Characterization of additional components of the environmental pH-sensing complex in the pathogenic fungus Cryptococcus neoformans

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ABSTRACT

Pathogenic microorganisms must adapt to changes in their immediate surroundings, including alterations in pH, to survive the shift from the external environment to that of the infected host. In the basidiomycete fungal pathogen Cryptococcus neoformans, these pH changes are primarily sensed by the fungal-specific, alkaline pH-sensing Rim/Pal pathway. The C. neoformans Rim pathway has diverged significantly from that described in ascomycete fungi. We recently identified the C. neoformans putative pH sensor Rra1, which activates the Rim pathway in response to elevated pH. In this study, we probed the function of Rra1 by analyzing its cellular localization and performing protein co-immunoprecipitation to identify potential Rra1 interactors. We found that Rra1 does not strongly colocalize or interact with immediate downstream Rim pathway components. However, these experiments identified a novel Rra1 interactor, the previously uncharacterized C. neoformans nucleosome assembly protein 1 (Nap1), which was required for Rim pathway activation. We observed that Nap1 specifically binds to the C-terminal tail of the Rra1 sensor, likely promoting Rra1 protein stability. This function of Nap1 is conserved in fungi closely related to C. neoformans that contain Rra1 orthologs, but not in the more distantly-related ascomycete fungus Saccharomyces cerevisiae. In conclusion, our findings have revealed the sophisticated, yet distinct, molecular mechanisms by which closely and distantly related microbial phyla rapidly adapt to environmental signals and changes such as alterations in pH.

As any cell transitions from one environment to another, it must adapt to variations in its extracellular conditions. Specifically, microbial pathogens must rapidly sense and adapt to the many changes in environment that are encountered upon entering a host. For example, a human host might present many extreme stresses relating to the external environment for a pathogen, including an elevated temperature, alterations in pH, limited nutrient availability, and other stresses such as those associated with an activated immune response. The pathogen must respond to these many stressful conditions in order to survive in the host.

One of the most important ways in which fungi sense their external environment is through the Rim/Pal pH response pathway. This fungal-specific pathway is involved in cellular signaling in response to alkaline pH, ultimately regulating gene expression in order to adapt to this environmental stressor. This pathway is required for survival at elevated pH across many fungal species (1–3). First identified in Saccharomyces cerevisiae and Aspergillus nidulans, the Rim/Pal pathway, respectively, was further explored for roles in fungal pathogens, such as Candida albicans,
Cryptococcus neoformans, and A. fumigatus (4–8). In these pathogenic organisms, this alkaline pH-activated signaling pathway controls the expression of many genes required for full virulence (1, 9, 10). Indeed, many pathogenic species seem to have co-opted this pathway for use as a sensor of host conditions. For example, as it transitions to the neutral to alkaline pH of the host, the human fungal pathogen C. albicans requires its Rim signaling pathway to activate genes involved in the yeast-hyphal transition, a process required for tissue invasion (2, 6). This alkaline response pathway is also required for tissue invasion and pathogenesis of the filamentous opportunistic fungus, A. fumigatus (8).

The yeast-like fungus C. neoformans is a neuropathogen in immunocompromised hosts, and the RIM101 transcription factor gene is one of the most highly induced genes in the setting of cryptococcal infection (11). Interestingly, C. neoformans Rim pathway signaling is required for full expression of the most important cell feature associated with Cryptococcus pathogenesis, the polysaccharide capsule (7). Together, these observations underscore the importance for microbial pathogens to correctly interpret host signals, such as elevated pH, and translate these signals to adaptive microbial responses.

The Rim pathway has been most extensively studied in fungi from the Ascomycete phylum, including S. cerevisiae, C. albicans, A. nidulans, and A. fumigatus (12–14). In more distantly related fungi, such as the basidiomycete fungus C. neoformans, many of the Rim pathway signaling elements are conserved, including the Endosomal Sorting Complex Required for Transport (ESCRT) machinery which acts a scaffold for Rim pathway proteins during pathway activation, the Rim proteolysis complex (Rim13, Rim20, Rim23), and the Rim101 transcription factor (7, 15). These Rim pathway effectors not only represent homologs by sequence, but these proteins also function within the Rim pathway to transduce signals in response to alkaline pH. However, many orthologs of the upstream signaling components of the Rim pathway, including the pH sensor, are not present in the genomes of most basidiomycetes as assessed by direct sequence similarity.

Previously, we identified the Rra1 protein as a putative pH sensor required for activation of the C. neoformans Rim pathway (15). Like the canonical Rim pathway pH sensor, Rra1 has seven predicted transmembrane domains and functions upstream of the Rim101 processing complex, including the ESCRT and Rim proteolysis complexes. Additionally, though no basidiomycetous fungi appear to have homologs of the Rim21 pH sensor, many—such as Cryptococcus gattii, Ustilago maydis, and Tremella mesenterica—have Rra1 homologs (15). While Rra1 may share structural similarities with the canonical Rim pathway pH sensor, it is unclear whether it activates downstream Rim pathway components in a similar manner. In ascomycetes, the Rim21/PalH pH sensor directly interacts with downstream Rim pathway components to induce Rim101/PacC processing complex assembly at the plasma membrane (13, 14, 16, 17). Additionally, we functionally placed the C. neoformans Rra1 protein within the Rim pathway as the most upstream component yet identified. However, efforts to identify homologs of other upstream effectors of the Rim pathway in C. neoformans through purely genetic means have not yet revealed other potential components of the Rim membrane sensing complex, including the Rim8/PalF arrestin-like protein that bridges the interaction between the pH sensor and the Rim proteolysis complex in ascomycetes (15, 18, 19). Therefore, it is still unclear how Rra1 functions in the C. neoformans Rim pathway.

In this work, we searched for novel upstream regulators of the Rim pathway in C. neoformans through the identification of specific interactors of the Rra1 pH sensor. We employed proteomic methods to identify potential Rra1 interactors, subsequently using genetic techniques to determine whether these potential interactors regulated the activation of the Rim pathway in C. neoformans. We functionally placed one of these identified interactors, nucleosome assembly protein 1 (Nap1), in the Rim pathway as a Rra1 signaling partner. Based on our evidence, we propose that C. neoformans Nap1 displays a novel, basidiomycete-specific function in Rim pathway activation through its interaction with the Rra1 pH sensor.

Results

Rra1, an upstream regulator of the Rim pathway, localizes to punctate structures at the plasma membrane
We previously identified a seven-transmembrane domain-containing protein, Rra1 (Required for Rim101 Activation 1), that acts upstream of the *C. neoformans* Rim pathway proteolysis complex (15). To determine if this Rra1 protein might serve as a sensor of extracellular pH, we created a Rra1-GFP fusion protein to assess its subcellular localization. This pHIS3-RRA1-GFP fusion protein construct consists of the RRA1 gene fused to GFP under control of the constitutively-active Histone H3 promoter. The pHIS3-RRA1-GFP construct was expressed in the rra1Δ mutant and shown to be functional, effectively rescuing the phenotypic defects demonstrated by the rra1Δ mutant strain, such as deficient growth on YPD pH8 and YPD + 1.5 M NaCl (Fig. S1A). When incubated at pH 4 or pH 8, the Rra1-GFP protein localized diffusely within endomembrane-like structures. This endomembrane pattern of fluorescence resembles perinuclear ER localization, which can be visualized by the ER marker Sec63 (Fig. 1A, B). Strikingly, the Rra1-GFP protein localized most intensely in puncta on the cell surface, particularly at pH 4 (Fig. 1A). These puncta are present near the plasma membrane regardless of pH. However, upon pathway activation at pH 8, these cell surface puncta appear to decrease in number and/or intensity (Fig. 1A). These data suggest that Rra1-GFP is located and is potentially functioning at the cell surface. Moreover, the Rra1-GFP fusion protein might be endocytosed or degraded upon pathway activation.

The *C. neoformans* Rim pathway proteolysis complex consists of the ESCRT machinery (as a membrane-bound scaffolding component) and the Rim pathway-specific proteolysis complex components Rim13, Rim20, and Rim23 (15). To probe the spatial relationship between the Rra1 protein and other Rim pathway components, we expressed the Rra1-GFP construct in a strain expressing the ESCRT machinery component Vps23 tagged with mCherry (pHIS3-mCherry-VPS23). When visualized using Z-stacked microscopic images, mCherry-Vps23 localizes to large, rare punctate structures within the cytosol, with most cells displaying only one of these structures (Fig. 1C). This strikingly focal, punctate pattern of mCherry-Vps23 fluorescence at pH 4 became more diffuse after a shift to pH 8, but it retained its cytosolic location (Fig 1C). Although the Rra1-GFP signal also included distinct punctate structures, the Rra1-GFP puncta were present at the cell surface as opposed to the cytosol. Additionally, we did not observe colocalization of Rra1-GFP with the mCherry-Vps23 structures when incubated in either pathway-inactivating (pH 4) or pathway-activating (pH 8) conditions (Fig. 1C, S1B). We also expressed a Rra1-mCherry construct in a strain expressing the Rim proteolysis complex component Rim23 tagged with GFP (eRIM23-GFP). Rim23-GFP forms puncta at the cell surface at pH 8 (pathway-activating conditions), similar to the Rra1-GFP strain. However, we did not observe consistent colocalization between the Rra1- and Rim23-containing puncta at pathway-activating pH 8 (Fig. 1D). These results suggest that Rra1 primarily localizes in specific cellular sites that are spatially separated from more downstream Rim pathway components. Moreover, the Rra1 protein may be activating the Rim pathway without directly interacting with the ESCRT or Rim pathway proteolysis complexes.

**Identification of potential Rra1 interacting proteins**

Our prior genetic studies to identify upstream activators of the Rim pathway revealed that Rra1 is required for Rim pathway activation. However, functional orthologs of additional upstream activators of the pathway in other species, including the Rim8/PalF arrestin and Rim9/PalI membrane protein, were not identified in *C. neoformans* using either genetic homology or genetic screens for Rim pathway activators (15). We therefore sought to identify upstream activators of the Rim pathway by characterizing proteins that interact with Rra1 using co-immunoprecipitation techniques. We specifically sought potential Rra1-interacting proteins under pathway-activating (alkaline) pH conditions. The Rra1-GFP protein was immunoprecipitated from cell lysates using a GFP-Trap resin, and the proteins isolated in this manner were analyzed using tandem MS-MS.

To exclude potential false-positive interactions, we focused on those proteins with at least 3 exclusive peptides that were present only in the Rra1-GFP sample and not in the control condition (Table 1). A full list of the potential interactors can be found in Table S1. This process yielded 41 proteins that were enriched in the Rra1-GFP sample. Of these, 25 represented proteins that

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were not ribosomal components or that were not highly expressed predicted members of metabolic pathways.

Consistent with the above colocalization experiments, none of the ESCRT proteins nor any other known Rim pathway components were represented among the putative Rra1-GFP interactors. This observation further corroborates the hypothesis that there is spatial separation between the Rra1-containing pH-sensing complex and the more downstream Rim pathway proteolysis complex in C. neoformans.

Of the remaining candidate Rra1 interactors, the most highly represented protein was C. neoformans nucleosome assembly protein 1 (Nap1), with 18 exclusive unique peptides identified by tandem MS. To confirm the Rra1-Nap1 interaction implied by the proteomics data, we performed co-immunoprecipitation (co-IP) experiments using strains expressing Rra1-GFP (15). We immunoprecipitated Rra1-GFP using GFP-Trap beads as described above (Fig. 2). For western blot analysis of these samples, we used an antibody directed against Saccharomyces cerevisiae Nap1. In our western blots, we found that this antibody recognized two bands of appropriate sizes in C. neoformans. Importantly, these bands are absent in nap1Δ mutant strains, confirming that this antibody is able to specifically detect the C. neoformans Nap1 protein. To determine whether Nap1 interacts with downstream Rim pathway components, we repeated this process using a strain expressing Rim23-GFP. These results verified our MS data, showing that Nap1 co-immunoprecipitates with Rra1-GFP, but not with Rim23-GFP, confirming a specific interaction between Nap1 and Rra1. (Fig. 2).

**Nap1 localizes strongly to the cell cytosol**

In order to further characterize the function of the Nap1 protein, we created a fusion protein expression construct in which Nap1 was tagged with GFP at its C-terminus. When this construct was expressed in the nap1Δ mutant strain, all nap1Δ phenotypes were rescued (Fig. S1C), indicating that the addition of the GFP tag did not significantly affect Nap1 function. We assessed the localization of Nap1-GFP at both pH 4 and pH 8. Like S. cerevisiae Nap1, C. neoformans Nap1-GFP appears to localize largely within the cytosol and displays similar localization at both pH 4 and pH 8 (Figure 3A) (20). Nap1-GFP is excluded from cytosolic vesicles, as well as being present at much lower levels in the nucleus and large vacuoles (Fig. 3B). Unlike the Rra1-GFP protein, Nap1-GFP is not isolated to membranes. These data suggest that Nap1 is largely localized and functioning in the cytosol.

In order to assess potential sites of Rra1 and Nap1 interaction, we created a strain expressing both the Rra1-GFP construct described above and a C-terminally tagged Nap1-mCherry expression construct. We assessed the localization of Rra1-GFP and Nap1-mCherry by capturing Z-stacked images (Fig. 3C). We did not observe significant colocalization of Rra1-GFP cell surface puncta and Nap1-mCherry in either inactivating (pH 4) or activating conditions (pH 8). However, on rare occasions, we did observe some colocalization between intracelluar Rra1-GFP punctate structures and Nap1. These results suggest that Rra1 and Nap1 interact most intensely when Rra1 is present on endomembranous structures or cytoplasmic puncta rather than at the plasma membrane, or at more diffuse subcellular sites that are not appreciated by these microscopy methods.

**Characterization of the role of NAP1 in Rim pathway-dependent and -independent phenotypes.**

To determine whether the C. neoformans Nap1 protein is involved in Rim pathway activation, we created a strain with a complete deletion of the NAP1 gene (CNAG_02091). The resulting nap1Δ mutant strain demonstrated a slow-growth phenotype under otherwise permissive conditions—rich medium (YPD) at 30°C (Fig. 4A). The nap1Δ strain grows even more slowly compared to the wild-type (WT) at 37°C on YPD (Fig. 4A). This growth impairment is not shared by strains with mutations in established Rim pathway components.

We also tested the nap1Δ mutant for phenotypes characteristic of Rim pathway mutants. Consistent with other Rim pathway mutants, the nap1Δ strain failed to grow at alkaline pH and on high salt medium (Fig. 4A). This growth defect was not ameliorated with extended growth time to accommodate the slow growth of the nap1Δ mutant strain. Importantly, like other Rim pathway mutants, the alkaline pH growth defect in the nap1Δ mutant strain was suppressed by the expression of a truncated, constitutively-active form of the
Rim101 transcription factor (GFP-Rim101T) (Fig. 4B) (15). The truncated GFP-Rim101T protein mimics the cleaved and activated form of Rim101, the downstream target protein of Rim pathway signaling. Here, we conditionally expressed GFP-Rim101T under the control of the galactose-inducible GAL7 promoter. The pH-specific growth defect of the nap1Δ strain was partially suppressed on media containing galactose (GFP-Rim101T inducing condition), but growth was not restored on media containing glucose (GFP-Rim101T non-inducing condition) (Fig. 4B), similar to the effect observed for other Rim pathway mutants (15). Importantly, the more general nap1Δ mutant growth defect that is not affected by pH was not rescued by GFP-Rim101T expression. The nap1Δ mutant strain also had a capsule defect when compared to the WT under capsule-inducing conditions, similar to other Rim pathway mutants (Fig. 4C). Therefore, C. neoformans Nap1 regulates both Rim pathway-dependent and Rim pathway-independent cellular processes.

Nap1 functions upstream of the Rim pathway proteolysis complex

The ultimate output of Rim pathway signaling is the cleavage and activation of the Rim101 transcription factor, which is then translocated to the nucleus to regulate gene expression in response to alkaline pH (7, 21). To conclusively determine whether Nap1 is required for Rim101 activation, we examined Rim101 protein cleavage in the WT and nap1Δ strains using a previously described western blot-based assay for GFP-Rim101 truncation (Fig. 5A) (7, 15). In the absence of Nap1, the proteolytically cleaved forms of GFP-Rim101 that can be seen in the WT background are not evident in response to alkaline pH, similar to prior findings in the rim23Δ and rra1Δ Rim pathway mutant strains (15). We also examined the localization of GFP-Rim101 in both inactivating and activating conditions. In the WT strain, the GFP-Rim101 fusion protein localizes strongly to the nucleus under activating pH conditions (7, 15). However, there was reduced nuclear localization of GFP-Rim101 at pH 8 in the nap1Δ mutant, similar to other Rim pathway mutants, such as rim23Δ (Fig. 5B). These results indicate that Nap1 expression is specifically required for the cleavage and activation of Rim101 in response to alkaline pH.

To further place Nap1 within the Rim pathway, we examined the requirement for Nap1 in the formation of the Rim proteolysis complex. We have previously demonstrated that, under activating conditions, GFP-tagged Rim23 (Rim23-GFP) forms distinct puncta on the plasma membrane (15). Presumably, this represents the site of assembly of the Rim23/Rim20/Rim13 complex required for Rim101 proteolysis. Previously, it was demonstrated that mutants with Rim pathway defects upstream of Rim23 fail to form these puncta (15). We therefore assessed Rim23-GFP puncta formation in the nap1Δ strain (Fig. 5C). Similar to a mutant of the Rra1 protein, which is the most upstream Rim pathway component yet identified in C. neoformans, the nap1Δ strain fails to form Rim23-GFP puncta when grown at alkaline pH, placing Nap1 functionally upstream of Rim proteolysis complex formation. Therefore, the Nap1 protein is required for the two most downstream functions of Rim pathway activation—proper assembly of the Rim101 proteolysis complex and accumulation of the proteolytically cleaved and activated form of the Rim101 protein itself.

Nap1 specifically interacts with Rra1 and directs Rra1 stability

To further explore the interaction of Rra1 and Nap1, we disrupted the NAP1 gene in a strain expressing the Rra1-GFP fusion protein and assessed Rra1-GFP localization in the nap1Δ mutant strain. We observed that, in the nap1Δ mutant strain, Rra1-GFP is largely localized to endomembranes, with complete absence of the brightly fluorescent puncta at the plasma membrane (Fig. 6A). In order to test whether this was due to a change in localization or a change in protein levels, we assessed the levels of both the Rra1-GFP and Rim23-GFP proteins in the WT and nap1Δ mutant strain backgrounds. Interestingly, when Rra1-GFP is expressed in a nap1Δ strain, we observed a significant decrease in Rra1-GFP protein levels at both inactivating and activating pH (pH 4 and pH 8, respectively) (Fig. 6B). This decrease in protein levels was specific to Rra1-GFP, with no apparent changes in Rim23-GFP protein levels in the nap1Δ strain (Fig. 6B).

Since Nap1 has been shown to modulate gene expression in S. cerevisiae through its role in nucleosome assembly and movement (22), we
explored whether the change in Rra1-GFP protein level was due to altered gene expression or due to differences in protein expression or stability. Therefore, we assessed RRA1 mRNA transcript levels by qRT-PCR in the WT and nap1Δ strains at both pH 4 and pH 8. There was no significant difference in RRA1 transcript abundance between the two strains at either pH (Fig. 6C). These results indicate that Nap1 specifically promotes Rra1 protein expression or stability rather than RRA1 transcription.

*Nap1 specifically interacts with the Rra1 C-terminus*

To further probe the Rra1-Nap1 protein-protein interaction, we utilized C. neoformans strains expressing serially truncated forms of the Rra1 protein tagged with GFP (illustrated in Fig. 7A). Rra1-296T-GFP is truncated such that the most distal portion of the C-terminus is deleted, but that a more proximal highly charged region (HCR) of the C-terminal tail is intact. This potentially charged region is similar to the C-terminal region in the *S. cerevisiae* Rim21 protein, which is involved in the pH sensing activity of this protein (23). Rra1-273T-GFP represents a further truncation in which the entire C-terminus, including the HCR, has been deleted immediately following the predicted seventh transmembrane domain of the protein. The Rra1 C-terminus (GFP-Rra1Ct) strain expresses the region of the Rra1 protein that is C-terminal to the seventh transmembrane domain (the portion that has been deleted in the Rra1-273T-GFP construct).

To assess the interaction of Nap1 with the various domains of the Rra1 protein, we performed GFP-Trap co-IP experiments with whole cell lysates prepared from each of these strains after incubation in alkaline, Rim pathway-activating conditions. Western blot analysis of these samples demonstrated that the Nap1 protein specifically co-immunoprecipitates with the full-length Rra1 protein as well as the Rra1 C-terminus (Fig. 7B). However, Nap1 protein was not detected in the IP sample for strains expressing either the complete or partial truncations of the Rra1 C-terminal tail. These results suggest that Nap1 specifically interacts with domains present in the Rra1 C-terminus, particularly those present after residue 296.

**Nap1 is required for virulence and survival in macrophages**

Since the nap1Δ strain displays significant temperature and pH stress-induced growth defects, we assessed nap1Δ mutant survival in macrophages using the J774 macrophage-like cell line. This *in vitro* model of fungal-host cell interaction often predicts virulence in more complex models of infection (24). While the rim101Δ mutant strain showed no defect in survival in macrophages compared to the WT strain, the nap1Δ mutant had a significant decrease in growth and survival in J774 cells (Fig. 8). These results indicate that the Nap1 protein is required for growth and/or survival in host-like conditions.

When bone marrow-derived macrophages (BMDMs) are co-incubated with the rim101Δ mutant that has been grown in tissue culture medium, these BMDMs produce increased levels of the pro-inflammatory cytokine TNF-α compared to BMDMs incubated with WT *C. neoformans*. To determine whether the nap1Δ mutant similarly hyperactivates macrophages, we assessed mouse BMDM activation in response to co-incubation with *C. neoformans* cells. During co-incubation with the nap1Δ mutant strain, BMDMs produced similar levels of TNF-α to cells incubated with WT *C. neoformans*, a markedly different phenotype from the hyperinflammatory rim101Δ mutant (Fig. S2). However, these results are consistent with the fact that the nap1Δ mutant strain displays many phenotypes that are not restricted to characteristic Rim pathway mutants.

**Nap1 is not required for Rim pathway activation in S. cerevisiae**

While Nap1 orthologs have not previously been described to be involved in the Rim/Pal pathway in ascomycetes, it is possible that its additional cellular functions may obscure its involvement in this signaling pathway. We therefore hypothesized that Nap1 would not be required for Rim pathway activation in *S. cerevisiae* (20, 25). We performed qRT-PCR for the SMP1 and NRG1 genes, which are well-characterized targets of activated Rim101 in *S. cerevisiae* (9). Both of these targets are upregulated in WT cells in response to alkaline pH in a Rim101-dependent manner. We assessed expression of these genes in the *S. cerevisiae* rim20Δ, rim21Δ, and nap1Δ mutants compared to expression in WT at
activating pH 8. Under these pathway-activating conditions, both the \textit{rim20Δ} and \textit{rim21Δ} mutant strains displayed constitutively decreased expression of the \textit{NRG1} and \textit{SMP1} target genes relative to the WT (Fig. S3). However, the \textit{nap1Δ} mutant strain displayed no significant difference in expression level of these two genes when compared to the WT. These results suggest that, unlike in \textit{C. neoformans}, the Nap1 protein is not required for Rim pathway activation in \textit{S. cerevisiae}.

\textbf{C. gattii nap1Δ displays similar phenotypes to Rim pathway mutants}

To determine whether this novel function of Nap1 translated to other basidiomycete fungi or is specific to \textit{C. neoformans}, we assessed the role of Nap1 in Rim pathway activation in the distinct pathogenic species \textit{Cryptococcus gattii}. Similar to the \textit{C. neoformans nap1Δ} mutant, the \textit{C. gattii nap1Δ} mutant displays a significant growth defect when compared to WT under normal growth conditions and at elevated temperature (Fig. 9). Additionally, the \textit{C. gattii nap1Δ} mutant strain displays a significant growth defect on YPD pH 8, similar to both the \textit{rim101Δ} and the \textit{rra1Δ} mutants in this background (Fig. 9, (15)). Therefore, while Nap1 does not appear to be required for Rim101 function in the ascomycete yeast \textit{S. cerevisiae}, Nap1 plays a role in Rim pathway activation in a related but divergent \textit{Cryptococcus} species.

\textbf{Discussion}

In this study, we have further characterized mechanisms by which a fungal pathogen interprets extracellular host-relevant conditions. Specifically, we explored the interaction between localization and function for the \textit{C. neoformans} Rra1 protein, a novel activator of the cryptococcal Rim alkaline-response signaling pathway (15). We have demonstrated that Rra1 primarily localizes to the cell surface in punctate structures, and that these structures are dynamically regulated by external pH. Interestingly, the subcellular sites of Rra1 enrichment are spatially distinct from more downstream components of the pathway, such as the Rim proteolysis and ESCRT complexes. Furthermore, we discovered a novel role for the \textit{C. neoformans} Nap1 protein to interact with Rra1 and promote Rim pathway activation. We characterized the significant role that Nap1 plays in promoting the stability of the Rra1 protein, thereby controlling the activation of the Rim pathway.

Based on our subcellular localization studies, Rra1-GFP is localized both to punctate structures at the plasma membrane and to endomembranous structures including the ER. These Rra1-GFP puncta seem to change both in number and localization based on extracellular pH, suggesting that a dynamic alteration in Rra1 localization accompanies its activation at alkaline pH. Rra1-GFP localization changes from small, cell-surface puncta to larger intracellular regions of accumulation, suggesting that Rra1 might be endocytosed in response to pathway activation, perhaps as a mechanism for regulating or dampening the signaling output of the pathway. Alternatively, Rra1 might undergo reduced trafficking and delivery to the cell surface in pathway-activating pH conditions. This cell surface cycling model is supported by the larger, less well-defined appearance of the intracellular Rra1-GFP puncta. Though the pathway seems to be activated at the plasma membrane in ascomycete fungi (16, 17), the phosphorylation and endocytosis of PalH (in \textit{A. nidulans} (26)) and degradation of Rim21 (in \textit{S. cerevisiae} (27) aid in the turnover of these signaling proteins in these organisms. Future studies will determine the role of cycling of Rra1 to and from the plasma membrane in the regulation of Rim pathway signaling. Additionally, our experiments assessing the similar patterns of localization between the Rra1 protein and an ER marker, Sec63, suggests that Rra1 is also partially localized to the ER. This intracellular site of enrichment might simply represent the movement of a highly processed protein to its site of action, or it might also be an additional site of Rra1 function that has yet to be explored.

Notably, Rra1-GFP does not co-localize with the ESCRT machinery component mCherry-Vps23. Rim pathway function requires an intact ESCRT pathway as mutations in ESCRT components result in failed activation of the Rim101 transcription factor (15, 16, 28, 29). Our data suggest that the Rim pathway pH-sensing complex containing Rra1 is spatially separate from the ESCRT/Rim proteolysis complex. If this is the case, future studies will determine the mechanism by which Rra1 communicates with the Rim proteolysis complex in order to activate the response to alkaline pH.
To begin to address the regulation of Rra1 signaling, we performed a proteomics screen to identify interactors of the Rra1 pH sensor in *C. neoformans*. In previous work, we identified many of the Rim pathway components by sequence homology with orthologs in ascomycete fungi (7, 15). However, these studies suggested that the most upstream components of the pathway, including the Rra1 pH sensor, may have evolved separately in the basidiomycete fungi, including *C. neoformans*. For this reason, we sought to identify novel pathway regulators through proteomics approaches. In doing so, we hypothesized that we would find proteins such as adaptor proteins or messengers that could act as links involved in signal transduction between the Rra1 surface sensing protein and the more downstream Rim pathway components such as the ESCRT/Rim proteolysis complex. This type of unbiased interactant screen might even identify potential negative regulators of Rra1 activity that would not have been found in our previous screens that were specifically designed to identify Rim pathway activators. So far, we have successfully validated the interaction between Rra1 and the Nap1 protein, which represented the most abundant protein in the putative interactome of Rra1. Future work will determine whether the other potential interactors of Rra1 identified in this proteomics study are required for either the activation or downregulation of the Rim pathway.

Nap1 was first described to play a role in nucleosome assembly through Nap1 dimer binding of histone proteins, acting as a chaperone for histone proteins and a scaffold for the assembly of the nucleosome *in vitro* (30, 31). However, Nap1 has been implicated in many other cellular processes aside from nucleosome assembly. For example, Nap1 has repeatedly been determined to play a significant role in cell division and cell cycle control through its binding to cell cycle regulators, such as the mitotic cyclin Clb2 and the cyclin-dependent kinase Cdc28 (32–34). During cell division, Nap1 is proposed to act as a scaffold protein for septin formation through its binding to the Gin4 kinase and septin proteins in *S. cerevisiae* (35). This role was demonstrated to be crucial for regulation of filamentous growth in *C. albicans* (36). None of the previously described scaffolding roles for Nap1 in cell division easily explain its requirement in *C. neoformans* for pH-dependent growth and Rim101 activation. However, the previously described function for Nap1 as a scaffold and chaperone suggests and supports its role as a potential Rra1 scaffolding protein in the context of Rim pathway signaling.

Nap1 has not previously been determined to play a role in the response to environmental and cellular stressors, including extracellular pH. However, like the *C. neoformans* Nap1 protein, *C. albicans* Nap1 and its phosphoregulation are required for full virulence and survival of this fungus in mouse infection models through its regulation of the hyphal growth (36). This study in *C. neoformans* is the first instance in which Nap1 has been shown to play a role in alkaline pH signaling in fungi. Additionally, based on our preliminary studies in *S. cerevisiae*, this role in the Rim pathway may be specific to *Cryptococcus* species, or perhaps be the case more broadly within the biologically diverse basidiomycete fungi.

We propose a potential mechanism in which Nap1 acts as a scaffold to regulate Rra1 protein levels in response to changes in pH. In this model, Nap1 regulates Rra1 protein levels both by stabilizing Rra1 at acidic pH to maintain levels of this pH sensor in the membrane, and then by promoting Rra1 internalization, and perhaps degradation, once the Rim pathway has been activated. This hypothesis is further supported by the specificity of the protein interaction between Nap1 and the Rra1-C-terminus, and not with other Rim pathway components such as Rim23. In *A. nidulans*, the PalF arrestin-like protein specifically binds to the C-terminus of the PalH pH sensor, and this binding is required for pH-mediated signal transduction in this fungus (19, 37). Additionally, Rim23 protein levels are not affected in the *nap1Δ* strain, again supporting the hypothesis that the role of Nap1 in the Rim pathway is specifically through Rra1 regulation. However, due to the historical role of Nap1 as a histone scaffold and regulator, it is possible that Nap1 could be playing a dual role within the Rim pathway, both through regulation of the Rra1 pH sensor, as demonstrated here, and through its interaction with histones. This second function would represent a potential, but as of yet unexplored, role for Cn Nap1 in the regulation of transcription through interaction or cooperation with the Rim101 transcription factor in the nucleus.

*Cryptococcus neoformans* and *S. cerevisiae* Nap1 seem to vary significantly. In terms of protein similarity, there is only 43% sequence identity
between the \textit{S. cerevisiae} and \textit{C. neoformans} Nap1 proteins. Also, the \textit{C. neoformans} Nap1 protein has a shorter C-terminus relative to the \textit{S. cerevisiae} protein. The phenotypes of the respective mutants also vary significantly. The major phenotype associated with the nap1Δ mutant strain in \textit{S. cerevisiae} is an elongated budding phenotype, with no effect on growth rate (20, 32, 33). However, in this study, we demonstrated a significant growth defect of the \textit{C. neoformans} nap1Δ strain under normal growth conditions (rich medium at permissive temperatures), in addition to sensitivities to alkaline pH and elevated salt concentrations. It is therefore likely that these similar proteins from two distinct fungal species diverged not only in sequence, but in function. We have previously demonstrated that other components of the Rim pathway, namely the pH sensor, have evolved in a distinct manner in ascomycetous and basidiomycetous fungi, yet the general predicted structure of the pH sensors is the same between the two fungal phyla. It follows that a divergent form of Nap1 could mediate conserved and new functions compared to the conserved protein in model yeasts and molds. These and future studies will continue to inform us about ways in which fungal pathogens have co-opted central signaling mechanisms to sense and respond to their hosts.

\section*{Materials and Methods}

\subsection*{Strains, media, and growth conditions}

The strains used in this study and their genotypes are listed in Table 2, and they were constructed in the H99 or KN99a strain backgrounds. Unless otherwise noted, \textit{C. neoformans} cells were grown in Yeast extract-Peptone-Dextrose (YPD) medium (1% yeast extract, 2% peptone, 2% dextrose; plus 20% Bacto Agar for solid medium). YPGal medium contains 1% yeast extract, 2% peptone, and 3% galactose. YPD or YPGal pH 4 or pH 8 was prepared by adding 50 mM HEPES for liquid medium or 150 mM HEPES for solid medium and adjusting pH using either HCl or NaOH. Synthetic complete medium (SC) pH 4 or 8 consisted of 1X Yeast Nitrogen Base plus Ammonium Nitrate + complete amino acid mix, which was then buffered with McIlvaine’s buffer (0.1M sodium citrate and 0.2M sodium phosphate) to the appropriate pH. Cells were grown at 30°C unless otherwise noted.

\subsection*{Molecular biology and strain creation}

To create the mutants used in this study, deletion constructs were created to replace the entire open reading frame with the neomycin (\textit{NEO}) dominant selectable marker. The knockout constructs were created using PCR overlap-extension as described previously (38). All constructs were transformed into the \textit{C. neoformans} H99 strain by biolistic transformation as previously described (39). For endogenous C-terminally tagged strains (p\textit{NAP1-GFP-\textit{NEO}} and p\textit{NAP1-mCh-\textit{HYG}}), plasmids were created using In-Fusion cloning into the pUC19 vector (Clontech). The Safe Haven galactose-inducible truncated \textit{RIM101} construct (p\textit{GAL7-RIM101-70T}) was created by inserting the p\textit{GAL7-RIM101-70T} construct from pTO22 into the Safe Haven vector containing a dominant nourseothricin (\textit{NAT}) resistance marker using In-Fusion cloning (40). The \textit{RRA1} full-length and truncation GFP-fusion constructs (p\textit{His3-RRA1-GFP-NAT}, p\textit{His3-RRA1-4XFLAG-NAT}, p\textit{His3-RRA1-mCherry-NAT}, p\textit{His3-RRA1-296T-GFP}, p\textit{His3-RRA1-273T-GFP}, and p\textit{His3-GFP-RRA1-Cterm}) were created by In-Fusion cloning the truncated \textit{RRA1} genes along with \textit{GFP}, 4XFLAG tag, or \textit{mCherry} and the \textit{RRA1} terminator into the pcN20 plasmid, which contains the histone H3 promoter and a \textit{NAT} resistance marker (41). The \textit{NAP1} reconstitution construct was created by cloning the \textit{NAP1} gene with terminator into the pcN20 vector using restriction digestion and ligation. The \textit{pHis3-mCherry-VPS23-\textit{NEO}} construct was constructed by cloning the \textit{VPS23} gene with its terminator into the pcN52 plasmid, which contained the histone H3 promoter and the \textit{NEO} resistance marker (42). The endogenous \textit{RIM23-GFP} construct was created using overlap-extension PCR and co-transformed with a plasmid containing a selectable marker. All primers used to create the KO constructs and fusion constructs, confirmation primers, and Southern blot primers are listed in Table S2.

Upon transformation, strains were selected on medium containing nourseothricin, neomycin, or hygromycin. Deletion mutants were confirmed with a combination of positive and negative confirmation PCRs, assessing for the presence or absence, respectively, of the allele of interest. PCR confirmation was followed by Southern blot to confirm single integration of the deletion or reconstitution constructs (data not shown).
For creation of strain KS356 (nap1Δ rra1Δ pHIS3-RRA1-GFP) and KMP91 (pHRR1-mCherry-NAT eRIM23-GFP) by a genetic cross, colonies of strains KS352 (nap1Δ MATα) and KS310 (rra1Δ pHIS3-RRA1-GFP MATα) for KS356 and strains KS289 (rim23::NEO + endogenous RIM23-GFP + pCH233) and KMP87 (KN99a + pHRR1-mCherry-NAT) were individually suspended in sterile water, combined, and plated on MS medium. Mating reactions were placed in the dark at room temperature for one week, and meiotic spores were isolated by micromanipulation and germinated on YPD.

Proteomics

C. neoformans cells (WT or rra1Δ + pHIS3-RRA1-GFP) were incubated in conditions as described above, pelleted at 5000 rpm for 5 minutes at 4°C, washed in 1 mL sterile water, pelleted at 13,000 rpm for 2 minutes at 4°C, and then were flash frozen at -80°C. 500 µL of 500 µm acid-washed glass beads were added to samples, and cells were mechanically lysed by bead beating. Cell lysates were separated from beads using 3 washes of 0.4 mL lysis buffer (50 mM Tris-HCl, pH 7.5, 1mM EDTA, 1 mM β-mercaptoethanol, 1X Complete Mini EDTA-free protease inhibitor cocktail (Roche), and 1 mM phenylmethane sulfonyl fluoride (PMSF)). Lysates were precleared by centrifugation at 15,000 rpm for 5 min at 4°C (whole lysate sample). Rra1-GFP was solubilized from membranes by addition of a final concentration of 1% Fos-14-Choline and inversion at 4°C for 1 hour. Since Rra1 is predicted to be an integral membrane protein with seven transmembrane domains, pilot studies were conducted using various detergents to determine conditions that might best solubilize this fusion protein from membranes while minimizing disruption of protein-protein interactions (Fig. S4). The insoluble fraction was removed by ultracentrifugation at 100,000xg. Proteins were immunoprecipitated by addition of 50 µL GFP-Trap resin (Chromotek) and inversion at 4°C for 2 hours.

Mass spectrometry experiments were performed at an n of 1 by the Duke Proteomics Core Facility. Resin-bound proteins were treated with 1% RapiGest (Waters) at 50°C for 10 minutes, then reduced by addition of 10 mM final concentration dithiothreitol and heating at 32°C for 35 minutes and alkylated by addition of 20 mM final concentration iodoacetamide and incubation at room temperature for 30 minutes. Samples were then trypsin digested on-resin overnight at 32°C. Samples were then subjected to a 90-minute chromatographic separation on a nanoscale capillary reverse phase ultra-performance liquid chromatography system (Waters) coupled to a Q-Exactive Plus high resolution accurate mass tandem mass spectrometer (Thermo) via a nanoelectrospray ionization source. Data was analyzed as described in the Supplementary Methods. A full peptide list with parameters used for identification can be found in Table S3.

We restricted the list of identified potential interactors to those present in the Rra1-GFP sample and absent in the untagged control, with at least 3 exclusive unique peptides detected in the sample (Table S1). From these, we selected proteins from among those with the highest differential between the Rra1-GFP and WT control samples for further study.

Protein extraction, immunoprecipitation, and western blot

For confirmatory co-immunoprecipitation experiments, the whole-cell lysates were prepared for immunoprecipitation as described above, but with 10 µL GFP-Trap per sample. These immunoprecipitation samples were eluted from the GFP-Trap resin by the addition of 20 µL of 2X Laemmli buffer (Bio-Rad) and heating at 80°C for 5 minutes. Whole lysates were prepared for western blot by addition of 2X Laemmli buffer to a 1X concentration and heating at 80°C for 5 minutes. For GFP-Rim101 western blots, samples were prepared as described above, but with the addition of 1X PhosStop (Roche) to the lysis buffer. Western blots were performed as described previously by separation of proteins on 4-12% NOVEX NuPage Bis-Tris gels (Thermo Fisher Scientific). GFP-tagged proteins were detected with anti-GFP monoclonal primary antibody (Roche 11814460001; Lot no. 14717400) at a 1:5000 dilution in StartingBlock (TBS) Blocking solution (Thermo Scientific), then with secondary anti-mouse horseradish peroxidase (HRP)-conjugated antibody (Jackson ImmunoResearch Laboratories, Inc. 111-035-008; Lot no. 128022; 1:25,000 in StartingBlock) with final detection using Amersham ECL Prime Western Blotting Detection Agent (GE Healthcare). FLAG-tagged Rra1 was detected with anti-FLAG monoclonal antibody M2.
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(Sigma-Aldrich F3165; Lot no. SLBQ7119V). Nap1 was detected with an anti-Nap1 polyclonal primary antibody (Santa Cruz Biotechnology sc-7165; Lot no. H0604) that was developed against Saccharomyces cerevisiae Nap1 at a 1:200 dilution, then detected with a secondary HRP-conjugated anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Inc. 115-035-174; Lot no. 127837) at a 1:25,000 dilution. Control blots were probed with anti-PSTAIR monoclonal antibody (Sigma P7962; Lot no. 015M4840V) (43).

Microscopy

Microscopy images were captured using a Zeiss Axio Imager A1 fluorescence microscope equipped with an Axio-Cam MRM digital camera for DIC and fluorescence images. The high-resolution fluorescent images for Rra1-GFP colocalization and Nap1 localization studies were captured using a DeltaVision Elite deconvolution microscope equipped with a CoolSnap HQ2 high-resolution charge-coupled-device (CCD) camera. Deconvolution of DeltaVision images was performed using the softWoRx Imaging Workstation (Applied Precision), using the Enhanced Ratio method at 10 cycles of deconvolution. For assessment of capsule production, cells were counterstained with India ink. For images with nuclear staining, C. neoformans cells were incubated with NucBlue Live Ready Probes Reagent for 30 minutes prior to imaging (Thermo Fisher Scientific).

Quantitative Real-Time PCR

For quantitative real-time PCR (qRT-PCR) experiments, C. neoformans and S. cerevisiae cells were grown under described conditions. Cultures were pelleted and lyophilized. RNA was prepped using the RNeasy Plant Mini Kit (Qiagen). 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 (44) using primers listed in Table S2.

Macrophage survival assay

Survival of C. neoformans cells in macrophages was assessed as previously described (7), except that J774 cells were activated for 1 hour by the addition of 10 nM phorbol myristate acetate (Fisher BP6851) prior to the addition of C. neoformans cells at an MOI of 1. C. neoformans cell were opsonized prior to coincubation with an anti-capsule monoclonal antibody at 1 μg/mL for 1 hour (18B7 (45)). Each fungal strain was tested with at least 3 biological replicates per experiment.

Macrophage activation assay

To assess macrophage activation, bone marrow-derived macrophages (BMDMs) were isolated and prepared as previously described (46). The harvested BMDMs were plated in 96-well plates in DMEM with 10% FBS, 1 U/mL penicillin/streptomycin at a concentration of 5 x 10^4 cells/well (FBS: Sigma F2442). 5 x 10^5 C. neoformans cells (MOI of 10) were added to each well, and the cells were co-incubated at 37˚C and 5% CO2. After 6 hours of coincubation, the medium from each well was harvested, and secreted TNF-α was quantified by enzyme-linked immunosorbent assay (ELISA) (Biolegend 43904; Lot no. B187908). Experiments were performed in biological triplicates. Data represented are TNF-α in picograms/mL for each biological triplicate.
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FOOTNOTES
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Proteomics data can be accessed on the Mass Spectrometry Interactive Virtual Environment (MassIVE) through the University of California at San Diego, accession number MSV000082127. Three analyses performed by the Duke Proteomics and Metabolomics Shared Resource in support of this project. Sample ID21519 was called Rra1-GFP, a 1D coomassie-stained gel band from a GFP-Trap pulldown of Rra1-GFP from the membrane protein fraction of Cryptococcus neoformans. Sample ID21570 was called no-GFP control, and consisted a GFP-Trap pulldown from the insoluble fraction of C. neoformans H99 lysate (non GFP expressing). ID21571 was called Rra1-GFP, and consisted of a GFP-Trap pulldown from the insoluble fraction of C. neoformans H99 lysate containing Rra1-GFP.

The abbreviations used are: Rra1, required for Rim101 activation 1; Nap1, nucleosome assembly protein 1; YPD, yeast extract-peptone-dextrose medium; SC, synthetic complete medium; ESCRT, endosomal sorting complex required for transport; WT, wild-type; qRT-PCR, quantitative real-time polymerase chain reaction; HCR, highly charged region.

Table 1: Potential Rra1-GFP Interacting Proteins. Represented are proteins which were present in the Rra1-GFP co-immunoprecipitation mass spectrometry samples and absent in the negative control samples with at least 3 exclusive unique peptides represented in the Rra1-GFP sample.

| Gene locus tag | Gene Product                                      | Peptide Count |
|---------------|---------------------------------------------------|---------------|
| CNAG_03488    | conserved hypothetical protein (Rra1)              | 62            |
| CNAG_02091    | nucleosome assembly protein                        | 18            |
| CNAG_00237    | 3-isopropylmalate dehydratase                     | 8             |
| CNAG_02928    | 60s ribosomal protein 15-b                         | 6             |
| CNAG_05823    | conserved hypothetical protein                     | 6             |
| CNAG_00774    | d-3-phosphoglycerate dehydrogenase 2              | 5             |
| CNAG_00605    | cytoplasmic protein                               | 5             |
| CNAG_02100    | fatty-acid synthase complex protein                | 5             |
| CNAG_06112    | carbamoyl-phosphate synthase arginine-specific large chain | 4 |
| CNAG_06061    | eukaryotic translation initiation factor 3 subunit 6 | 4 |
| CNAG_02814    | glycerol-3-phosphate dehydrogenase                | 4             |
| CNAG_00891    | ATP-binding cassette transporter                   | 4             |
| CNAG_01655    | dynamin                                           | 4             |
| CNAG_03298    | hypothetical protein                              | 4             |
| CNAG_05650    | ubiquitin carboxyl-terminal hydrolase 5           | 4             |
| CNAG_04984    | pleiotropic drug resistance protein               | 4             |
| CNAG_02403    | conserved hypothetical protein                     | 4             |
| CNAG_03202    | adenylate cyclase                                 | 3             |
| CNAG_02991    | coflin                                            | 3             |
| CNAG_02485    | ATP synthase gamma chain                          | 3             |
| CNAG_04803    | structural molecule                               | 3             |
| CNAG_01682    | mitochondrial outer membrane 72K protein          | 3             |
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| Gene ID      | Description                          | Source |
|--------------|--------------------------------------|--------|
| CNAG_02110   | N-terminal acetyltransferase         | 3      |
| CNAG_00990   | CIPB                                 | 3      |
| CNAG_05292   | alpha,alpha-trehalose-phosphate synthase | 3    |
| CNAG_03904   | endopeptidase                        | 3      |
| CNAG_00686   | conserved hypothetical protein       | 3      |
| CNAG_07628   | translation initiation factor elf3c  | 3      |
| CNAG_02099   | fatty acid synthase beta subunit     | 3      |
| CNAG_01000   | nucleoporin Nup157/170               | 3      |
| CNAG_00104   | mRNA export factor elf1              | 3      |
| CNAG_03904   | endopeptidase                        | 3      |
| CNAG_00686   | conserved hypothetical protein       | 3      |
| CNAG_05292   | alpha,alpha-trehalose-phosphate synthase | 3    |
| CNAG_03904   | endopeptidase                        | 3      |
| CNAG_00686   | conserved hypothetical protein       | 3      |
| CNAG_05292   | alpha,alpha-trehalose-phosphate synthase | 3    |
| CNAG_03904   | endopeptidase                        | 3      |
| CNAG_00686   | conserved hypothetical protein       | 3      |
| CNAG_05292   | alpha,alpha-trehalose-phosphate synthase | 3    |
| CNAG_03904   | endopeptidase                        | 3      |
| CNAG_00686   | conserved hypothetical protein       | 3      |
| CNAG_05292   | alpha,alpha-trehalose-phosphate synthase | 3    |
| CNAG_03904   | endopeptidase                        | 3      |
| CNAG_00686   | conserved hypothetical protein       | 3      |
| CNAG_05292   | alpha,alpha-trehalose-phosphate synthase | 3    |
| CNAG_03904   | endopeptidase                        | 3      |
| CNAG_00686   | conserved hypothetical protein       | 3      |
| CNAG_05292   | alpha,alpha-trehalose-phosphate synthase | 3    |
| CNAG_03904   | endopeptidase                        | 3      |
| CNAG_00686   | conserved hypothetical protein       | 3      |
| CNAG_05292   | alpha,alpha-trehalose-phosphate synthase | 3    |
| CNAG_03904   | endopeptidase                        | 3      |
| CNAG_00686   | conserved hypothetical protein       | 3      |
| CNAG_05292   | alpha,alpha-trehalose-phosphate synthase | 3    |
| CNAG_03904   | endopeptidase                        | 3      |
| CNAG_00686   | conserved hypothetical protein       | 3      |
| CNAG_05292   | alpha,alpha-trehalose-phosphate synthase | 3    |
| CNAG_03904   | endopeptidase                        | 3      |
| CNAG_00686   | conserved hypothetical protein       | 3      |
| CNAG_05292   | alpha,alpha-trehalose-phosphate synthase | 3    |
| CNAG_03904   | endopeptidase                        | 3      |
| CNAG_00686   | conserved hypothetical protein       | 3      |
| CNAG_05292   | alpha,alpha-trehalose-phosphate synthase | 3    |
| CNAG_03904   | endopeptidase                        | 3      |
| CNAG_00686   | conserved hypothetical protein       | 3      |
| CNAG_05292   | alpha,alpha-trehalose-phosphate synthase | 3    |
| CNAG_03904   | endopeptidase                        | 3      |
| CNAG_00686   | conserved hypothetical protein       | 3      |
| CNAG_05292   | alpha,alpha-trehalose-phosphate synthase | 3    |
| CNAG_03904   | endopeptidase                        | 3      |
| CNAG_00686   | conserved hypothetical protein       | 3      |
| CNAG_05292   | alpha,alpha-trehalose-phosphate synthase | 3    |
| CNAG_03904   | endopeptidase                        | 3      |
| CNAG_00686   | conserved hypothetical protein       | 3      |
| CNAG_05292   | alpha,alpha-trehalose-phosphate synthase | 3    |
| CNAG_03904   | endopeptidase                        | 3      |
| CNAG_00686   | conserved hypothetical protein       | 3      |
| CNAG_05292   | alpha,alpha-trehalose-phosphate synthase | 3    |
| CNAG_03904   | endopeptidase                        | 3      |
| CNAG_00686   | conserved hypothetical protein       | 3      |
| CNAG_05292   | alpha,alpha-trehalose-phosphate synthase | 3    |
| CNAG_03904   | endopeptidase                        | 3      |
| CNAG_00686   | conserved hypothetical protein       | 3      |

Table 2: Strains used in this study

| Strain   | Genotype                          | Source |
|----------|-----------------------------------|--------|
| H99      | MATα                              | (47)   |
| KN99a    | MATα                              | (48)   |
| KS310    | rra1::NEO + pKS85 (pHIS3-RRA1-GFP-NAT) MATα | This study |
| KS317    | rra1::NEO + pKS82 (pRRA1-4XFLAG-NAT) MATα | This study |
| KMP67    | H99 + pKS58 (pHIS3-mCherry-VPS23-NEO) + pKS85 (pHIS3-RRA1-GFP-NAT) MATα | This study |
| KMP91    | H99 + pKP27 (pHIS3-RRA1-mCherry-NAT) + endogenous RIM23-GFP | This study |
| KMP87    | KN99a + pKP27 (pHIS3-RRA1-mCherry-NAT) MATα | This study |
| KMP42    | nap1::NEO + pKS58 (pHIS3-RRA1-GFP-NAT) + pKP12 (pUC19-eNAP1-GFP-HYG) | This study |
| TOC35    | rim101::NAT MATα                   | (7)    |
| KS336    | rra1::NEO MATα                     | This study |
| KMP31    | nap1::NEO MATα                     | This study |
| KS352    | nap1::NEO MATα                     | This study |
| KMP70    | nap1::NEO + pKP23 (pHIS3-NAP1-NAT) MATα (NAP1R) | This study |
| KMP64    | H99 + pKP18 (pGAL7-RIM101-70T) MATα | This study |
| KMP54    | nap1::NEO + pKP18 (pSDMA25-pGAL7-RIM101-70T) MATα | This study |
| KS354    | nap1::NEO + pKP12 (pUC19-eNAP1-GFP-HYG) | This study |
| TOC106   | eGFP-RIM101 MATα                   | (1)    |
| KMP33    | nap1::NEO + eGFP-RIM101 + pCH233 (NAT) MATα | This study |
| KS87-2   | rim23::NEO + eGFP-RIM101 + pCH233 (NAT) MATα | (15)   |
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| Strain   | Description                                                                 | Source         |
|----------|-----------------------------------------------------------------------------|----------------|
| KS289    | rim23::NEO + eRIM23-GFP + pCH233 (NAT) MATα                                  | (15)           |
| KS301    | rra1::NEO + eRIM23-GFP + pCH233 (NAT) MATα                                  | (15)           |
| KS353    | nap1::NEO + eRIM23-GFP + pCH233 (NAT) MATα                                  | This study     |
| KS356    | nap1::NEO + rra1::NEO + pKS85 (pHIS3-RRA1-GFP-NAT) MATα                    | This study     |
| KS338    | rra1::NEO + pHIS3-RRA1-296T-GFP-NAT MATα                                    | This study     |
| KS340    | rra1::NEO + pHIS3-RRA1-273T-GFP-NAT MATα                                    | This study     |
| KS234    | H99 + pKS50 (pHIS3-GFP-RRA1 C-terminus) MATα                               | This study     |
| BY4743   | Saccharomyces cerevisiae MATα/h; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; lys2Δ0/lys2Δ0; MET15/met15Δ0; ura3Δ0/ura3Δ0 | (49)           |
| 37333    | S. cerevisiae rim20::KANMX4 MATα/h; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; lys2Δ0/lys2Δ0; MET15/met15Δ0; ura3Δ0/ura3Δ0 | (49)           |
| 31150    | S. cerevisiae rim21::KANMX4 MATα/h; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; lys2Δ0/lys2Δ0; MET15/met15Δ0; ura3Δ0/ura3Δ0 | (49)           |
| 35119    | S. cerevisiae nap1::KANMX4; MATα/h; his3Δ1/his3Δ1; leu2Δ0/leu2Δ0; lys2Δ0/lys2Δ0; MET15/met15Δ0; ura3Δ0/ura3Δ0 | (49)           |
| R265     | Cryptococcus gattii                                                         | (50)           |
| KS259    | C. gattii rra1::NEO                                                         | (15)           |
| KS261    | C. gattii rim101::NEO                                                        | (15)           |
| KMP56    | C. gattii nap1::NEO                                                         | This study     |

Supplementary Table S1. Full Proteomics table (excel sheet)
Supplementary Table S2. List of primers used in this study.
Supplementary Table S3. Full list of peptides identified in this proteomics study, along with peptide identification probabilities.
FIGURE 1. **Rra1 localization in C. neoformans.**

A. Rra1-GFP localization in the *rra1Δ + pHIS3-RRA1-GFP* strain. *C. neoformans* cells were cultured in SC pH 4, then shifted to SC pH 4 or SC pH 8 for 30 minutes before imaging. 

B. Sec63-GFP localization. A strain expressing an endogenous *SEC63-GFP* construct was incubated in SC overnight prior to imaging. 

C. Rra1-GFP and mCherry-Vps23 localization. Cells were cultured overnight in SC pH 4, then shifted to SC pH 4 or SC pH 8 prior to imaging. 

D. Rim23-GFP and Rra1-mCherry localization. *C. neoformans* cells were cultured in SC pH 4, then shifted to SC pH 8 for 30 minutes before imaging. All scale bars are 5 microns. Images for A, B, and D were taken using an AxioVision microscope, and images for C were taken using a DeltaVision deconvolution microscope.
FIGURE 2. Nap1 specifically interacts with Rra1. A. Western blot analysis of Nap1 protein in cell lysates and IP samples. *C. neoformans* strains expressing Rra1-GFP (98 kDa) or Rim23-GFP (80 kDa) in the presence or absence of Nap1 (45 kDa) were cultured overnight, then shifted to YPD pH 8 for 1 hour prior to harvest. Cells were lysed, membranes solubilized as described, and IP was performed with GFP-Trap beads. Markers on blot represent molecular weight in kDa.
FIGURE 3. Nap1 localizes to the cytosol. A. Nap1-GFP localizes to the cytoplasm. Nap1-GFP localization was assessed on a DeltaVision deconvolution microscope, and images were deconvolved to assess Nap1-GFP localization. B. Nap1-GFP partially localizes to the nucleus. Cells were cultured overnight in SC, then incubated with NucBlue live cell stain for 30 minutes in the dark prior to imaging on an AxioVision microscope. C. Nap1-mCherry and Rra1-GFP localization. Cells were incubated in SC pH 4 overnight, then shifted to SC pH 4 or SC pH 8 for 30 minutes before imaging on a DeltaVision deconvolution microscope. All scale bars are 5 microns.
FIGURE 4. The nap1Δ mutant strain phenotypes. A. The nap1Δ strain displays growth defects under various growth conditions. Serial dilutions of *C. neoformans* were plated on various media, including YPD, YPD pH 8, or YPD + 1.5 M NaCl and incubated at 25°C (YPD), 30°C (YPD, pH 8, and NaCl), or 37°C (YPD). YPD plates were imaged on day 3. pH 8 and NaCl plates were imaged on day 6. B. The nap1Δ mutant strain is rescued by galactose-inducible RIM101T. Strains containing a galactose-inducible truncated form of *RIM101* (GAL-RIM101T) were incubated on YPD, YPGal, YPD pH 8, and YPGal pH 8 to assess growth. C. Capsule production in the nap1Δ mutant. *C. neoformans* cells were incubated in CO2-independent tissue culture medium for 3 days and counterstained with India ink to assess capsule formation. Images were captured using an AxioVision microscope.
FIGURE 5. The nap1Δ mutant strain displays Rim pathway-associated phenotypes. A. Western blot (WB) of total cell lysates with an anti-GFP antibody was used to assess GFP-Rim101 cleavage in response to alkaline pH. *C. neoformans* cells expressing the endogenous GFP-RIM101 construct in a WT, rim101Δ, or nap1Δ background were incubated overnight in SC pH 4, then shifted for 30 minutes into SC pH 4 or SC pH 8 prior to lysis and WB analysis. GFP-Rim101 is 140 kDa, and the final, smaller GFP-Rim101 cleavage product is 70 kDa. Marker sizes indicated are in kDa. B. GFP-Rim101 localization was observed in response to pH shift in WT and indicated mutant strains. *C. neoformans* cells were incubated as described above for 30 minutes prior to imaging on an AxioVision microscope. C. Rim23-GFP localization in response to pH in WT and mutant strains. *C. neoformans* cells containing the endogenous RIM23-GFP construct in the WT, rra1Δ, or nap1Δ backgrounds were incubated overnight in SC pH 4, then shifted to SC pH 8 for 30 minutes prior to imaging on an AxioVision microscope.
FIGURE 6. Nap1 is required for Rra1 protein stability. A. Rra1-GFP localization in the presence and absence of Nap1. Cells were incubated in SC and imaged on an AxioVision microscope. Scale bars represent 5 microns. B. Western blot analysis of Rra1-GFP and Rim23-GFP protein levels in the presence or absence of Nap1. Cells were cultured overnight, then shifted to YPD pH 4 or YPD pH 8 for 1 hour prior to lysis. Samples represent whole lysates. Markers indicate size in kDa. The expected size of the PSTAIR (Cdc2) control is 34 kDa. C. RRA1 mRNA expression levels in the WT and nap1Δ mutant backgrounds. Cells were incubated for 1 hour in YPD pH 4 or YPD pH 8, followed by RNA isolation. Bars represent the fold-change expression over WT pH 4 levels for each strain. qRT-PCR was performed in triplicate, and error bars represent the SD. Significance was analyzed by two-way ANOVA with Tukey’s multiple comparisons test.
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FIGURE 7. Nap1 binds specifically to the Rra1 C-terminus. A. Schematic of the Rra1 truncations used in this study, with insert representing the residues making up the HCR. Alignment of basidiomycete Rra1 protein sequences was performed using T-Coffee (51). B. Western blot analysis of Rra1-GFP IPs. Strains expressing each of the truncations listed in Fig 7A were incubated in YPD pH 8 for 1 hour prior to lysis. GFP-tagged Rra1 proteins were immunoprecipitated with GFP-Trap beads. GFP WB contains whole lysate samples, and Nap1 WB contains IP samples. Rra1-296T-GFP expected size is 60 kDa, Rra1-273T 57 kDa, and GFP-Rra1Ct 69 kDa. Markers represent expected size in kDa.
FIGURE 8. **Nap1 is required for survival in macrophages.** WT, rim101Δ, nap1Δ, and NAP1R cells were cultured overnight, then added to J774 macrophages at an MOI of 1:1. After 24 hours, J774 cells were lysed and C. neoformans cells were harvested and CFUs counted. Data represents percent of the WT input/output ratio (WT set to 100%). Error bars represent standard deviation. Significance was assessed by two-way ANOVA with Tukey’s multiple comparisons test. ** p<0.01.
FIGURE 9. *C. gattii* NAP1 is required for growth at pH 8. Serial dilutions of *C. gattii* WT, rim101Δ, rra1Δ, and nap1Δ strains were plated on YPD, YPD pH 8, or YPD + 1.5 M NaCl and incubated at 25°C (YPD), 30°C (YPD, pH 8, and NaCl), and 37°C (YPD). YPD plates were imaged on day 3; pH 8 and NaCl plates were imaged on day 6.
Characterization of additional components of the environmental pH-sensing complex in the pathogenic fungus *Cryptococcus neoformans*

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