. We then validated these 94 mutants by re-plating them onto the same media (YPD pH 8 and YPGal pH 8) and onto YPD or YPGal media alone (to control for the varied carbon sources).
source) or medium containing 1.5 M NaCl, as an additional phenotypic test. This second pass screening technique identified 36 mutant strains for further consideration and analysis. We submitted pooled genomic DNA to the Duke sequencing core for whole-genome sequencing using Illumina MiSeq. To identify the genomic location of the insertions in these mutants, we used the AIM-Seq bioinformatics pipeline that allowed us to rapidly identify genomic sites of insertion in a high throughput manner (Esher et al., 2015). AIM-Seq revealed 27 unique sites of genomic integration.

**Lipidomics analysis**

WT strains were incubated in 50 mL YPD overnight (~18 h) at 30°C with 150 rpm shaking. These cultures were used to seed 250 mL YPD cultures in a 1 L flask and allowed to incubate overnight (~18 h) at 30°C with 150 rpm shaking. Cells were pelleted, normalized to an OD 600 of 3, and incubated in either YPD or CO2-independent media for 1.5 h at either 30°C or 37°C respectively. Cells were pelleted and washed once with dH2O. Samples were then resuspended in 1 mL ice-cold ammonium bicarbonate (50 mM). Samples were pelleted again and the ammonium bicarbonate was removed. Pellets were flash frozen and lysed. Lysis was performed by bead beating (0.5 mL of 3 μM glass beads in a Mini-BeadBeater-16 (BioSpec), 6 cycles of 2 min each with a one-minute ice incubation between bead-beating cycle for cell recovery). Supernatants were transferred to new tubes and washed 3 times with .4 mL of Phosphate Buffered Saline (PBS). Samples were prepared in triplicate. Samples were allowed to settle, and then pelleted at 13,000 ×g for 10 min to pellet the whole lysate. Supernatant, mostly containing PBS, was removed. Whole lysate pellets were resuspended in 800 μL PBS and stored at −80°C until extraction and analysis.

Lipid extraction was performed using an acidic Bligh Dyer method (xx, yy). Specifically, half (0.4 mL) of each above lysate solution was transferred into a 17-mL glass tube (with a Teflon-lined cap), to which 1 mL of chloroform, 2 mL of methanol and 0.4 mL of PBS were added to create a single-phase solution consisting of chloroform/methanol/PBS (1:2:0.8, v/v/v). This solution was incubated for 20 min at room temperature with intermittent mixing. After centrifugation at 3000 ×g for 10 min at room temperature, the supernatant was transferred to a fresh 17-mL glass tube, followed by the addition of 50 μL of concentrated HCl (37%) to acidify the solution. Afterwards 1 mL of chloroform and 1 mL of PBS were added to convert the single phase into a two-phase solution consisting of chloroform/methanol/PBS (2:2:1.8, v/v/v). After centrifugation at 3000 ×g for 10 min at room temperature, the lower phase was recovered and dried under a stream of nitrogen. The dried lipid extract was re-suspended in chloroform/methanol (2:1, v/v) before LC/MS analysis.

Normal phase LC-ESI MS of the lipid extracts was performed using an Agilent 1200 Quaternary LC system coupled to a high resolution TripleTOF5600 mass spectrometer (Sciex, Framingham, MA). Chromatographic separation was performed on an Ascentis Silica HPLC column, 5 μm, 25 cm × 2.1 mm (Sigma-Aldrich, St. Louis, MO). Elution was achieved with mobile phase A, consisting of chloroform/methanol/aqueous ammonium hydroxide (800:195:5, v/v/v), mobile phase B, consisting of chloroform/methanol/water/aqueous ammonium hydroxide (600:340:50:5, v/v/v/v) and mobile phase C, consisting of chloroform/methanol/water/aqueous ammonium hydroxide (450:450:95:5, v/v/v/v), over a 40 min long run, performed as follows: 100% mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 11 min. The mobile phase composition was then changed to 100% mobile phase C over 3 min and held at 100% C for 3 min, and finally returned to 100% A over 0.5 min and held at 100% A for 5 min. The LC eluent (with a total flow rate of 300 μl/min) was introduced into the ESI source of the high resolution TF5600 mass spectrometer, with MS settings as follows: Ion spray voltage (IS) = −4500 V (negative ion mode) or +5000V (positive ion mode), Curtain gas (CUR) = 20 psi, Ion source gas 1 (GS1) = 20 psi, De-clustering potential (DP) = 55 V, and Focusing Potential (FP) = 150 V. Samples were analyzed in negative-ion mode, with the full-scan spectra being collected in the m/z 300–2000 range. Nitrogen was used as the collision gas (collision energy = 40 eV) for tandem mass spectrometry (MS/MS) experiments.

**RNA-Sequencing preparation and analysis**

Three biological replicates of WT, rim101Δ and cdc50Δ were incubated overnight (~18 h) at 30°C with 150 rpm shaking in YPD media. Cells were pelleted and resuspended in CO2-independent media and incubated for 1.5 h at 37°C with 150 rpm shaking. Cells were then pelleted, pellets were flash frozen on dry ice, and lyophilized overnight. RNA was extracted using the Qiagen RNeasy Plant Minikit with optional on column DNase digestion (Qiagen, Valencia, CA).

RNA samples were submitted to the Duke Sequencing and Genomic Technologies Shared Resource for library preparation and RNA sequencing. Sequencing was carried out on an Illumina HiSeq 4000 instrument with 50 bp single end reads. All raw and analyzed data has been submitted to the NCBI GEO database (GSE110723).

Alignment and differential expression analysis were performed following an RNA-Seq Bioconductor workflow in R (Love et al., 2015; Team, 2017). Reads were mapped to the C. neoformans reference genome (obtained from
NCBI, accessed July 2017) using STAR alignment software (Dobin et al., 2013). Differential gene expression analysis was performed using the DESeq2 package for R with a false discovery rate (FDR) of 10% (Love et al., 2014). Genes were considered statistically differentially expressed if they had an adjusted p-value < 0.05.

Venn diagrams were generated in R using the Vennerable package (Swinton, 2009) and Gene IDs (CNAG number) as inputs.

A modified GO term analysis using the FungiDB database was performed to identify genes that were significantly regulated in a given process as previously reported (Brandão et al., 2018). A list of 675 C. neoformans H99 genes with membrane-associated functions (using the search term ‘membrane’) was generated and compared with the significantly differentially regulated genes for each strain. This list generated was based on Interpro protein product descriptions, user comments, PubMed citations and phenotypic data included in the FungiDB database. This list was compared to the significantly differentially regulated genes identified by RNA-Seq. The proportion of membrane-associated genes that were differentially expressed in each strain was calculated and enrichment was determined by a Chi-square test with a 5% expected frequency.

The shared differentially expressed membrane-associated genes between cdc50Δ and rim101Δ were plotted on a heatmap to visualize the expression of these transcripts. Heatmap generation and hierarchical clustering was performed in R using the Pretty Heatmap package (Kolde, 2015). Gene IDs (CNAG number) and associated log2 fold changes were used as inputs. A complete list of the RNA-Seq datasets containing differentially expressed genes in each strain can be found in Table S1.

RNA extraction and quantitative real time PCR

Three biological replicates of WT, rra1Δ, rra1Δ +Rra1-GFP and rra1Δ +Rra1-273T-GFP were prepared, RNA-extracted and submitted for RNA-sequencing. Strains were incubated overnight (~18 h) at 30°C with 150 rpm shaking in YPD media. Cells were pelleted and resuspended in CO2-independent media and incubated for 1.5 h at 37°C with 150 rpm shaking. Cells were then pelleted, pellets were flash frozen on dry ice, and lyophilized overnight. RNA was extracted using the Qiagen RNeasy Plant Minikit with optional on column DNase digestion (Qiagen, Valencia, CA). cDNA was prepared by reverse-transcriptase PCR using the AffinityScript cDNA QPCR Synthesis kit (Agilent Technologies) according to the manufacturer’s protocol, using the oligo dT primers to bias for mRNA. qRT-PCR reactions were performed as previously described (Cramer et al., 2006) using the primers listed in Table 3.

Assessment of plasma membrane asymmetry

Annexin V staining demonstrated inconsistent and insensitive staining patterns that could not reproduce or differentiate between strains with known plasma membrane defects in asymmetry. Therefore, cinnamycin susceptibility was used to analyze lipid asymmetry in the membrane of WT strains exposed to varied extracellular pH. WT strains were incubated in YPD overnight (~18 h) at 30°C with 150 rpm shaking. Cells were normalized to an OD 600 of 0.25 and a 1:100 dilution was made of the cell suspension in either YPD pH 4 or pH 8. Cinnamycin (1 mg Santa Cruz Biotechnology, Inc) was resuspended in ethanol and diluted in either pH 4 or pH 8 YPD to reach desired concentration. Minimum Inhibitory Concentrations (MICs) was measured after 48 h of growth at 30°C. Three replicates of the experiment were performed for WT and cdc50Δ at pH 4 and pH 8.

Microscopy

To analyze Rra1-GFP, Rra1-296T-GFP, Rra1-273T-GFP and GFP-CtRra1 localization, strains were incubated overnight (~18 h) at 30°C with 150 rpm shaking in pH 4 Synthetic Complete media buffered with McIlvaine’s buffer (SC). Cells were then pelleted and resuspended in either pH 4 or pH 8 YPD media. Strains were shaken at 150 rpm, 30°C for 60 min. The high-resolution fluorescent images of Rra1-GFP were captured using a Delta Vision Elite deconvolution microscope equipped with a Coolsnaph Q2 high resolution charge-coupled-device (CCD) camera. All other differential interference (DIC) and fluorescent images were captured using a Zeiss Axio Imager A1 fluorescence microscope equipped with an Axio- Cam MRM digital camera. Puncta per cell was quantified using ImageJ Software (Fiji) software (Schindelin et al., 2012) and a blinded identification of cells with membrane associated puncta in each condition. Approximately 600 cells per condition/strain were analyzed in this way.

To analyze localization patterns of Rra1-GFP with Filipin III, cells were incubated in 25 mL YPD cultures overnight shaking at 150 rpm at 30°C. A 5 mL volume of the cells was then pelleted and washed with PBS. Cells were pelleted again and resuspended in either 1 mL PBS + 5 μL Filipin III (2 mg/mL from Cayman Chemical) or PBS alone. Cells were incubated at room temperature in the dark for 10 min. Cells were then spun slowly (5000 rpm) for 2 min, washed with 500 μL PBS, spun at 5000 rpm for 2 min again and resuspended in 200 μL PBS. Cells were
imaged immediately using a Zeiss Axio Imager A1 fluorescence microscope equipped with an Axio-Cam MRM digital camera. The high-resolution fluorescent images of Rra1-GFP both treated and untreated with Filipin III were captured using a Delta Vision Elite deconvolution microscope equipped with a CoolSnap HQ2 high resolution charge-coupled-device (CCD) camera.

To analyze GFP-Rim101 localization, strains were incubated overnight (~18 h) at 30°C with 150 rpm shaking. Cells were then pelleted and resuspended in either pH 4 or pH 8 Synthetic Complete media buffered with McIlvaine’s buffer. Strains were shaken at 150 rpm, 30°C for either 5, 10, 20, 30 or 60 min. Nuclear staining was assessed by applying NucBlue directly to the sample slide and incubated in the dark for 30 min before imaging. Differential interference (DIC) and fluorescent images were captured using a Zeiss Axio Imager A1 fluorescence microscope equipped with an Axio-Cam MRM digital camera. Fluorescence intensity plots were created using ImageJ software (Fiji) (Schindelin et al., 2012).

Protein extraction, membrane fractionation and western blot

Protein extracts were prepared as in a similar manner to what was previously described (Ost et al., 2015). Briefly, strains were incubated overnight (~18 h) at 30°C with 150 rpm shaking in YPD media buffered to pH 4. Cells were then pelleted and resuspended in YPD media buffered to pH 7 with NaOH. These cells were incubated for 60 min and immediately pelleted and flash frozen. Cells were then lysed using 0.4 mL lysis buffer containing 2x protease inhibitors (Complete, Mini, EDTA-free; Roche), 1x phosphatase inhibitors (PhosStop; Roche) and 1 mM phenylmethanesulfonyl-fluoride (PMSF). Lysis buffer was buffered to either pH 4 or 8 using Tris buffer. Lysis was performed as described above. Lysates were transferred to new tubes, washed 3 times with 0.4 mL of appropriately buffered lysis buffer, and the crude pellet was collected by centrifugation at 15,000 rpm, 4°C, for 5 min. The supernatant (crude lysate) was transferred to a new tube, an aliquot was collected from this supernatant and set aside. To separate the soluble from the insoluble, crude lysates were separated by ultracentrifugation (60,000 xg) 1 h at 4°C. The soluble fraction was transferred to a new tube and the insoluble pellet was resuspended in the equivalent volume of appropriately buffered lysis buffer containing 1% Triton X-100. All samples were normalized to the total protein concentration in the crude lysate using bicinchoninic acid assay (BCA) and western blots were performed as described above.

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Author contributions

HEB, KSO, SKE, ZG and JAA were involved with the conception and design of experiments and the writing process. HEB, KSO, SKE, KMP, JWS and ZG were involved in the acquisition, analysis and interpretation of the data.

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