Cryptococcus neoformans transcriptional regulation of the host-pathogen interface

by

Teresa Rodgers O'Meara

University Program in Genetics and Genomics
Duke University

Date: __________________________
Approved:

___________________________
J. Andrew Alspaugh, Supervisor

___________________________
Joseph Heitman

___________________________
Sue Jinks-Robertson

___________________________
Fred Dietrich

___________________________
Meta Kuehn

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University Program in Genetics and Genomics in the Graduate School of Duke University

2013
ABSTRACT

_Cryptococcus neoformans_ transcriptional regulation of the host-pathogen interface

by

Teresa Rodgers O'Meara

University Program in Genetics and Genomics
Duke University

Date:_______________________

Approved:

___________________________

J. Andrew Alspaugh, Supervisor

___________________________

Joseph Heitman

___________________________

Sue Jinks-Robertson

___________________________

Fred Dietrich

___________________________

Meta Kuehn

An abstract of a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University Program in Genetics and Genomics in the Graduate School of Duke University

2013
Abstract

*Cryptococcus neoformans* is a human fungal pathogen that is also ubiquitous in the environment. To cause disease inside a human host, *C. neoformans* must be able to sense and respond to a multitude of stresses. One of the major responses to the host is the induction of a polysaccharide capsule, which allows the fungus to resist damage and evade the host immune response. This capsule is regulated by a number of signal transduction cascades, but a major contributor is the conserved cAMP/PKA pathway.

Using genetic and molecular biology techniques, I identified Gcn5 and Rim101 as key transcriptional regulators of capsule within the host. I determined that *C. neoformans* Rim101 is activated by a combination of the canonical pH sensing pathway and the cAMP/PKA pathway. This novel connection potentially gives the pathogen greater flexibility in responding to environmental stimuli, thus allowing for a greater capacity for disease.

I determined that the Rim101 transcription factor regulates cell wall remodeling in the context of the host by deep mRNA sequencing, electron microscopy, and biochemical assays. Using chromatin immunoprecipitation, I confirmed that these cell wall changes are under direct control of Rim101. I then confirmed the importance of cell wall changes in the host by nanoString profiling of fungal RNA in the context of a murine lung infection. I also examined the lungs of infected mice for cytokine and immune cell infiltrate and determined that *C. neoformans* cell wall changes are important in avoiding triggering an aberrant host response. I hypothesize that this cell wall remodeling via Rim101 activation is required for full capsule attachment and for masking immunogenic molecules from the host immune system.
Dedication

To Matt and Robin
# Contents

Abstract .............................................................................................................................................. iv

List of Tables ....................................................................................................................................... xi

List of Figures ...................................................................................................................................... xii

Acknowledgements ............................................................................................................................. xiv

1. Introduction ...................................................................................................................................... 16

  1.1 Cryptococcus neoformans is an opportunistic human fungal pathogen ................................. 16

  1.2 Human colonization requires adaptation to multiple stresses .............................................. 18

    1.2.1 Responses to host environmental stresses ........................................................................ 18

    1.2.2 Mechanisms to combat host immune responses ................................................................. 19

      1.2.2.1 Macrophages .................................................................................................................... 19

      1.2.2.2 Capsule ........................................................................................................................... 20

  1.3 Conservation and divergence of the cAMP/ PKA pathway ..................................................... 22

  1.4 Summary of the work included in this thesis .............................................................................. 23

2. Cryptococcus neoformans histone acetyltransferase Gcn5 regulates fungal adaptation
   to the host ........................................................................................................................................ 28

  2.1 Introduction .................................................................................................................................... 28

  2.2 Results .......................................................................................................................................... 30

    2.2.1 Identification of the C. neoformans homologue of S. cerevisiae Gcn5 .......................... 30

    2.2.2 C. neoformans Gcn5 complements a S. cerevisiae histone acetyltransferase mutant ....... 32

    2.2.3 Gcn5 is involved in stress tolerance .................................................................................... 33

    2.2.4 Gcn5 is localized to the nucleus .......................................................................................... 34

    2.2.5 Gcn5 regulates capsule in C. neoformans ............................................................................ 37
4.2 Importance ................................................................................................................. 92
4.3 Introduction ............................................................................................................... 92
4.4 Results ..................................................................................................................... 95
  4.4.1 Infection with the rim101Δ strain results in increased host inflammation ...... 95
  4.4.2 Altered cytokine and cellular immune response to the rim101Δ mutant ...... 102
  4.4.3 Rim101 is involved in cell wall remodeling under host-relevant conditions.. 104
  4.4.4 Rim101 transcriptionally regulates cell wall genes................................. 106
  4.4.5 Rim101-regulated gene expression is reflected in altered cell wall composition ................................................................................................................. 108
  4.4.6 Altered cell walls affect capsule attachment and phagocytosis ............. 109
  4.4.7 Rim101 induces TNF-α production in macrophages.............................. 113
4.5 Discussion ............................................................................................................... 114
  4.5.1 The rim101Δ mutant strain causes a fundamentally different disease....... 114
  4.5.2 Rim101 regulates remodeling of the cell wall upon entry into the host ...... 118
4.6 Methods and Materials ......................................................................................... 122
5. The Cryptococcus neoformans Rim101 transcription factor directly regulates genes required for adaptation to the host. ................................................................. 128
  5.1 Introduction ........................................................................................................... 128
  5.2 Results .................................................................................................................... 130
    5.2.1 Pka1 and Rim101 share downstream targets ........................................... 130
    5.2.2 Rim101 binds a conserved motif .............................................................. 135
    5.2.3 Rim101 binds a conserved motif in vivo .................................................. 138
    5.2.4 nanoString profiling of virulence gene expression in vitro .................. 139
    5.2.5 Rim101 regulates expression changes in animal models of infection...... 144
  5.3 Discussion ............................................................................................................. 146
5.4 Methods: ........................................................................................................149

6. Cryptococcus neoformans capsule: a sword and a shield.............................153
  6.1 Introduction ..................................................................................................153
  6.2 The biology of cryptococcal capsule .......................................................156
    6.2.1 Capsule Structure ...............................................................................156
    6.2.2 Capsule Synthesis ..............................................................................157
      6.2.2.1 Capsule monomers: .................................................................157
      6.2.2.2 Modification of capsule monomers: ..........................................158
      6.2.2.3 Location of capsule synthesis: ..................................................161
    6.2.3 Capsule Secretion ...............................................................................163
    6.2.4 Attachment .........................................................................................164
      6.2.4.1 Cell wall glucans ......................................................................167
      6.2.4.2 Chitin/Chitosan ........................................................................167
      6.2.4.3 Cell Wall Proteins ......................................................................168
  6.3 Regulation of capsule induction in specific environments ......................169
  6.4 Signal transduction pathways that induce capsule ...................................170
    6.4.1 Low Iron ............................................................................................170
    6.4.2 Host CO$_2$ levels ..............................................................................177
    6.4.3 Ambient pH .......................................................................................179
    6.4.4 Low glucose and low nitrogen ............................................................181
    6.4.5 Stress ..................................................................................................183
    6.4.6 Hypoxic stress ....................................................................................190
    6.4.7 Unconnected Genes and Conditions ..................................................192
      6.4.7.1 $TUP1$ .........................................................................................192
List of Tables

Table 1. Strains used in this thesis ................................................................. 27
Table 2: Identification of \textit{C. neoformans} components of the SAGA complex .............. 32
Table 3: Representative genes regulated by Gcn5 that may be involved in host response. .................................................................................................................. 45
Table 4: Subset of Rim101-dependent gene expression in capsule-inducing conditions. .......................................................................................................................... 72
Table 5: Differentially regulated cell wall genes between wild type and \textit{rim101}Δ strains. .............................................................................................................................. 107
Table 6. Normalized RNA counts from nanoString analysis ........................................ 141
Table 7. Genes involved in capsule synthesis ........................................................ 160
Table 8. Genes involved in cell wall biogenesis and capsule attachment ................. 165
List of Figures

Figure 1: Identification of *C. neoformans* histone acetyltransferase Gcn5 .......................... 31

Figure 2: *C. neoformans* Gcn5 has specific roles in high temperature growth and FK506 resistance .................................................................................................................................................................................. 36

Figure 3: Gcn5 is required for capsule attachment to the cell ......................................................... 38

Figure 4: Virulence analysis of the gcn5Δ mutant strain ................................................................. 40

Figure 5. Garcinol phenocopies the gcn5Δ mutant ........................................................................ 42

Figure 6: *C. neoformans* Rim101 is required for capsule attachment ........................................... 64

Figure 7: Rim101 retains conserved phenotypes from other fungal species ......................... 66

Figure 8: Rim101 localization is dependent on PKA and Rim20 ...................................................... 69

Figure 9: Western blot analysis of Rim101 in rim101 and pka1Δ mutant backgrounds .................... 71

Figure 10: Rim101 is involved in the response to low iron stress .................................................... 74

Figure 11: Virulence analysis of the rim101Δ mutant strain ........................................................... 76

Figure 12: Rim101 effects on *C. neoformans* virulence, immune cell infiltration, and inflammation .................................................................................................................................................................................. 98

Figure 13: Rim101 regulates *C. neoformans* cell size in vivo ....................................................... 101

Figure 14: Rim101 mediates innate immune cell responses ............................................................ 103

Figure 15: Rim101 regulates cell wall components ........................................................................ 105

Figure 16: Rim101 regulates capsule attachment ............................................................................. 112

Figure 17: The rim101Δ strain induces different TNF-α responses than the wild type ................. 114

Figure 18. Pairwise correlation analysis of the entire transcriptomes of the rim101Δ and pka1Δ mutant strains .................................................................................................................................................................................. 132

Figure 19. The rim101Δ and pka1Δ strain have both overlapping and divergent phenotypes .................................................................................................................................................................................. 134

Figure 20. Rim101 binds a conserved motif ..................................................................................... 137
Figure 21. Chromatin immunoprecipitation to detect GFP-Rim101 DNA binding ........ 139

Figure 22. Variation in gene expression and localization due to induction in tissue culture medium .................................................................................................................................................. 143

Figure 23. *In vivo* profiling of gene expression ......................................................................................................................... 145

Figure 24. Different inducing conditions result in varying degrees of encapsulation in the wild type strain. .................................................................................................................................................. 170

Figure 25. *C. neoformans* signal transduction networks that respond to iron, glucose, physiological CO2, and host pH signals. .................................................................................................................................................. 173

Figure 26. Elements of MAPK cascades in *C. neoformans* and their roles in capsule regulation. .................................................................................................................................................. 185

Figure 27. Signal transduction cascades that regulate titan cell formation ............. 203
Acknowledgements

Graduate school has been a tremendous and wonderful experience and my happiness levels have been higher than I could ever have hoped for. Firstly, I am grateful to Andy, who has been the best mentor. He has been a role model for me, both as a scientist and as a full human being and I feel very lucky for the opportunity to be in his lab and learn from him. I knew, after a week, that I wanted to be in the Alspaugh lab, and I have never regretted this decision.

I am thankful for all of the Alspaugh lab members, who have all contributed to an amazing lab feeling. I am grateful for Connie, who kept me on track and laughed when my life was in shambles from blank western blots and empty clones. She helped me make good choices in life and in science. I am grateful for Kyla, who did not flinch from a mouse-filled summer. I was so happy to have a partner on the dark side of the lab. I am grateful for Diana, whose sense of adventure led her to willingly get in my broken down car and drive into the country before she even knew me. I am grateful for Liz, who paved the way and introduced me to Crypto. I am grateful for Mike, who taught me how to work with Cryptococcus when I was just starting. I am so grateful that these wonderful people were in my life.

I am also grateful for all of the scientific advice I received from my committee. After every meeting, I was thankful for the chance to have such excellent scientists looking at my research and helping me think of ways to ask interesting questions and push the science further. I am also thankful for our amazing collaborators, especially Stephanie, who taught me so much and allowed me to do things I could not have otherwise.
I am thankful for my family. Graduate school was never a question, and their support and love has been unwavering. Lastly, I am thankful for Matt, who has been my partner in all things. None of this would have been possible without him.
1. Introduction

1.1 Cryptococcus neoformans is an opportunistic human fungal pathogen

*Cryptococcus neoformans* is a human fungal pathogen that causes nearly 1 million new infections in immunocompromised individuals every year, leading to over 600,000 deaths (281). The fungus is environmentally ubiquitous; a study of New York children revealed that all of the children, regardless of HIV status, had antibodies against *C. neoformans* capsule (2). However, the public health burden from *C. neoformans* is expanding due to the increasing spread of the HIV/AIDS epidemic and the use of immunosuppressive drugs. Isolates of *C. neoformans* have been collected globally from soil, trees, and pigeon guano, all of which provide drastically different environmental stimuli than a human host (316). The capacity of *C. neoformans* to sense and respond to the host environment is key for its ability to cause disease.

Individuals are usually colonized with *C. neoformans* through inhalation of either spores or desiccated yeast cells from the environment (353). There have been no documented cases of human-to-human transmission. In many cases, individuals who inhale cryptococcal cells will clear the infection without any symptoms. However, there is evidence that in some cases, *C. neoformans* persists as granulomas in the lungs for years, and the host produces an antibody response to these fungal cells (122). These dormant infections can then reactivate after a change in the immune status of the host, but it is still unclear whether most disease is due to reactivation or to a new infection.

During active infection in an immunocompromised host, the impaired immune system cannot contain *C. neoformans* within the lungs. The cells are then able to disseminate throughout the body and cross the blood-brain barrier. An important aspect
of cryptococcal disease is the ability of this fungus to proliferate in the brain and cerebrospinal fluid. This proliferation leads to meningoencephalitis that is uniformly fatal if untreated. Even in developed countries where patients are treated with first line antifungal and antiretroviral therapies, the mortality rate is 12%, and the antifungal drugs have significant side effects (210, 281). In resource-limited regions of the world, the mortality rate can reach 50 to 70% (281).

To understand the pathogenesis of C. neoformans, it is important to look at both the host and the fungal responses during infection. In mouse models of cryptococcosis, wild type cryptococcal strains produce a strong Th2-biased immune response, with increased levels of interleukin 4 (IL-4) and IL-5, favoring persistence of the infection (374). In contrast, cryptococcal strains that induce Th1 immune responses are generally cleared from the lung (273). Artificially biasing the immune response towards Th1 cytokines, using a cryptococcal strain that produces murine interferon-γ, results in increased clearance of cryptococcal cells and immunity against subsequent cryptococcal infections (375). The decrease in Th1-type cell mediated immune responses in AIDS patients may explain their increased susceptibility to cryptococcal infections compared to patients with other types of immune deficiencies (374).

These immunological studies demonstrate that microbial persistence and continued fungal proliferation can lead to symptoms of disease. However, an exuberant inflammatory response to cryptococcal cells can also result in host damage. In AIDS patients treated with antifungals and antiretrovirals, the recovered immune system can over-react to the remnants of a cryptococcal infection. This overactive inflammation is known as immune reconstitution inflammatory syndrome (IRIS), and it is a major consideration in the treatment of AIDS patients with cryptococcosis. Recent studies have
noted that between 8 and 50% of AIDS patients develop IRIS (30, 35, 321). The inflammatory immune response in these IRIS patients is characterized by increased levels of IL-6 and C-reactive protein (CRP) during the IRIS events and a lack of Tumor Necrosis Factor-α (TNF-α) and other Th1 cytokines prior to the start of antiretroviral therapies (35). Treatment of IRIS can include administration of steroids to dampen the systemic inflammatory response and prevent further damage to host tissues (321). This illustrates the necessity of careful examination of host responses in the treatment of cryptococcal disease.

1.2 Human colonization requires adaptation to multiple stresses

1.2.1 Responses to host environmental stresses

There are a number of stresses present in the human host that require significant adaptation on the part of the fungal cell to allow for proliferation. For example, most fungi are unable to grow at human body temperatures, thus precluding them from pathogenesis under normal circumstances. C. neoformans uses the Ras, MAPK, Protein kinase C, and calcineurin signaling cascades to adapt to high temperature growth, allowing for proliferation at 37°C. Among other processes, this proliferation involves careful regulation of actin and septin localization, trehalose production, and chitin and chitosan content in the cell wall (5, 20, 24, 25, 196, 256).

Another mechanism of host resistance to microbes is the sequestration of iron. The human host is able to keep iron levels extremely low (10^{-18} M) through constitutively expressing iron-binding proteins such as transferrin and lactoferrin, thus depriving microbes of this essential metal (177, 367). Additionally, ferric iron is insoluble at physiological pH. To adapt to these low iron conditions, C. neoformans uses the Cft1 iron permease, Cfo1 ferroxidase, and Cig1 mannoprotein to allow for growth on heme,
transferrin, and lactoferrin as iron sources (44, 180, 181). It can also use the Sit1 siderophore transporter to obtain iron captured by siderophores from other organisms (340). To reduce ferric iron to ferrous iron, C. neoformans uses cell surface reductases, extracellular 3-hydroxyanthranilic acid, and melanin (177, 260).

1.2.2 Mechanisms to combat host immune responses

1.2.2.1 Macrophages

C. neoformans is a facultative intracellular pathogen, and intracellular proliferation and survival have also been connected with the ability of C. neoformans to cross the blood-brain barrier via intracellular transport across the endothelial cell layer (62, 223). The intracellular proliferation rate has also been correlated with virulence levels of a related species, C. gattii (226). However, C. neoformans is still subject to attack and killing by immune cells (110). Therefore, the interactions of cryptococcal cells with macrophages and other phagocytes are important aspects of pathogenesis.

Recent studies have hypothesized that cryptococcal cells are present in the soil and subject to predation by amoeba (334, 335). Survival and resistance to predation may explain the evolutionary pressures behind the ability of C. neoformans cells to resist killing by macrophages within the host. The ability of Cryptococcal cells to proliferate within macrophages or amoeba requires resistance to oxidative stress, nitrosative stress, and acidic pH (9, 105, 386). To adapt to oxidative stress, C. neoformans produces superoxide dismutases and peroxidases to degrade hydrogen peroxide. Additionally, the cell produces melanin, which is induced via the cAMP, Hog1 and Pkc1 signaling cascades (18, 81, 154, 363). Melanin can act as a sink for reactive oxygen species. Nitrosative stress also activates the Hog1 and Pkc1 pathways (39, 129). Additional input from the Atf1 and Yap4 transcription factors induce the expression of the
thioredoxins that prevent damage from the reactive nitrogen (241, 242). Because of these adaptations, the cryptococcal cells can proliferate in the phagolysosome instead of being killed, often leading to lysis of the macrophage.

The ability to escape macrophages without killing either the macrophage or the fungal cell is a newly described phenomenon called vomocytosis (225). During this non-lytic exit, the cryptococcal cell induces phagosome fusion with the plasma membrane, allowing for escape (172). This method of escape also prevents the release of cytokines from a lysed macrophage, thus preventing activation of a more inflammatory immune response.

1.2.2.2 Capsule

The major mechanisms for avoiding activation of immune responses and evading phagocytosis are related to the induction of a polysaccharide capsule. This polysaccharide capsule is one of the major virulence factors of C. neoformans and most strains without capsule are unable to cause disease in murine models of infection (54-57). C. neoformans induces capsule in response to a number of host-specific environmental stimuli such as serum, low iron, physiological CO₂ levels, and physiological pH (pH 7.4) (17, 140, 218, 350, 385, 387). The primary components of capsule are glucuronoxylomannan (GXM) and glucuronoxylomannogalactan (GXMGal), which are long mannose polysaccharides that are modified with xylose and O-acetyl residues (92). Further description of the structure of capsule can be found in Chapter 6.

The capsule plays multiple roles during infection, both actively and passively modulating immune responses and host damage. Accumulation of capsule around the cell prevents phagocytosis, as evidenced by the increased rate of phagocytosis of acapsular strains (193, 223). The capsule may also increase antioxidant defenses within
the macrophage (386). During cryptococcal infections, capsule is also thought to prevent leukocyte migration into the lungs, dampening the host immune response to the cryptococcal cells (94). Shed GXM can also act as a potent anti-inflammatory component; bacterially induced inflammation in mice can be ameliorated by treatment with cryptococcal capsule (291). Additionally, capsule can also be used as a treatment for arthritis (240, 245, 343). However, soluble capsule has also been identified as a direct cause of host damage. Accumulation of capsule in the cerebrospinal fluid can lead to increased intracranial pressure, thus exacerbating the cryptococcal meningoencephalitis (118).

In addition to its active roles in preventing inflammation, capsule also plays an important role in masking cell wall structures from the host immune system. Many fungal cell wall components, including glucans, mannoproteins, and chitin, are pathogen-associated molecular patterns that are potent stimulators of inflammation (204). Although the exact immune response to the various cryptococcal cell wall components has not been thoroughly examined, Chapter 4 contains evidence of the importance of masking during cryptococcal infections.

This thesis is focused on examining the ability of *C. neoformans* to transcriptionally induce capsule accumulation around the cell in response to the human host environment. One of the major pathways involved in the transcriptional regulation of capsule induction is the cyclic AMP / Protein Kinase A (cAMP/PKA) pathway. A discussion of the other pathways involved in the transcriptional regulation of capsule can be found in Chapter 6.
1.3 Conservation and divergence of the cAMP/PKA pathway

Almost all organisms, ranging from bacteria to higher eukaryotes, use cAMP as a secondary messenger to translate signals from environmental stimuli into activation of downstream effector proteins (233). In fungi, cAMP signals through Protein Kinase A (PKA), which then phosphorylates downstream targets to allow for adaptation to external signals. Despite maintaining a high degree of conservation in the core elements of this signaling pathway, the upstream environmental signals that activate PKA and the downstream responses to this stimulus are highly divergent between different fungal species (120).

In S. cerevisiae and C. albicans, adenylyl cyclase is activated by signaling through G-protein coupled receptors and Ras in response to low glucose (120). C. albicans also uses the cAMP pathway to respond to serum, N-acetylglucosamine, and CO₂ levels (120, 233). In C. neoformans, however, the cAMP pathway does not respond to activation of Ras, and the phenotypes of a ras1Δ mutant are not rescued by addition of exogenous cAMP (5, 6, 8). Instead, the C. neoformans cAMP pathway responds to physiological CO₂ levels via a carbonic anhydrase (Can2) and amino acids via the Gpr4 G-protein coupled receptor (17, 243, 379). In all of these organisms, production of cAMP causes the release of the catalytic subunits of PKA from the inhibitory subunits, allowing the freed PKA to phosphorylate a conserved motif (R/K-R/K-X-S/T) in its downstream target proteins.

The downstream target proteins of Pka1 vary widely between different fungal species. Each pathogenic fungus that uses cAMP/PKA signaling to respond to the host requires different phenotypic outputs to cause disease. C. albicans uses the cAMP/PKA pathway to regulate proteins involved in hyphal growth, which is a necessary component
of invasive candidiasis (219). The *C. neoformans* cAMP/PKA pathway induces two important virulence phenotypes, melanin and encapsulation (81). This is just one example of how pathogens have adapted conserved signaling cascades, resulting in highly specific responses to the host.

### 1.4 Summary of the work included in this thesis

At the beginning of this project, we had evidence that the cAMP/PKA pathway in *C. neoformans* transcriptionally regulates some of the genes required for capsule synthesis. PKA phosphorylation of transcription factors had also been observed in other fungal species, including in *S. cerevisiae*, suggesting that a similar network structure could also occur in *C. neoformans* (278). We hypothesized that there would be specific transcription factors that respond to PKA phosphorylation to regulate the induction of capsule around the cell. The goal of this thesis was to identify these potential targets and examine their regulation by PKA.

The first approach was to perform a bioinformatic search of the *C. neoformans* genome database to identify potential transcriptional regulators that have the consensus sequence for PKA phosphorylation. At the time, the *C. neoformans* genome was incompletely annotated, which limited our ability to identify potential transcription factors. We were able to obtain a set of putative DNA binding proteins that we then examined for the presence of the conserved R/K-R/K-X-S/T PKA phosphorylation motif. This analysis resulted in 34 candidate downstream targets of Pka1.

The next step was to examine the capsule phenotype of these target genes using the deletion collection created by the Madhani lab (221). We initially examined the capsule phenotype of each candidate mutant strain by India ink counterstaining after incubation in tissue culture conditions. I then generated independent mutations of any
targets that demonstrated a change in capsule phenotype, with the goal of connecting these genes to the PKA phosphorylation pathway. I hypothesized that examining these transcription factors would allow me to extend the cAMP/PKA network and identify the mechanisms for the transcriptional regulation of capsule in response to host conditions.

Through this process, I identified two transcriptional regulators that play an important role in capsule regulation. The first, a histone acetyltransferase, was important for regulating transcription during host stress conditions (264). The identification and analysis of this protein are discussed in Chapter 2. Despite the presence of a PKA phosphorylation consensus sequence, the Gcn5 protein did not appear to be regulated by PKA. Using a GFP-tagged fusion protein, we attempted to detect phosphorylation on Western blots directly by using an anti-phosphoserine antibody and indirectly by treating the isolated protein with phosphatases (data not shown). However, we were unable to observe phosphorylation of Gcn5. Additionally, the localization of this protein was not dependent on the presence of Pka1. Although Gcn5 was not a direct target of Pka1, this was the first work examining the role of histone modifications on transcription in C. neoformans. Subsequently, other investigators have built upon this work to characterize an extensive system for histone modification that is required for C. neoformans stress response and pathogenesis (148).

The second candidate target of Pka1 was a homolog of the conserved Rim101 transcription factor. In many fungi, including the model S. cerevisiae, C. albicans, and A. nidulans species, Rim101 is proteolytically cleaved in response to neutral or alkaline pH (37, 153, 215, 238, 287, 378). The signaling cascade that cleaves Rim101 is also highly conserved. We demonstrated that the C. neoformans Rim101 homologue has conserved functions in regulating growth in alkaline and acidic pH, growth in low iron, and ionic
stress (266). Additionally, CnRim101 is required for capsule attachment to the cell (265, 266). Unlike other fungi, the *C. neoformans* Rim101 protein contains a PKA phosphorylation consensus sequence. Moreover, this PKA phosphorylation is required for localization and functionality of Rim101 (266). Despite activation through the cAMP/PKA pathway, Rim101 also requires elements of the conserved pH-sensing pathway, such as the Rim20 protein, for full function (37, 378). Chapter 3 discusses the experiments demonstrating the integration of the cAMP/PKA pathway and the pH-sensing pathway.

In many fungi, the Rim101/PacC protein is essential for virulence, especially during adaptations to human physiological pH; however, the targets of Rim101 signaling vary widely. In *C. albicans*, the Rim101 protein is a major regulator of the yeast-hyphal transition in response to physiological pH, and it is essential for virulence (13, 27, 85, 220). PacC in *A. nidulans* is required for the production of penicillin and aflatoxin (31, 43, 103). Unlike these species, in which mutation of the *RIM101* gene resulted in reduced virulence, the *C. neoformans* rim101Δ mutant displayed increased virulence in animal models of cryptococcosis (31, 83, 259, 266). In Chapter 4, I discuss how the Rim101 transcription factor is important in remodeling the cell wall in response to host environmental conditions. We hypothesize that during infection, the wild type strain undergoes cell wall changes that promote capsule binding and prevent the exposure of PAMPs. Without these changes, the host immune system overreacts, and the majority of the damage caused in a rim101Δ infection is due to the host immune responses.

We also sought to extend the cAMP/PKA network by examining the downstream targets of Rim101 signaling. This work is contained in Chapter 5. To approach this problem, we performed comparative transcriptional profiling of the wild type and rim101Δ
and pka1Δ mutants, which revealed both direct and indirect targets of the Rim101 and Pka1 proteins. Using gel shift assays and chromatin immunoprecipitation, we were able to define the Rim101 binding site in C. neoformans and show that it is highly conserved with other species. From this analysis, we were able to demonstrate direct Rim101 regulation of cell wall processes, including those identified in Chapter 4.

By investigating the downstream elements of the cAMP/PKA pathway that control capsule induction, we hoped to uncover potential targets for new anti-fungal therapies. Examining these elements allowed us to better understand how a conserved pathway can be used for a wide variety of functions, giving us insight into the range and flexibility of cAMP signaling, including the integration of a new pathway. Moreover, we gained a better understanding of how pathogenic fungi are able to interpret host-specific signals and co-ordinate the factors that cause their pathogenicity. Additionally, we found that the ability to cause disease is predicated on both the proliferation of a microbe and the response of the host.
<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>H99</td>
<td>Wild-type</td>
</tr>
<tr>
<td>TOC1</td>
<td>gcn5::nat</td>
</tr>
<tr>
<td>TOC3</td>
<td>gcn5::nat GCN5-neo</td>
</tr>
<tr>
<td>TYC33</td>
<td>ade2 cap59::ADE</td>
</tr>
<tr>
<td>TOC9</td>
<td>gcn5::nat pHIS-GFP-GCN5-neo</td>
</tr>
<tr>
<td>YKO</td>
<td>BY4741 Yeast KO collection (KAN1) MATa set gcn5::KAN1</td>
</tr>
<tr>
<td>TOY1</td>
<td>gcn5::KAN1 pYES-GAL-CNGCN5</td>
</tr>
<tr>
<td>TOY2</td>
<td>gcn5::KAN1 pYES-GAL</td>
</tr>
<tr>
<td>TOC 2</td>
<td>rim101::nat</td>
</tr>
<tr>
<td>TOC 4</td>
<td>rim101::nat RIM101-neo</td>
</tr>
<tr>
<td>TOC 10</td>
<td>rim101::nat + pHIS-GFP-Rim101-neo</td>
</tr>
<tr>
<td>TOC 12</td>
<td>ura5 pka1::URA5 + pHIS-GFP-Rim101-neo</td>
</tr>
<tr>
<td>TOC 13</td>
<td>ura5 pkr1::URA5 + pHIS-GFP-Rim101-neo</td>
</tr>
<tr>
<td>TOC 17</td>
<td>rim20::nat</td>
</tr>
<tr>
<td>TOC 21</td>
<td>rim20::nat + pHIS-GFP-Rim101-neo</td>
</tr>
<tr>
<td>TOC 18</td>
<td>rim101::nat + pHIS-GFP-Rim101-S773A-neo</td>
</tr>
<tr>
<td>TOC 20</td>
<td>ura5 pkr1::URA5 + pHIS-GFP-Rim101-S773A-neo</td>
</tr>
<tr>
<td>TOC 22</td>
<td>rim20::nat: + pHIS-GFP-Rim101-S773A-neo</td>
</tr>
<tr>
<td>JKH 7</td>
<td>ura5 pka1::URA5</td>
</tr>
<tr>
<td>CDC 7</td>
<td>ura5 pkr1::URA5</td>
</tr>
<tr>
<td>TYCC 33</td>
<td>ade2 cap59::ADE</td>
</tr>
<tr>
<td>TOC 106</td>
<td>rim101::nat + pRim101-GFP-Rim101</td>
</tr>
</tbody>
</table>
2. Cryptococcus neoformans histone acetyltransferase Gcn5 regulates fungal adaptation to the host

Chapter 2 was modified from a manuscript (of the same title) published in Eukaryotic Cell 9 (8): 1193-1202 (2012). The authors were Teresa R O'Meara, Christie Hay, Michael S. Price, Steve Giles, and J. Andrew Alspaugh.

2.1 Introduction

Microorganisms must rapidly induce and repress various transcriptional networks in order to adapt to the stressful conditions of the infected host. One aspect of this rapid transcriptional response is the ability to remodel chromatin, allowing transcription factors access to the promoters of important stress response genes. Acetylation of specific lysine residues of histone proteins is one mechanism for chromatin remodeling that leads to altered transcription (360). Acetylated histones are preferentially associated with regions of active transcription, and they are thought to recruit the assembly of transcriptional complexes (for reviews, see (23) and (141)). Mutation of histone proteins to prevent acetylation results in defects in transcription and cell growth (144, 224, 329, 392). In eukaryotes, histone proteins are acetylated by the Gcn5 protein as part of larger, multi-subunit, chromatin remodeling complexes (47, 97, 141, 311).

These large histone acetyltransferase complexes are recruited to specific gene promoters to modify local chromatin structure and regulate transcription. In the budding yeast Saccharomyces cerevisiae, the Spt3-Ada2-Gcn5 (SAGA) complex is involved in global transcriptional regulation. However, further studies on SAGA function, through mapping the localization of the complex to specific loci, has shown that binding is enriched at particular stress-responsive genes. In S. cerevisiae, Gcn5 is involved in regulating transcriptional responses to common environmental stresses, such as high
temperature, oxidative damage, high osmolarity, and nutrient deprivation. In the fission yeast *Schizosaccharomyces pombe*, Gcn5 is involved in a narrower group of stress responses and plays an important role in resistance to salt. Microarray studies in both of these species revealed that acetylation by the SAGA complex is able to remodel the chromatin to transcriptionally regulate genes important for responding to environmental stresses, and that these genes tend to be coordinately regulated (161).

*Cryptococcus neoformans* is an opportunistic human fungal pathogen that causes meningoencephalitis in immunocompromised hosts. Inside the host, *C. neoformans* lives extracellularly as well as within phagocytic cells. One way in which it responds to these varied stresses is by inducing important virulence factors such as the antioxidant melanin and antiphagocytic polysaccharide capsule (7, 81, 393). It also adapts to grow under conditions of high temperature, oxidative stress, high concentrations of salt, human physiological pH, and high levels of CO₂. Previous work in *C. neoformans* determined that the Gpa1/cAMP and Hog1/MAPK pathways, among others, are important in regulating responses to these common stresses by leading to the transcriptional activation of stress-related genes (192). However, the role of chromatin remodeling in the transcriptional response of *C. neoformans* to stress is still unknown. Therefore, in this work we examine the function of *C. neoformans* Gcn5 in adaptation to the host environment, paying particular attention to its role in transcriptional regulation of host stress-response genes. We demonstrate that histone acetyltransferase activity is necessary for *C. neoformans* response to specific conditions associated with the host environment, and it is therefore essential for virulence.
2.2 Results

2.2.1 Identification of the *C. neoformans* homologue of *S. cerevisiae* Gcn5

In a search of the *C. neoformans* genome for transcriptional regulators that may be involved in the response to host stresses, we identified the *C. neoformans* homologue of the *S. cerevisiae* acetyltransferase protein Gcn5 (Gene ID CNAG_00375). Compared with *S. cerevisiae*, the *C. neoformans* Gcn5 protein has a conserved histone acetyltransferase domain (71% identity) and a conserved bromodomain (53% identity) (Figure 1A). The bromodomain of Gcn5 is thought to be involved in the interactions between the acetyltransferase and the histone, and is required for stabilizing the association between other components of the SAGA complex and the promoters of target genes (38, 339). Of the 19 predicted *C. neoformans* proteins containing an acetyltransferase domain, only the putative Gcn5 also contains a bromodomain. Comparison of the *C. neoformans* protein with *C. albicans* Gcn5 showed similar sequence conservation for these two domains. Further *in silico* analysis revealed that the *C. neoformans* genome also contains orthologues for the majority of the other elements of the SAGA complex, including the major components Spt3 and Ada2p (Table 2). Notably, it is missing a clear orthologue of Spt20, which is essential for the structural integrity of the SAGA complex in *S. cerevisiae*. ClustalW alignment showed that the *S. cerevisiae* and *C. neoformans* orthologues of Gcn5 have the highest degree of sequence similarity among all of the SAGA complex proteins.
Figure 1: Identification of C. neoformans histone acetyltransferase Gcn5

A. Alignment of the Gcn5 proteins of S. cerevisiae, C. albicans and C. neoformans shows highest homology in the acetyltransferase domain and the bromodomain. The C. neoformans protein has a diverged N-terminal region.

B. C. neoformans GCN5 complements the S. cerevisiae gcn5Δ mutant. The CnGCN5 cDNA was cloned into the yeast expression vector pYES under a GAL1 promoter, and transformed into the S. cerevisiae gcn5Δ mutant strain. Cells containing the CnGCN5 gene or an empty vector were streaked onto inducing (galactose) or non-inducing (glucose) medium and incubated at 25°C for 2 days or 30°C for 3 days.
Table 2: Identification of C. neoformans components of the SAGA complex

<table>
<thead>
<tr>
<th>S. cerevisiae protein</th>
<th>C. neoformans Gene ID</th>
<th>Percent Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gcn5</td>
<td>CNAG_00375</td>
<td>44</td>
</tr>
<tr>
<td>Spt3</td>
<td>CNAG_05290</td>
<td>34</td>
</tr>
<tr>
<td>Tra1</td>
<td>CNAG_07377</td>
<td>29</td>
</tr>
<tr>
<td>Spt7</td>
<td>CNAG_03850</td>
<td>16</td>
</tr>
<tr>
<td>Ada3</td>
<td>CNAG_04674</td>
<td>18</td>
</tr>
<tr>
<td>Taf5</td>
<td>CNAG_05428</td>
<td>27</td>
</tr>
<tr>
<td>Spt8</td>
<td>CNAG_06597</td>
<td>30</td>
</tr>
<tr>
<td>Spt20</td>
<td>no homologue</td>
<td></td>
</tr>
<tr>
<td>Taf12</td>
<td>CNAG_06861</td>
<td>19</td>
</tr>
<tr>
<td>Taf6</td>
<td>CNAG_02536</td>
<td>19</td>
</tr>
<tr>
<td>Ada1</td>
<td>no homologue</td>
<td></td>
</tr>
<tr>
<td>Ada2</td>
<td>CNAG_01626</td>
<td>37</td>
</tr>
<tr>
<td>Sgf29</td>
<td>CNAG_06392</td>
<td>16</td>
</tr>
<tr>
<td>Taf10</td>
<td>CNAG_01972</td>
<td>21</td>
</tr>
<tr>
<td>Taf9</td>
<td>CNAG_07565</td>
<td>29</td>
</tr>
</tbody>
</table>

2.2.2 C. neoformans Gcn5 complements a S. cerevisiae histone acetyltransferase mutant

Despite conserved functional domains, the CnGcn5 protein has a predicted extended N-terminal region that is quite divergent from the S. cerevisiae Gcn5 protein (27% identity). To determine if CnGcn5 is a functional Gcn5 orthologue, we generated a transgenic S. cerevisiae gcn5Δ strain expressing CnGcn5. The CnGcn5 cDNA was expressed under the ScGAL7 inducible promoter, using the pYES yeast expression vector. The S. cerevisiae gcn5Δ mutant is unable to grow at 35°C, and exhibits slow growth even at 25°C. Under inducing conditions, the transgenic strain expressing CnGcn5 was able to grow well at 25°C after two days, unlike the Scgcn5Δ mutant strain. At 30°C, expression of CnGCN5 also supported growth of this strain after 3 days. The transgenic strain failed to grow at 30°C on glucose-containing medium, in which CnGCN5 expression from the GAL7 promoter would be repressed (Figure 1B).
established a conserved function between \textit{CnGCN5} and \textit{ScGCN5}, suggesting that CnGcn5 is also a histone acetyltransferase.

### 2.2.3 Gcn5 is involved in stress tolerance

The \textit{S. cerevisiae} and \textit{S. pombe gcn5Δ} mutants display defects in the cellular response to many stresses, including elevated temperatures, high salt concentrations, and nutrient deprivation. The effect of Gcn5 on the cell response to stress is linked to its activity as a histone acetyltransferase and transcriptional regulator. The SAGA complex is recruited by activator proteins such as Gcn4 to the promoters of stress-response genes, which leads to the transcriptional induction of these genes (161, 383). During osmotic stress, Gcn4 has been shown to bind to the promoter of HAL1, a regulator of the ion transporter ENA1, and is necessary for the induction of HAL1 gene expression (283).

To investigate the role of Gcn5 in \textit{C. neoformans}, we created the \textit{gcn5Δ} strain TOC1 in which we replaced the entire gene coding region with the nourseothricin resistance marker. Deletion of the gene was confirmed by both PCR and Southern blot analysis. To ensure that all mutant phenotypes observed were due to deletion of \textit{GCN5}, we complemented the strain by integrating the wild-type allele into the \textit{gcn5Δ} mutant strain, creating \textit{gcn5Δ+GCN5} strain TOC3. This strain complemented all observed mutant phenotypes.

To determine if the \textit{C. neoformans} Gcn5 protein controls responses to stresses that are relevant for pathogenesis, we examined growth of the mutant strain under various host-stress conditions. One of the major stresses that \textit{C. neoformans} encounters in the host is high temperature, and strains with defects in high temperature growth are generally avirulent (288). When incubated on rich medium at 37°C, the \textit{gcn5Δ} strain had a significant delay in growth compared to wild type (Figure 2A). In contrast,
the C. neoformans gcn5Δ strain had no defect in growth at 30°C, suggesting a role in stress-specific responses, instead of general transcriptional activation.

Concomitantly, we observed that the gcn5Δ mutant strain has increased sensitivity to oxidative stress at 30°C. Reactive oxygen species (ROS) are a primary mechanism of host defense against microbial invasion (134, 386). Hydrogen peroxide disc diffusion assays showed a larger zone of inhibition for the gcn5Δ mutant (50mm) compared to the wild type (45mm). Incubation of cells with 5mM hydrogen peroxide in liquid culture also showed that the gcn5Δ mutant strain is more sensitive than wild type. When strains were incubated in Dulbecco’s modified Eagle’s medium then subjected to 5mM hydrogen peroxide for 3 hours, the wild type cells had a 83.2% survival rate, whereas the gcn5Δ mutant cells had 74.9% survival rate. In contrast, the gcn5Δ strain had no growth defect compared to wild type when incubated with other stresses, such as low nutrient (YNB) or high salt concentrations (Figure 2B), or with the cell wall stressors SDS and caffeine (data not shown). There was also no defect for the gcn5Δ mutant strain in melanin production, an important virulence factor, when incubated on the melanin-inducing Niger seed medium at 30°C (data not shown).

2.2.4 Gcn5 is localized to the nucleus

In some protozoan parasites, the N-terminal region of Gcn5 orthologues are used as a nuclear importation signal, and nuclear localization is necessary for function as a histone acetyltransferase (29). Therefore, In order to determine whether Gcn5 function is regulated by localization, we created a GFP-Gcn5 fusion protein and examined localization under inducing and non-inducing conditions. We fused the green fluorescent protein gene to the N-terminus of the GCN5 gene and expressed it under control of a
constitutive histone promoter. We introduced this plasmid (pTO1) by biolistic transformation into the gcn5Δ mutant strain to create the gcn5Δ+GFP-GCN5 strain TOC5. We showed that the fusion protein was functional by complementation of temperature sensitivity and other phenotypes (data not shown). Using epifluorescent microscopy, we observed a nuclear pattern of localization after growth for 24 hours in YPD at both 30°C, and under high temperature stress at 37°C. (Figure 2C). Therefore, in contrast to some other pathogens, the CnGcn5 localization appears to be constitutively nuclear.
**Figure 2: C. neoformans Gcn5 has specific roles in high temperature growth and FK506 resistance**

**A. Gcn5 is involved in high temperature growth.** 1x10⁵ cells were 5-fold serially diluted on YPD plates and incubated at 30°C, 37°C, and 39°C for 2 days.

**B. CnGcn5 is not involved in low nutrient or high salt growth.** 1x10⁵ cells were 5-fold serially diluted on YNB plates, or YPD plates containing 1.5M KCl or 0.5M CaCl₂ and incubated at 30°C for 2 days.

**C. Gcn5 is localized to the nucleus at both 30°C and 37°C.** The pattern of GFP-Gcn5 localization was visualized by DIC and fluorescent microscopy, and the nucleus was stained with DAPI.

**D. Gcn5 is involved in resistance to FK506, but not fluconazole.** 1x10⁵ cells of wild type and gcn5Δ cells were tested for MIC of FK506 and fluconazole in YPD at 30°C.
2.2.5 Gcn5 regulates capsule in *C. neoformans*

Upon entering the infected host, *C. neoformans* induces a large polysaccharide capsule. This structure protects the fungal cells in many ways, including inhibiting phagocytic cell function and activating complement distant from the cell surface (60, 308, 352). To determine whether Gcn5 is involved in regulating this host-specific phenotype, we incubated wild type and *gcn5*Δ mutant cells in Dulbecco’s modified Eagle’s medium (DMEM) at 30°C as an inducing condition for polysaccharide capsule. To observe the capsule size, we stained the cell background with India ink and examined the zone of exclusion around the cells. Microscopic analysis of the cells established that the *gcn5*Δ mutant strain demonstrated a significantly decreased capsule size compared to wild type (Figure 3A). When the cells were incubated in DMEM at 37°C and 5% CO₂ to more accurately mimic human physiological conditions, the *gcn5*Δ mutant strain did not display visible capsule by India ink exclusion. This capsule defect is not due to the previously demonstrated growth defects at 37°C, as the *gcn5*Δ strain eventually grows to saturation at this temperature, albeit at a slower rate. The *gcn5*Δ mutant displayed markedly decreased capsular phenotype even when incubated to saturation. Additionally, it also failed to produce capsule when incubated in either low iron medium or 10% Sabourad medium, two distinct growth conditions that also induce capsule in wild type strains (data not shown).

Recent work has shown that mutants that do not demonstrate surface capsule by direct light microscopy and India ink staining may still secrete wild type levels of exopolysaccharide, presumably due to defects in capsule attachment instead of capsule secretion or biosynthesis (266). Therefore, the acapsular phenotype was further explored by testing the culture filtrate for secreted exopolysaccharide. This allowed us to
differentiate between defects in capsule production, secretion, and attachment. Strains were incubated to equal cell density in DMEM for one week at 30°C with shaking to induce capsule, after which the medium was heated to denature enzymes, and filtered to remove cellular debris and to isolate secreted capsular polysaccharides. The filtrate was separated by electrophoresis on a 0.6% agarose gel, transferred to a Nylon membrane, and immunoblotted with an anti-GXM capsular antibody (mAb18b7) (382). Compared to the wild type, the \( gcn5\Delta \) mutant strain may secrete a smaller length capsular polysaccharide, but still significantly more than the secretion-deficient and acapsular \( cap59\Delta \) strain (Figure 3B). This suggests that the capsule defect of the \( gcn5\Delta \) strain is due to a modest defect in capsule production and a more severe defect in capsule attachment.

**Figure 3: Gcn5 is required for capsule attachment to the cell**

**A. \( gcn5\Delta \) cells have a capsule defect.** Cells were incubated in Dulbecco’s modified Eagle’s medium at 30°C for 24 hours. Capsule was assessed by staining with India ink and visualizing the zone of exclusion at 63x magnification (scale bar = 10\( \mu \)M).
B. The *gcn5Δ* mutant strain is able to secrete polysaccharide. Electrophoretic mobility and quantity of shed polysaccharide from 3 *C. neoformans* strains was assessed by an immunoblotting technique of culture medium filtrate, using an anti-GXM antibody to probe for capsule as previously described. Cells were incubated in DMEM for 1 week at 30°C before filtering. Arrow indicates direction of electrophoresis. The *cap59Δ* mutant is included as a negative control as this strain releases no discernable capsular polysaccharide.

2.2.6 *C. neoformans gcn5Δ* mutant strain is avirulent

In order to be virulent, pathogens must be able to respond to the stresses encountered within the human host. To test whether the *gcn5Δ* mutant strain is able to cause disease despite defects in host stress response and capsule production, we used the murine inhalation model of cryptococcosis. 10 female A/J mice per strain were inoculated intranasally with 5x10⁵ CFU of the wild type (H99), *gcn5Δ* (TOC1) or *gcn5Δ+GCN5* (TOC3) strains. Mice were monitored for survival and sacrificed at predetermined clinical endpoints for mortality. Mice infected with the wild type or complemented *gcn5Δ+GCN5* strains had complete mortality after 18 days; there was no statistically significant difference between the strains. As expected from the *in vitro* stress sensitive phenotypes of the *gcn5Δ* mutant, mice infected with this strain survived the entire length of the experiment with little evidence of clinical disease (Figure 4).
Figure 4: Virulence analysis of the $gcn5\Delta$ mutant strain.

AJ mice were inoculated intranasally with $5 \times 10^5$ cryptococcal cells and monitored for survival.

2.2.7 A histone acetyltransferase inhibitor phenocopies the $gcn5\Delta$ mutant strain for temperature sensitivity.

In a wide range of systems from yeast to human, histone acetyltransferase activity can be inhibited by specific drugs. We examined whether one of these drugs, garcinol, a polyisoprenylated benzophenone derivative, inhibits *C. neoformans* Gcn5. Addition of this drug to wild type *C. neoformans* cells phenocopied the temperature sensitivity of the $gcn5\Delta$ mutation. Addition of 5 µM of garcinol to wild type cells caused a statistically significant growth defect at 37°C, and not at 30°C, after 24 hours incubation. At 50 µM, growth at 37°C was completely inhibited (Figure 5A). We further investigated garcinol-induced temperature sensitivity by performing a growth curve at 37°C for both the wild-type and the $gcn5\Delta$ mutant strain. Addition of 8µM garcinol caused a severe defect in growth for the wild type strain, such that after 24 hours, there was no
statistically significant difference in growth between the garcinol-treated cells and the
<code>gcn5Δ</code> mutant cells (Figure 5B). However, at previous time points, the garcinol-treated
cells had an increased growth defect compared to the <code>gcn5Δ</code> mutant strain. Treatment of
the <code>gcn5Δ</code> mutant cells with 8µM garcinol led to death of the strain after 24 hours. This
suggests that garcinol has off-target effects in addition to Gcn5 inhibition. Therefore, we
examined the effect of a second histone acetyltransferase inhibitor, anacardic acid.
Treatment with 10µM anacardic acid caused wild type cells to grow similar to the <code>gcn5Δ</code>
mutant strain for 8 hours, but the cells were eventually able to recover (data not shown).

We also tested whether compounds with antifungal activity displayed additive
effects with Gcn5 inhibition. The <code>gcn5Δ</code> strain was more sensitive than wild type to the
antifungal effects of FK506, even at 30°C (Figure 2D). FK506 acts by binding to FKBP12
and actively inhibiting the calcineurin pathway (267). FK506 has maximum efficacy at
37°C, as blocking the calcineurin pathway leads to abolishment of growth at elevated
temperatures(267, 268). This increased sensitivity to drug was not due to a general
defect in drug resistance, as the <code>gcn5Δ</code> mutant strain and wild type had similar MICs
against the antifungal drug fluconazole at 30°C.
Figure 5. Garcinol phenocopies the gcn5Δ mutant.

A. Treatment with garcinol inhibits the growth of wild type cells at 37°C. 1x10^5 cells were treated with 0, 0.05, 0.10 and 0.50μM garcinol and incubated at 37°C for 24 hours before spotting onto YPD.

B. Treatment of wild type cells with 5mM garcinol phenocopies the gcn5Δ mutant temperature sensitivity. 2x10^5 cells of each strain were incubated at 37°C in YPD for 48 hours, then counted with a haemocytometer.
2.2.8 Gcn5 regulates the expression of a number of genes involved in host stress response.

Phenotypic analysis demonstrated that the *C. neoformans gcn5Δ* mutant is defective in responding to several host-related stresses, including elevated temperature, oxidative stress, and capsule inducing conditions. These defects are likely due to an inability to regulate adaptive transcriptional responses through histone acetyltransferase activity. To determine which processes are transcriptionally regulated by Gcn5, we performed comparative transcriptional profiling between the wild type and the *gcn5Δ* mutant strain. Both strains were incubated for three hours in DMEM at 37°C to attempt to mimic host conditions, after which RNA was purified and hybridized against a *C. neoformans* Serotype A/D genome microarray, which uses 7738 70-mer probes to represent all predicted genes in the *C. neoformans* genome. Our results indicate that 417 putative genes in this growth condition are significantly differentially regulated in the *gcn5Δ* mutant strain (Table 3).

We examined the transcriptional profiling data to identify genes that may offer mechanisms for the observed phenotypic defects in the *gcn5Δ* mutant strain. When examining mechanisms for the capsule defect, we found that Gcn5 regulates the expression of multiple glucosidases, including Kre61, which are involved in cell wall biosynthesis and capsule architecture (131, 132). RT-PCR results confirmed a 6-fold decreased expression of Kre61 in the *gcn5Δ* strain compared to wild type (data not shown). β-glucan synthases also had lower expression in the mutant strain, suggesting that alterations in cell wall structure may occur in the *gcn5Δ* mutant strain, with resulting defective capsule attachment. Interestingly, these potential cell wall changes did not affect resistance to SDS, caffeine or sorbitol.
Two categories of genes that demonstrated increased transcription in the wild type strain compared to the \textit{gcn5}Δ mutant strain (potential Gcn5-dependent induction) were those encoding transcription factors and kinases. Therefore, in addition to directly regulating gene expression by histone acetylation, Gcn5 may also indirectly regulate gene expression and protein activity. In contrast, several mitochondrial and ribosomal genes showed decreased expression in the wild type, suggesting that Gcn5 normally acts to repress these genes.

Two putative catalase A proteins demonstrated decreased expression in the \textit{gcn5}Δ mutant strain, perhaps leading to the increased sensitivity of the \textit{gcn5}Δ mutant to hydrogen peroxide. In addition, FKBP12, the binding target of the drug FK506, showed increased expression in the \textit{gcn5}Δ mutant compared to wild-type, perhaps resulting in increased sensitivity to FK506 since FK506-FKBP12 binding is the mechanism of this drug’s toxicity to the cell.
Table 3: Representative genes regulated by Gcn5 that may be involved in host response.

This list contains representative functionally annotated genes whose expression was at least 2-fold different in wild type vs. gcn5Δ mutant strains after induction in DMEM for 3 hours, with p-value < 0.05.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Annotation</th>
<th>Fold change (Wild type/gcn5Δ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capsule</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CND06160</td>
<td>glucosidase, β-glucan synthesis-associated protein Kre61</td>
<td>8.27</td>
</tr>
<tr>
<td>CND00120</td>
<td>mannosyltransferase, putative</td>
<td>4.21</td>
</tr>
<tr>
<td>CNJ03090</td>
<td>Glycogen debranching enzyme, putative</td>
<td>2.43</td>
</tr>
<tr>
<td>CNH02490</td>
<td>glycoside hydrolase, cytoplasm protein</td>
<td>2.38</td>
</tr>
<tr>
<td>CNE02630</td>
<td>glucan 1,4-α-glucosidase, putative</td>
<td>2.21</td>
</tr>
<tr>
<td>CNA07240</td>
<td>CAP64 gene product - related</td>
<td>2.18</td>
</tr>
<tr>
<td>CND04970</td>
<td>glycosyltransferase family 31</td>
<td>2.07</td>
</tr>
<tr>
<td>CNH03390</td>
<td>α,α-trehalose-phosphate synthase (UDP-forming)</td>
<td>2.05</td>
</tr>
<tr>
<td>CNC01640</td>
<td>glucosidase, putative</td>
<td>2.03</td>
</tr>
<tr>
<td>CNAG_01341</td>
<td>mannose-6-phosphate_isomerase</td>
<td>-2.05</td>
</tr>
<tr>
<td>CND04560</td>
<td>mannose 6-phosphate isomerase</td>
<td>-2.16</td>
</tr>
<tr>
<td>CNAG_04574</td>
<td>dolichol phosphate-mannose biosynthesis</td>
<td>-4.44</td>
</tr>
<tr>
<td>Oxidative stress and drug sensitivity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNL06340</td>
<td>Catalase A</td>
<td>2.58</td>
</tr>
<tr>
<td>CNK00780</td>
<td>oxidoreductase</td>
<td>2.22</td>
</tr>
<tr>
<td>CNA05600</td>
<td>catalase A, putative</td>
<td>2.19</td>
</tr>
<tr>
<td>CNB01800</td>
<td>macrolide-binding protein FKBP12</td>
<td>-2.92</td>
</tr>
<tr>
<td>CNF03740</td>
<td>Protein kinase TOR1</td>
<td>2.73</td>
</tr>
<tr>
<td>Transcription factors and signaling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNC03320</td>
<td>transcription factor</td>
<td>7.58</td>
</tr>
<tr>
<td>CNAG_01611</td>
<td>regulator of G-protein signaling</td>
<td>6.98</td>
</tr>
<tr>
<td>CNAG_03346</td>
<td>prib protein, putative transcription factor</td>
<td>3.3</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNAG_00456</td>
<td>Na-K-Cl transporter, putative</td>
<td>7.12</td>
</tr>
<tr>
<td>CNA05130</td>
<td>Transporter ENA1</td>
<td>2.12</td>
</tr>
<tr>
<td>CNAG_01455</td>
<td>Ribosomal L39 protein</td>
<td>-5.04</td>
</tr>
<tr>
<td>CNA07960</td>
<td>Ribosomal S30 superfamily</td>
<td>-3.63</td>
</tr>
<tr>
<td>CNE01150</td>
<td>NADH-ubiquinone oxidoreductase 12 kda subunit, mitochondrial precursor, putative mitochondrial import inner membrane</td>
<td>-4.59</td>
</tr>
<tr>
<td>CNG02760</td>
<td>translocase subunit tim13, putative</td>
<td>-4.29</td>
</tr>
</tbody>
</table>
2.3 Discussion

Our results indicate that Gcn5 plays a critical role in *C. neoformans* adaptation to the host. In many organisms, Gcn5 orthologues serve as histone acetyltransferases within large, multi-subunit chromatin remodeling complexes, such as the SAGA complex. In *S. cerevisiae*, the SAGA complex is important in global transcriptional activation and in regulating the expression of highly inducible stress response genes (161). This complex is directed to the promoters of target genes by activators such as Gcn4p, Snf1p, and Gal4p. Once at the promoters, the histone acetylation of H3 and H2B allows transcriptional activation of these target genes (138, 165, 252). We have found putative homologues for the majority of the components of the yeast SAGA complex in the *C. neoformans* genome, with two exceptions: Spt20 and Ada1 were not readily identifiable by sequence homology. Of those genes present, the Gcn5 protein showed the most sequence identity, with the acetyltransferase and bromodomains sharing 71% and 53% identity respectively. *C. neoformans* has a unique extended N-terminal domain. This sequence divergence did not prevent the CnGcn5 protein from complementing the *S. cerevisiae* gcn5Δ mutant defects in high temperature growth, thus establishing a conserved histone acetyltransferase activity for CnGcn5. Although the N-termini of other Gcn5 orthologues determine regulatable subcellular localization, we have demonstrated that CnGcn5 appears to be constitutively localized to the nucleus.

*C. neoformans* exists in a very different niche from *S. cerevisiae*. As both an environmental fungus and an opportunistic pathogen, *C. neoformans* must be able to respond to a wide variety of stresses. Inside a human host, the pathogen is subjected to high CO₂, high temperatures, low iron, nutrient starvation, and osmotic stress. As a facultative intracellular pathogen, it also has to survive inside the macrophage, where it
is subjected to the acidity of the phagolysosome and the oxidative bursts of the host cells. In response to the host, \textit{C. neoformans} induces the virulence factors melanin and polysaccharide capsule, and it additionally initiates multiple signaling pathways for adapting to host stress. The Hog1/MAPK pathway uses a two-component system to sense extracellular signals and regulate expression of genes involved in osmotic, antifungal, and heat stresses, among others \cite{14, 16, 19, 192}. The cAMP pathway uses G-protein coupled receptors to sense the host environment and to activate several transcription factors, such as Nrg1 and Rim101, to regulate stress gene expression and production of capsule and melanin \cite{7, 78, 266, 300}. Additionally, the Ras and calcineurin signaling pathways are required for adaptation to high-temperature growth \cite{24, 196, 257, 268}. This demonstrates that this pathogenic microorganism uses numerous pathways to regulate adaptation to the host. However, the role of chromatin remodeling in \textit{C. neoformans} response to the host was previously unknown.

In other pathogens, the elements of the SAGA complex have recently been implicated in regulating pathways that are essential for virulence. Sellam, \textit{et al.} demonstrated that defects in the Ada2 protein in \textit{Candida albicans} leads to poor protein folding, hypersensitivity to oxidative stress and antifungals, and defects in virulence \cite{319}. In addition, although the Spt3 protein is required for pseudohyphal and invasive growth in \textit{S. cerevisiae}, \textit{spt3Δ/spt3Δ} mutants in \textit{C. albicans} are hyperfilamentous. Interestingly, the \textit{spt3Δ/spt3Δ} mutant is avirulent in animal models of candidiasis, confirming the importance of reversible yeast-hyphal transitions in this fungal pathogen, and showing the involvement of the SAGA complex regulation of gene expression in \textit{C. albicans} virulence \cite{203}. These data demonstrate that the gene targets of conserved histone acetyltransferase complexes may differ according to the environmental niche,
but the overall importance of gene regulation by histone acetylation is maintained in very divergent species. Other pathogens, such as the malaria parasite *Plasmodium falciparum*, also maintain homologues of the SAGA complex. Moreover, treatment with histone acetyltransferase inhibitors causes changes in global gene expression and parasite development, indicating that chromatin remodeling as a mechanism for responding to the host environment is not limited to fungal pathogens (80, 104).

Given these prior results, we hypothesized that chromatin remodeling and transcriptional regulation via Gcn5-mediated histone acetyltransferase activity would be an important way for *C. neoformans* to rapidly adapt to host conditions. In this study, we show that *C. neoformans* Gcn5 regulates processes required for survival within a human host, such as growth at 37°C, resistance to ROS, and capsule attachment to the cell wall (39, 105, 133, 257, 267, 268). In light of the capsule and host-stress response defects, the *gcn5Δ* mutant strain had an expected attenuation in virulence in the mouse inhalation model of cryptococcosis. We also demonstrated that histone acetyltransferase inhibitors are able to result in temperature sensitivity of *C. neoformans*, similar to mutation of the GCN5 gene.

To better understand the mechanisms underlying *gcn5* mutant phenotypes, we examined the genes that were differentially transcribed in the mutant strain compared to the wild type after incubation under *in vitro* conditions that are similar to those encountered within the infected host. We found that two putative catalase A genes had decreased expression in the *gcn5Δ* mutant strain. Previous studies indicated that overexpression of *C. neoformans* catalase genes increases resistance to hydrogen peroxide, confirming their role in protection from oxidative stress. However, none of the catalase gene mutations, either singly or in combination, had significant effects on
virulence (134). Giles, et al. concluded that this was most likely due to redundancy in oxidative stress resistance from the presence of other oxidative stress response pathways. Other studies have also shown that capsule is also important in regulating resistance to oxidative stress, presumably by acting as a buffer against the reactive oxygen species (386). In liquid culture, shed wild type capsule acts to protect cells from killing by hydrogen peroxide. Therefore, a combination of alterations in capsule attachment, capsule structure, and catalase gene expression may explain the increased sensitivity to oxidative stress in the gcn5Δ mutant strain.

Our phenotypic analysis also revealed gcn5Δ mutant defects in polysaccharide attachment to the cell wall. When we examined shed polysaccharide content, we determined that the gcn5Δ mutant strain produces and secretes capsule, but India ink staining demonstrated that the secreted polysaccharide is not being maintained at the cell wall. This is similar to defects previously documented in the rim101Δ mutant strain, where the cell is unable to anchor the capsule (266). Previous studies on the capsule have implicated cell wall biosynthesis as important in capsule attachment to the cell. A monoclonal antibody against β-glucan inhibits capsule formation around the normally encapsulated wild-type C. neoformans (301). This effect is presumably caused by preventing capsule attachment to the cell wall, although interference with capsular export could not be excluded. The α-glucan synthase enzyme activity, which is important in developing cell wall structure, is also required for C. neoformans capsule attachment to the cell surface (306). Therefore, we examined the transcriptional profiling data for cell wall biosynthesis genes that may be involved in capsule attachment. Our microarray data indicated that several β-glucosidases were differentially expressed in the gcn5Δ mutant compared to wild type. Kre61, a member of the Skn1/Kre6 family, had 8.2-fold
higher expression in the wild type than in the mutant strain, and it is one of the most highly differentially regulated genes. β-glucosidases have previously been shown to be important in cell wall biosynthesis, capsular architecture, and virulence (131, 132), although Kre61 had no major defect as a single mutant. We also observed Gcn5-dependent expression of other putative glucosidases and β-glucan synthases that may also be involved in cell wall biosynthesis and capsule attachment.

When examining our microarray data for other genes involved in host response, we determined that Tor1 and a putative calcineurin A subunit had differential expression in the wild type compared to the gcn5Δ mutant strain. We observed a 2.2-fold decrease in a putative calcineurin A gene expression in the gcn5Δ strain, which may explain some of the phenotypes we observed. The calcineurin pathway activates the transcription of genes necessary for growth at high temperatures, high concentrations of carbon dioxide or salt, and growth in alkaline pH (114, 196, 268). We also observed a 2.9-fold increase in the expression of FKBP12 in the gcn5Δ mutant strain compared to the wild type. The drug FK506 targets FKBP12 and the accumulation of this complex inhibits the activity of calcineurin (268). An increase in FKBP12 expression is likely to cause the increased sensitivity to FK506 that we observed in the gcn5Δ mutant strain.

We also demonstrated that Gcn5 is important in negatively regulating a large number of genes involved in mitochondria and ribosome processes. This is likely due to acetylation and transcriptional activation of repressors. During host-stresses, such as high temperature, reactive oxygen species, or nutrient deprivation, a common cell response is to repress metabolism and protein biosynthesis. Previous work on C. neoformans gene expression during macrophage infection showed that this condition represses 38 known components of the translation machinery (105). In the absence of
Gcn5, *C. neoformans* is unable to repress the expression of many mitochondrial and ribosomal genes. Multiple ribosomal subunits, NADH-ubiquinone oxidase proteins, and cytochrome C oxidase proteins, among others, showed increased expression in the mutant strain relative to wild type response to inducing conditions.

In summary, CnGcn5 is able to transcriptionally regulate many genes through chromatin remodeling by acting as a histone acetyltransferase. This highly conserved protein function has been co-opted by numerous organisms to regulate the expression of distinct gene sets required for survival in unique microenvironments. Among microbial pathogens, divergent species use histone modification to control the expression of those phenotypes required for survival within their particular host environment. Therefore, histone acetyltransferase inhibitors may be useful antimicrobial drug targets.

### 2.4 Methods and Materials

**Strains and Media:**

*C. neoformans* and *S. cerevisiae* strains used in this study are listed in Table 1. All *C. neoformans* mutants were created in the H99 background unless otherwise stated. Strains were maintained on rich medium (1% yeast extract, 2% peptone, 2% dextrose), and selective medium contained either nourseothricin 100mg/L Werner BioAgents, Jena-Cospeda, Germany) or neomycin (G418) (200mg/L Clontech, Takara-Bio Inc.). Niger seed medium was made as previously described (198). Stress medium was created by adding 44.1 g calcium chloride, 8.4 g lithium chloride, 111.8 g potassium chloride, 9.71 g caffeine, 0.1g SDS, or 87.6g sodium chloride to 1 L rich medium before autoclaving.

**Molecular Biology Techniques:**
Standard techniques for Southern hybridization were performed as described (318). *C. neoformans* genomic DNA for Southern blot analysis was prepared using CTAB phenol-chloroform extraction as described (293).

**Gene disruption:**

The wild-type GCN5 allele was mutated by replacing the entire locus with a nourseothricin (nat) resistance allele. The *gcn5::nat* resistance allele was created by using PCR overlap extension and biolistically transformed into the H99 strain, using previously described methods (115, 232, 344). Several independent *gcn5Δ* mutant colonies had identical phenotypes *in vitro* and showed deletion of the gene by PCR. The TOC1 strain was chosen for further study, and Southern blot analysis confirmed deletion of the GCN5 allele.

To reconstitute the wild-type allele, the GCN5 locus was amplified from H99 genomic DNA and biolistically co-transformed into the *gcn5Δ* strain with a separately amplified neomycin resistance allele to create strain TOC3, as previously described (136). The wild-type GCN5 allele was amplified with primers 5’ ATGGTACCAGCAATGAAGAC 3’ and 3’ AAAGGATGCAGGTGAACCTTA 5’, and the neomycin allele was amplified with the M13 forward and reverse primers. The reconstituted strain was tested by PCR for presence of the wild-type allele, and for reversion of phenotypes.

**Capsule induction and blotting:**

To induce capsule for India ink visualization, cells were incubated in Dulbecco’s modified Eagle’s medium (DMEM) at 30°C with shaking for 24 hours to remove high temperature effects. Cells were also incubated under the standard capsule inducing conditions of DMEM at 37°C with 5% CO₂ for 72 hours (140). Estimation of shed
exopolysaccharide was determined using a technique developed by Yoneda and Doering (382). Conditioned medium was created by shaking cells at 30°C in DMEM for 1 week before incubating at 70°C for 15 minutes, centrifuging at 1600 x g for 3 minutes, and sterile filtering with a 0.2µ filter. 15µL of conditioned medium was run on a 0.6% agarose gel for 15h at 25V, then transferred to a nylon membrane using Southern blot techniques. The membrane was air dried, blocked in Tris buffered saline + Tween (Tbs-T) and 5% milk, and probed with monoclonal antibody 18b7 (1/1000 dilution) (48). The membrane was then washed with Tbs-T, incubated with an anti-mouse peroxidase conjugated secondary antibody (1/25,000 dilution, Jackson Labs) and detected using SuperSignal West Dura Substrate (Thermoscientific).

MIC testing:

In vitro susceptibility tests of C. neoformans strains, and determinations of MIC were performed by the National Committee for Clinical Laboratory Standards (NCCLS) broth macrodilution method (253). The fungal growth inhibition assay for garcinol was performed at 0, 5, 10 and 50mM drug concentrations in YPD at 37°C. FK506 growth inhibition assay was performed at 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03 and 0µg/mL concentrations in YPD at 30°C. Fluconazole growth inhibition assay was performed at 80, 40, 20, 10, 5, 2.5, 1.25, and 0µg/mL concentrations. Drug dilutions and inoculum preparation were by the NCCLS criteria (253). Cell density was read by haemocytometer.

GFP fusion:

We created a green fluorescent protein(GFP) –Gcn5 fusion protein by cloning the Gcn5 coding sequence and terminator region into pCN50 (266) to create pTO1. The coding sequence and the terminator region of Gcn5 was amplified by using primers
modified with BamHI sites (5’-GCTGAGGATCCATGGCGCCAAAACCACGTGTGC-3’)
and (5’-GCTGAGGATCCATGCGCCCGTCGAA-3’), and then cloned into the
pCN50 plasmid to create a GFP-Gcn5 fusion protein under the constitutive histone H3
promoter (266). pTO1 was then biolistically transformed into the TOC1 strain
background to create strain TOC9.

**Microscopy:**

Bright field, differential interference microscopy (DIC) and fluorescent images
were captured with a Zeiss Axio Imager.A1 fluorescent microscope equipped with an
AxioCam mrM digital camera. To visualize capsule, cells were grown in inducing
conditions, then stained with India ink on glass slides. Images were collected at x63
magnification. To visualize GFP and DAPI, cells were incubated in ice-cold methanol for
1 minute, washed three times in PBS, and stained with DAPI before images were
collected at x63 magnification, using 488 nm wavelength for GFP fluorescence and 350
nm wavelength for DAPI.

**RNA and cDNA preparation:**

Strains were incubated to mid-logarithmic phase in 5ml of YPD then washed
three times with sterile water before incubation in DMEM for 3 hours. Cells were washed
three times before centrifugation and freezing on dry ice and lyophilizing. RNA was
prepared from lyophilized samples using the RNeasy kit (Qiagen). 20 µL of 1 µg/µL RNA
was submitted to the Duke University Microarray Core Facility. cDNA for real time-PCR
was generated using RETROscript (Ambion) using oligo-dT primer. Quantitative real-
time PCR was performed as previously described, using the constitutive GPD1 gene to
normalize the samples (266).

**Microarray and Data Analysis:**
The microarray used in this study was developed by the Cryptococcus Community Microarray Consortium with financial support from individual researchers and the Burroughs Wellcome Fund (http://genome.wustl.edu/services/microarray/cryptococcus_neoformans). RNA labeling and hybridization were performed by the Duke University Microarray Core Facility according to their established protocols for custom spotted arrays (78, 266). Data was analyzed using JMP genomics v. 4.1 (SAS institute, Cary NC) and initial background subtraction was performed. We used ANOVA normalization and FDR analysis to calculate differences between treatment effects for pairs of inducing conditions. Genes were considered for further evaluation if they had log2-transformed fold changes greater than 2-fold with a p-value <0.001 (78, 373).

The .gal file for the Cryptococcus version 2 microarray contains probes specific for Serotype A and D strains of C. neoformans. Cryptococcus genome annotation for probes specific for Serotype D strains JEC21 and B3501 were obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Cryptococcus genome annotation for probes specific for Serotype A strain H99 were obtained from the Broad Institute (http://www.broadinstitute.org/). Chromosome coordinates for individual C. neoformans Serotype D probes are genome Build 2 Version 1 specific. The .gal file is provided in a tab delimited format.

**Virulence and Data Analysis:**

The virulence of the C. neoformans strains was assessed using a murine inhalation model of cryptococcosis as described previously (77). 10 female A/Jcr mice were inoculated intranasally with 5x10⁵ C. neoformans cells of wild-type (H99), gcνΔ mutant (TOC1), or gcν5+GCN5 complemented strain (TOC3). Mice were monitored daily
for signs of infection and were sacrificed at predetermined clinical endpoints predicting mortality. The statistical significance between the survival curves of all animals infected with each strain was evaluated using the log-rank test (JMP software, SAS institute, Cary NC). Cell counts were analyzed using Student's t-test. All studies were performed in compliance with the institutional guidelines for animal experimentation.

Acknowledgements

This work was funded by NIH grant R56 AI074677 (JAA), the Duke UPGG Genetics Training Grant 5 T32 GM007754-30, and an American Heart Association MAA Spring 09 Predoctoral Fellowship 09PRE2010039 (TRO). We thank Arturo Casadevall for mAb 18b7 and the Duke Core Microarray facility.
3. Interaction of *Cryptococcus neoformans* Rim101 and Protein Kinase A regulates capsule

*Chapter 3 was modified from a manuscript (of the same title) published in PLoS Pathogens 6(2) 2010. The authors were Teresa R. O'Meara, Diana Norton, Michael S. Price, Christie Hay, Meredith F. Clements, Connie B. Nichols, and J. Andrew Alspaugh*

### 3.1 Summary

*Cryptococcus neoformans* is an environmental fungus and an opportunistic human pathogen. Survival of this fungus within a human host depends on its ability to sense the host environment and respond with protective cellular changes. It is known that the cAMP/PKA signal transduction cascade is important for sensing host-specific environments and regulating the cellular adaptations, such as capsule and increased iron uptake, that are necessary for growth inside the infected host. Here we document that, unlike what has been described in other fungal species, a *C. neoformans* Rim101 homologue is directly regulated by PKA. The Rim101 signaling pathway is also involved in capsule regulation and virulence. Our study demonstrates that Rim101 integrates two conserved signal transduction cascades, and it is important in regulating microbial pathogenesis.

### 3.2 Introduction

All cells, including pathogenic microorganisms, must be able to sense and respond to changes in their environment. As these cells enter a human host, they need to protect themselves from the immune system and rapidly adapt to human physiologic conditions, such as low nutrient availability, varying pH, and mammalian concentrations of carbon dioxide (350). Therefore, they must coordinate multiple signaling pathways in order to control appropriate cellular responses.
One of the most common environmental stresses for pathogenic fungi is a change in the extracellular pH. Alterations in pH can affect a large number of cellular processes including membrane and cell wall stability, morphogenesis, protein stability and function, and nutrient uptake \((10, 27, 50, 84, 85, 200, 201)\). Many of these responses to pH are regulated by the Rim101 transcription factor and its homologues (PacC in filamentous fungi). Additionally, many pathogenic fungi respond to the neutral or slightly alkaline pH of the host by inducing virulence-associated phenotypes \((10, 31, 43, 73, 74, 337, 338)\). Therefore, in diverse fungi such as *Aspergillus* and *Candida* species, mutants defective in pH sensing/response no longer induce phenotypes associated with virulence in pathogenic species. For example, *C. albicans* rim101\(\Delta/\Delta\) mutants do not undergo pH-dependent dimorphic switching, do not appropriately increase uptake of iron, and do not secrete the proteases and phosphatases necessary for invasion of host tissues \((13, 27, 85, 197, 220, 259, 354)\). *A. nidulans* pacC (rim101) mutants display decreased growth, decreased secondary metabolite production, and defective invasive growth \((31, 43, 99, 103, 286)\). Although *A. nidulans* is non-pathogenic, these cellular processes have been associated with virulence in other *Aspergillus* species.

In addition to the direct effects of ambient pH on cell integrity and various metabolic processes, pH changes also affect nutrient uptake. For example, under alkaline conditions, the availability of free iron is greatly reduced as the iron equilibrium shifts from the bioavailable ferrous form to the insoluble ferric form. Studying iron flux is an important new horizon in fungal pathogenesis, as the human host keeps free iron levels at extremely low concentrations \((10^{-18}\) M\) through constitutively expressing iron-binding proteins such as transferrin and lactoferrin. In this way, the host protects against
invading microorganisms. Fungal pathogens unable to increase iron uptake in this iron-limited host environment often have severe defects in virulence (12, 85, 177, 202, 340). The pH-responsive Rim101 transcription factor is involved in the regulation of iron homeostasis, directly binding to the promoters of genes encoding high affinity iron uptake proteins: iron transporters, iron permeases and siderophore transporters (12, 99, 302).

_Cryptococcus neoformans_ is an opportunistic human fungal pathogen. Unlike the distantly related pathogens _Candida albicans_ or _Aspergillus fumigatus_, _C. neoformans_ grows within a very narrow range of pH values in the host. It grows well at the human physiological pH of the blood and cerebrospinal fluid (pH 7.4) as well as in acidic environments such as the phagolysosome of the macrophage (pH 5) (76, 261). However, unlike _C. albicans_ and _A. fumigatus_, _C. neoformans_ demonstrates a severe growth defect above pH 8. Despite this increased sensitivity to alkaline pH, there is still evidence that _C. neoformans_ responds to the slightly alkaline pH of the infected host blood by inducing virulence-associated phenotypes. Capsule production, a major virulence determinate, is optimal at human physiological pH (19, 76, 140, 243).

On a molecular level, _C. neoformans_ capsule synthesis is transcriptionally regulated by elements of the cAMP/PKA pathway. Strains with mutations in core cAMP signaling elements (such as the Gpa1 Gα protein, adenylyl cyclase Cac1, or the PKA catalytic subunit Pka1) display defective expression of capsule, and these mutant strains are attenuated for virulence in animal models of cryptococcosis. When the regulatory subunit of PKA is mutated in the _pkr1Δ_ strain, the cells have constitutive activation of PKA signaling and display high production of capsule, even in non-inducing conditions (6-8, 78, 81, 300). Other inducing conditions for capsule include iron deprivation, nutrient
limitation, and the presence of serum. The mechanisms by which these environmental
signals are sensed and subsequently transduced to specific intracellular signaling
pathways are not yet known. To explore the interaction of the inducing environmental
signals, signal transduction pathways, and downstream effectors controlling capsule
synthesis, we have begun to characterize specific transcription factors that are predicted
to be targeted by the cAMP pathway, and which also directly control capsule gene
expression. By examining the biological function and regulation of the *C. neoformans*
Rim101 homolog transcription factor, we have determined a new pathway for *C.
neoformans* regulation of capsule production. We have also defined a novel interaction
between the highly conserved Rim and PKA signaling pathways.

### 3.3 Results

#### 3.3.1 Identifying PKA-regulated transcription factors

To identify potential transcriptional regulators of *C. neoformans* capsule that are
also directly phosphorylated by PKA, we used a bioinformatic survey of the annotated *C.
neoformans* genome. We first searched the available annotation (GO terms, gene
names, homology designators) for proteins likely to be involved in transcriptional
regulation: transcription factors, DNA binding motifs, zinc finger domains, and other
transcriptional regulators. Given the incomplete annotation of the genome, we accepted
that many transcriptional regulators might be initially misidentified or excluded by this
approach.

Among this subset of proteins, we searched the predicted protein sequence for
consensus sequences for PKA phosphorylation (R/K R/K X S/T), to identify potential
direct targets of the Protein Kinase A enzyme. One of these proteins is a homologue of
Rim101/PacC, a conserved fungal C2H2 transcription factor (GenBank ID CNH00970).
The *C. neoformans* Rim101 protein contains a RRASSL motif at aa730, and has highest homology to *Aspergillus nidulans* PacC in the C2H2 domain. Analysis of the protein for conserved domains revealed that the only significant Pfam-A match is the zinc finger C2H2 domain (aa133-155).

### 3.3.2 Disruption of RIM101

To characterize the biological role of this *C. neoformans* transcriptional regulator, we disrupted the *C. neoformans* *RIM101* gene by homologous recombination. Southern blot analysis confirmed that the *rim101::nat* mutant allele precisely replaced the native gene in the *rim101Δ* deletion strain (TOC2)(Table 1) without additional ectopic integration events (data not shown). In addition to this mutant strain in which the entire *RIM101* open reading frame was deleted, we created an independent *rim101Δ* mutant with a partial gene deletion. All phenotypes between these strains were identical, and the TOC2 strain was therefore chosen as the representative *rim101Δ* mutant strain. To ensure that any phenotypes observed in the *rim101Δ* mutant strain were due to disruption of the *RIM101* gene, we created a *rim101Δ+RIM101* complemented strain (TOC4) by integrating a wild-type copy of the *RIM101* gene into the genome of the *rim101Δ* mutant strain.

The *rim101Δ* mutant strain grew at a similar rate as wild-type on rich medium (YPD) and minimal medium (YNB) at 30°C, 37°C and 39°C. We found no defect in melanin production on Niger-seed medium. We also examined the mutant strain for resistance to hydrogen peroxide and paraquat by disc diffusion assays and established that the zone of inhibition for the *rim101Δ* mutant strain was similar to that of wild-type, showing no additional sensitivity to reactive oxygen species.
3.3.3 Rim101 is required for capsule induction

The rim101Δ mutant strain has a major defect in polysaccharide capsule, an important virulence factor in *C. neoformans*. We incubated wild-type and rim101Δ mutant strains in a capsule inducing medium, Dulbecco's modified Eagle's medium (DMEM) containing 25mM NaHCO₃, for 72hrs at 37°C and 5% CO₂. Incubation in this medium usually leads to large polysaccharide capsules surrounding each cell which can be quantitatively measured by analyzing the percent packed cell volume (Cryptocrit analysis) (140). The rim101Δ mutant strain exhibits markedly reduced capsule around the cell (3.6% packed cell volume) compared to the wild-type strain (6.2% packed cell volume). The rim101Δ mutant capsule-defective phenotype was not noted in previous reports of the *C. neoformans* Rim101 protein (221), therefore, we confirmed our observation in several ways. We examined several independent rim101Δ mutants, including partial and complete gene deletions, which all displayed similar capsule defects in the inducing conditions. These differences in capsule were microscopically visualized by the exclusion of India ink (Figure 6A). Reintroduction of the wild-type allele fully complemented the capsule phenotype (6.8% packed cell volume).

To confirm the role of Rim101 and the Rim pathway in *C. neoformans* capsule production, we searched the *C. neoformans* genome for conserved elements of the Rim pathway, and we identified the RIM20 gene. Rim20 is a scaffold protein required for Rim101 cleavage/activation in other fungal species (32, 36, 84, 89, 287). When we mutated this gene, we observed a similar capsule defect in the rim20Δ mutant compared to the rim101Δ mutant strain (Figure 6A).

*C. neoformans* capsule is secreted out of the cell and subsequently bound to the cell wall (381). To determine whether rim101Δ mutant strains produce and secrete
capsular polysaccharide, we used a previously described gel electrophoresis technique to quantify this polymer (382). *C. neoformans* cells were incubated in capsule-inducing DMEM for 1 week, after which polysaccharide that was shed into the medium was analyzed for relative abundance and size by reactivity against an anti-GXM antibody (mAb18B7). We noted a similar amount of secreted polysaccharide in the *rim101Δ* mutant as wild-type (Figure 6B) suggesting that there is no significant GXM synthesis defect in the *rim101Δ* strain. We repeated this assay using low iron medium (140) as the capsule inducing condition instead of Dulbecco’s modified Eagle’s medium, and we again observed a similar amount of secreted capsule in wild-type and *rim101Δ* mutant strains (Figure 6B). As previously described, we used a *cap59* mutant strain that is unable to secrete capsule as a negative control (123) and detected no capsule by this assay in this strain. We also demonstrated the quantitative nature of this assay by analyzing the *pka1Δ* mutant strain, in which there is a previously documented decrease in capsule production compared to wild-type (81) (Figure 6B). Therefore, the hypocapsular phenotype of the *rim101Δ* mutant strain in the presence of intact polysaccharide production suggests a defect in capsule attachment. This result is similar to the phenotype of the *C. neoformans ags1Δ* mutant strain which can synthesize and secrete capsule but cannot bind it (305, 306).

### 3.3.4 CnRim101 retains conserved physiological roles with Rim101/PacC from other fungal species

In fungi as diverse as *Aspergillus, Saccharomyces, Candida, and Ustilago* species, the Rim101/PacC proteins control multiple pH-related phenotypes, including regulating iron homeostasis; maintaining membrane and cell wall-associated proteins; and secreting proteases, secondary metabolites, and phosphatases(10, 31, 83, 85).
Many of these factors are important in the virulence of pathogenic fungi.

Figure 6: *C. neoformans* Rim101 is required for capsule attachment

**A. rim101Δ mutants have a capsule defect.** Cells were incubated in capsule inducing conditions for 3 days. Capsule was assessed by staining with India ink and visualizing the zone of exclusion at 63x magnification (scale bar = 10µ).

**B. The rim101Δ mutant sheds equivalent capsule to wild-type.** Electrophoretic mobility and quantity of shed polysaccharide was assessed by a blotting technique of culture medium filtrate, using an anti-GXM antibody to probe for capsule. Cells were incubated in Dulbecco’s modified Eagle’s medium or low iron medium for 1 week before filtering. Arrow indicates direction of electrophoresis.

**C. GFP-tagged Rim101 is functional.** Cells were incubated in capsule inducing conditions for 3 days. Capsule was assessed by staining with India ink and visualizing the zone of exclusion at 63x magnification (scale bar = 10µ).

**D. Rim101-S773A does not complement capsule.** Cells were incubated in capsule inducing conditions for 3 days. Capsule was assessed by staining with India ink and visualizing the zone of exclusion at 63x magnification (scale bar = 10µ).
We tested the *C. neoformans rim101Δ* mutant strain to determine if this protein retains conserved physiological roles with Rim101/PacC proteins in other fungal species. On alkaline medium, the *rim101Δ* mutant strain exhibits a severe growth defect compared to the wild-type and the reconstituted strains at alkaline pH above 7.6 (*p* < 0.01) (Figure 7A). Similar to other *rim101*-defective fungal strains, the *C. neoformans rim101Δ* mutant also displayed sensitivity to medium containing 200mM LiCl or 1.5M NaCl (Figure 7B). To confirm that this growth defect was due to specific sensitivity to ionic stress as opposed to general sensitivity to osmotic stress, we tested the cells for growth on medium containing 2.5M sorbitol and detected no difference in growth between the *rim101Δ* mutant strain and wild-type. We also determined no defects in response to cell wall stress for the *rim101Δ* mutant strain during growth on calcofluor white, Congo red, or 0.05% SDS. These data suggest that, unlike in other fungal species, there are no major defects in cell wall integrity in the *C. neoformans rim101Δ* mutant strain.
Figure 7: Rim101 retains conserved phenotypes from other fungal species

A. The *rim101Δ* mutant is sensitive to alkaline pH. Cells were incubated in buffered YNB, and growth was determined by cell counts after 72 hours.

B. The *rim101Δ* mutant is sensitive to salt stress. 1x10⁵ cells from each strain were serially diluted (5-fold dilution) onto YPD plates containing 1.5M NaCl or 200mM LiCl. The plates were incubated at 30°C for 3 days. Cells were plated onto YPD plates for 48 hours as a control.

3.3.5 Rim101 localization is regulated by PKA and Rim20

In other fungal species, the Rim101 protein is activated by cleavage and subsequently localized to the nucleus, as expected for a transcription factor (239). To test whether *C. neoformans* Rim101 is cleaved and localized to the nucleus, we created a GFP-Rim101 fusion protein. We fused the green fluorescent protein gene to the N-terminus of the *RIM101* gene, expressing the new transgene under control of a constitutive histone promoter. Introduction of this plasmid (pTO2) by biolistic
transformation into the \textit{rim101}\textDelta mutant strains fully complemented the \textit{rim101}\textDelta mutant capsule phenotype, indicating that the GFP-Rim101 fusion protein is functional (Figure 6C).

Unlike \textit{A. nidulans}, in which localization is dependent on activation, \textit{C. neoformans} Rim101 is nuclear under all conditions tested. Using epifluorescent microscopy, we observed a nuclear pattern of localization for \textit{C. neoformans} GFP-Rim101 after 24 hours growth in various conditions, including YNB buffered at pH 8, Dulbecco’s modified Eagle’s medium, YNB (pH 5.4), and YPD (Figure 8). In contrast, the GFP-Rim101 protein in the \textit{pka1}\textDelta and \textit{rim20}\textDelta mutant backgrounds localized to both the nucleus and the cytoplasm under the same growth conditions, suggesting that PKA activity and Rim20-mediated cleavage are both important for complete nuclear localization (Figure 8). In addition, overexpression of the GFP-tagged Rim101 protein was not able to suppress the \textit{pka1}\textDelta or the \textit{rim20}\textDelta mutant capsule phenotype (Figure 6C).

To further explore the potential association of PKA and Rim101, we mutated the single Rim101 consensus sequence for PKA phosphorylation by changing serine 773 to an alanine, creating the Rim101-S773A mutant protein encoded in plasmid pTO3. When examining the localization of the GFP-Rim101-S773A protein, we observed both nuclear and cytoplasmic fluorescence, similar to the localization of wild-type GFP-Rim101 expressed in the \textit{pka1}\textDelta and \textit{rim20}\textDelta mutant backgrounds. Introduction of the GFP-Rim101-S773A protein into the \textit{rim20}\textDelta background also resulted in both nuclear and cytoplasmic localization of Rim10. Additionally, the Rim101-S773A mutant protein in the \textit{rim101}\textDelta mutant background did not complement the capsule phenotype (Figure 6D). This observation, coupled with our documentation that some GFP-Rim101-S773A
protein is localizing to the nucleus, indicates that serine 773 is necessary for full function of the Rim101 protein. When we transformed the Rim101-S773A mutant protein into the \textit{pkr1\Delta} mutant strain, in which PKA signaling and capsule production are constitutively active, multiple independent transformants had markedly attenuated capsule, even though the wild-type \textit{RIM101} gene was still present in these strains (Figure 6D). In contrast, introduction of the plasmid containing the wild-type Rim101 fused to GFP into the \textit{pkr1\Delta} strain had no effect on capsule. This suggests that the Rim101-S773A mutant protein is acting in a dominant negative manner on \textit{C. neoformans} capsule.
Figure 8: Rim101 localization is dependent on PKA and Rim20.

The pattern of GFP-Rim101 localization in the indicated strains was visualized by DIC and fluorescent microscopy at 63x magnification and by confocal microscopy at 100x magnification.

3.3.6 Rim101 cleavage is mediated by PKA and Rim20

To examine the interaction of PKA and the *C. neoformans* Rim pathway, we used western blotting techniques to compare Rim101 protein processing in multiple strain
backgrounds. Using an anti-GFP monoclonal antibody for detection, we identified bands corresponding to the GFP-Rim101 fusion protein from cell lysates of cultures incubated in YPD to mid-log phase (Figure 9A). The protein we detected when both Pka1 and Rim20 were wild-type had a molecular weight of approximately 120kD (Figure 9A). In contrast, expression of the identical GFP-Rim101 fusion protein in the pka1 or rim20Δ mutant backgrounds resulted in a protein band of approximately 140kD, which is the predicted size of the full length fusion protein. The GFP-Rim101-S773A protein also migrated with a reduced electrophoretic mobility in the rim101Δ or rim20Δ backgrounds, resulting in an approximately 140kD band. The 120kD processed form of GFP-Rim101 was dependent on both PKA and Rim20. Treatment with lambda phosphatases did not alter the mobility of any of these bands (data not shown). We also observed multiple smaller bands in the pka1Δ and rim20Δ mutant backgrounds. These may represent degradation products, suggesting that both Pka1 and Rim20 are necessary to prevent aberrant proteosomal involvement (153). In addition, the strains with a 140kD GFP-Rim101 protein were the same strains that had both cytoplasmic and nuclear patterns of GFP fluorescence. Only the 120kD processed form had predominantly nuclear localization (Figure 8).

Aspergillus nidulans PacC undergoes two successive cleavage events that regulate its function as an alkaline-responsive transcription factor (89, 287). To examine whether C. neoformans Rim101 also undergoes a second cleavage event under activating conditions, we incubated the rim101Δ +GFP-RIM101 strain to mid-log phase in either YPD or capsule inducing medium. When incubated in DMEM, we detected an additional band at approximately 70kD, corresponding to a potentially cleaved N-terminal fragment of the Rim101 protein (Figure 9B). This band was not present when PKA,
Rim20, or the PKA phosphorylation consensus sequence were mutated, or under non-inducing conditions, demonstrating that PKA phosphorylation and Rim20 are necessary for this further cleavage of Rim101 under inducing conditions.

**Figure 9:** Western blot analysis of Rim101 in rim101 and pka1Δ mutant backgrounds

**A. Rim101 cleavage is dependent on PKA and Rim20.** Immunoprecipitated GFP-Rim101 from rim101Δ GFP-rim101, rim101Δ +GFP-rim101-S773A, pka1Δ + GFP-rim101, rim20Δ + GFP-rim101, and rim20Δ + GFP-rim101-S773A strains was immunoblotted using anti-GFP antibody.

**B. Rim101 is cleaved after induction in capsule medium.** GFP-Rim101 was immunoprecipitated from rim101Δ, rim101Δ + rim101-S773A, rim20Δ + GFP-rim101, and rim20Δ + GFP-rim101-S773A cell lysates after incubation in either YPD medium or the capsule-inducing medium DMEM at 30°C to mid-log phase. Samples were run on Bis-Tris gels and immunoblotted using an anti-GFP antibody.

### 3.3.7 Rim101 downstream targets—Capsule and Iron

To define the downstream targets of the Rim101 transcription factor, we performed comparative transcriptional profiling between the rim101Δ mutant strain and wild-type using whole genome microarrays. We confirmed these results for several
genes using quantitative real-time PCR (data not shown). These results indicate that Rim101 controls the transcription of many genes involved in several categories of cellular function (Table 4).

**Table 4: Subset of Rim101-dependent gene expression in capsule-inducing conditions.**

This list contains functionally annotated genes whose expression was at least 2-fold different in wild-type vs. *rim101Δ* mutant strain after incubation in capsule-inducing medium for 3 hrs.

<table>
<thead>
<tr>
<th>Category</th>
<th>Gene ID</th>
<th>Description</th>
<th>Fold change (WT/rim101)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metal homeostasis</td>
<td>CNC01660</td>
<td>cytokine inducing-glycoprotein <em>CIG1</em></td>
<td>471.88</td>
</tr>
<tr>
<td></td>
<td>CNE04530</td>
<td>siderochrome-iron transporter <em>SIT1</em></td>
<td>81.14</td>
</tr>
<tr>
<td></td>
<td>CNM02420</td>
<td>acidic laccase, putative <em>FET3</em></td>
<td>20.75</td>
</tr>
<tr>
<td></td>
<td>CNM02430</td>
<td>ferric permease <em>CFT1</em></td>
<td>16.59</td>
</tr>
<tr>
<td></td>
<td>CNG00950</td>
<td>metalloreductase</td>
<td>16.09</td>
</tr>
<tr>
<td></td>
<td>CND01080</td>
<td>copper uptake transporter <em>CTR4</em></td>
<td>7.09</td>
</tr>
<tr>
<td>Cell Wall</td>
<td>CNA07540</td>
<td>mannoprotein <em>MP88</em></td>
<td>-5.98</td>
</tr>
<tr>
<td></td>
<td>CND03490</td>
<td>mannoprotein <em>MP98</em></td>
<td>-6.52</td>
</tr>
<tr>
<td></td>
<td>CNG04420</td>
<td>α -1,3-glucan synthase, <em>AGS1</em></td>
<td>-3.78</td>
</tr>
<tr>
<td></td>
<td>CNN00660</td>
<td>glucan 1,3 β-glucosidase protein</td>
<td>-2.07</td>
</tr>
<tr>
<td>Capsule biosynthesis</td>
<td>CNH00170</td>
<td>phosphomannomutase <em>PMM1</em></td>
<td>3.19</td>
</tr>
<tr>
<td></td>
<td>CNL06460</td>
<td>UDP-glucose 6-dehydrogenase <em>UGD1</em></td>
<td>2.87</td>
</tr>
<tr>
<td></td>
<td>CNF00920</td>
<td>mannosyltransferase <em>CMT1</em></td>
<td>-2.97</td>
</tr>
<tr>
<td>Other</td>
<td>CNA05130</td>
<td>sodium efflux pump <em>ENA1</em></td>
<td>6.92</td>
</tr>
</tbody>
</table>

Under capsule-inducing conditions, we were able to document differential expression for a limited number of genes that may be involved in capsule biosynthesis. We observed a 2.9-fold greater expression of *UGD1* in the wild-type compared to the *rim101Δ* mutant strain. *UGD1* encodes a UDP-glucose dehydrogenase that is necessary for UDP-glucuronic acid synthesis and thus capsule biosynthesis (142, 247). A
mannosyltransferase (Cmt1) was 2.97-fold greater expressed in the rim101Δ mutant strain than the wild-type. Mannosyltransferases such as Cmt1 have been implicated in the biosynthesis of GXM(330). In addition, we observed a 3.2-fold decrease in expression of a phosphomannomutase gene (PMM) in the rim101Δ mutant strain. PMM is involved in the biosynthesis of GDP-mannose, another nucleotide sugar essential for capsule production, and is transcriptionally regulated by PKA (75, 158, 177, 218, 372).

The data also show a small number of other nucleotide sugar-related genes that are differentially expressed and may be involved in capsule production. The fact that many highly inducible capsule genes are not transcriptionally regulated by Rim101 is consistent with our observation that the capsule defect is due to adherence, not production.

Transcriptional profiling also suggested that Rim101 controls the expression of several genes involved in iron or metal homeostasis, including the iron transporter gene CFT1, siderophore importer gene SIT1, and copper transporter gene CTR4 (158, 177, 218, 365). In addition, we documented Rim101-dependent expression of homologues of S. cerevisiae iron permeases (FRP1) and reductases (FET3), which are known to be regulated by PKA and by the Cryptococcus transcription factor Cir1 (158, 180, 181, 218).

To demonstrate that decreased expression of these genes was biologically relevant, we incubated the rim101Δ mutant strain in low iron medium, and we observed a distinct growth defect compared to wild-type or the reconstituted RIM101 strain (Figure 10A). C. neoformans strains typically induce capsule in response to growth in this medium. Although the rim101Δ mutant strain grew slowly in low iron medium, it eventually reached saturation phase. However, even when grown to saturation, the rim101Δ mutant strain did not exhibit capsule in low iron medium (data not shown). Further analysis of
these iron homeostasis genes revealed that the promoters of all of these iron regulating genes contain the potential Rim101 consensus binding sequence GCCAAG or the diverged sequence CCAAGAA, recognized by the *S. cerevisiae*, *C. albicans* and *A. nidulans* Rim101 orthologs (201, 302, 342). These results indicate that *C. neoformans* Rim101 retains conserved roles in regulating iron homeostasis and import.

**Figure 10: Rim101 is involved in the response to low iron stress**

**A. Rim101 is required for growth in low-iron medium.** Strains were incubated in low-iron medium and growth was quantified by monitoring the absorbance of the culture at 600nm.

**B. Iron genes contain Rim101 binding sites.** The 5'-untranslated regions of *C. neoformans* genes involved in iron regulation were evaluated for the presence of putative Rim101 binding sites (GCCAAG or CCAAGAA).

### 3.3.8 Rim101 and Virulence

The ability to produce capsule is important for the pathogenicity of *C. neoformans*, and other capsule-deficient strains are severely attenuated for virulence in animal models of cryptococcosis (6, 8, 55-57). In addition, the ability to obtain iron from
the host and to grow in low iron conditions is important for microbial survival in the host (158, 181, 368). We therefore hypothesized that the hypocapsular \textit{rim101}\textsuperscript{Δ} mutant would be avirulent in animal models of cryptococcosis. However, a recent manuscript in which the investigators tested virulence properties in a large collection of \textit{C. neoformans} mutants suggested that the \textit{rim101}\textsuperscript{Δ} might be more virulent than wild-type (221). We therefore tested the role of Rim101 in \textit{C. neoformans} pathogenicity. Female A/Jcr mice (10 per strain) were inoculated intranasally with 5\times10\textsuperscript{5} CFU of the wild type, \textit{rim101}\textsuperscript{Δ} mutant, or \textit{rim101}\textsuperscript{Δ}+RIM101 complemented strains. Mice were monitored for survival and sacrificed at predetermined clinical endpoints predicting mortality (Figure 11A). Infection with either the wild-type or the \textit{rim101}\textsuperscript{Δ}+RIM101 complemented strain resulted in complete mortality 18 and 19 days after infection respectively; there was no statistically significant difference between the survival of these two groups (\(p = 0.13\)). Mice infected with the \textit{rim101}\textsuperscript{Δ} mutant strain succumbed to the infection 16 days post-infection; this represents a statistically significant decrease in survival compared to animals infected with the wild-type strain (\(p < 0.002\)). We repeated the infection and sacrificed mice on day 2, day 9 and day 14 to determine fungal burden in the lungs, spleen, and brain. In all organs, we found no statistically significant difference in rates of dissemination among the 3 inoculated strains.

The subtle but reproducible increased virulence of the \textit{rim101}\textsuperscript{Δ} mutant cells in the inhalation model of \textit{C. neoformans} infection may be due to enhanced survival in the acidic environment of the alveolar macrophage. We specifically tested intracellular survival of the wild-type, \textit{rim101}\textsuperscript{Δ} mutant, and \textit{rim101}\textsuperscript{Δ}+RIM101 complemented strains. As described previously, we co-cultured \textit{C. neoformans} strains with IFN-\(\gamma\) and LPS-activated J774.1 macrophage-like cells (77). There was no significant difference in the
phagocytosis index of these strains by the macrophages, signifying that the altered capsule in the rim101Δ mutant did not affect fungal cell uptake into macrophages (41). In contrast, the rim101Δ mutant cells demonstrated increased intracellular survival ($p < 0.004$) within macrophages when normalized against the wild-type (Figure 11B).

Figure 11: Virulence analysis of the rim101Δ mutant strain

A. *rim101Δ* mutant strains are hypervirulent in the murine inhalation model of *cryptococcosis*. AJ mice were inoculated intranasally with 5x10⁵ cryptococcal cells and monitored for survival.

B. *rim101Δ* mutant cells survive better than wild-type within macrophages. The *rim101Δ* mutant and isogenic wild-type strains were co-incubated with J744.1 macrophage-like cells previously activated by INF-γ and LPS. Extracellular yeast cells were removed after one hour of co-incubation. After 24 hours of co-culture, the macrophages were lysed with SDS, and surviving yeast cells were quantitatively cultured. To precisely control for the number of added cells, the colony-forming units from each strain were normalized to that of wild-type cells. Data points represent the average of triplicate samples +/- standard error.

### 3.4 Discussion

Microbial pathogens use varied adaptive mechanisms to survive the harsh conditions of the infected host. *Cryptococcus neoformans* creates a polysaccharide capsule in response to host conditions such as low iron and high CO₂ concentrations (177, 350). The *C. neoformans* genome contains a number of genes involved in the
biosynthesis of this capsule, and many of these genes are highly transcriptionally regulated, at least partially in response to the PKA pathway (300). This led us to screen through the genome for transcription factors that are potentially regulated by PKA, and we previously found that the Nrg1 protein regulates capsule. Deletion of the \textit{Nrg1} gene resulted in a partial capsule reduction, and mutation of the putative PKA phosphorylation consensus sequence prevented full capsule induction. However, not all of the transcriptionally regulated capsule genes appeared to be targets for Nrg1, and many of the \textit{nrg1Δ} mutant phenotypes were not as severe as mutations in the more upstream components of the cAMP pathway (78). Therefore, we hypothesized that several transcriptional regulators would control capsule gene induction in response to the PKA pathway. Using a combination of bioinformatic and phenotypic screening, we identified the \textit{C. neoformans} Rim101 protein as another potential novel PKA-dependent transcriptional regulator of capsule genes.

We hypothesized that the \textit{C. neoformans} Rim101 protein may be a target of direct PKA phosphorylation due to the presence of a consensus sequence for PKA phosphorylation at amino acid positions 730-736. In contrast, the previously described \textit{C. albicans} and \textit{S. cerevisiae} Rim101 proteins do not contain potential PKA phosphorylation consensus sequences. However, there are multiple ways in which PKA can regulate downstream targets, including indirect activation of upstream regulatory proteins as well as by occupying the chromatin of the target genes (295). Our bioinformatic approach, therefore, does not identify all of the targets of PKA, but does allow us to potentially identify direct targets of PKA phosphorylation.

To determine the relationship between Rim101 and PKA, we used complementary genetic, biochemical, and protein localization experiments. Our results
suggest that PKA and Rim20 are necessary for maintenance of Rim101 nuclear localization by altering the cleavage of this transcription factor. Rim20 has been previously implicated in the first cleavage of Rim101, by binding to PEST domains, which are also present in \textit{C. neoformans} Rim101 (287, 378). In contrast to the predominantly nuclear localization of Rim101 in wild-type cells, we observed both nuclear and cytoplasmic localization of this protein in the \textit{pka1}\textsubscript{Δ} and \textit{rim20}\textsubscript{Δ} mutant strain backgrounds. We also observed both nuclear and cytoplasmic localization of the Rim101-S773A mutant protein with a putative PKA phosphorylation consensus sequence mutation. In addition, the GFP-tagged Rim101 protein in all of these strains had decreased electrophoretic mobility when compared to the \textit{rim101}\textsubscript{Δ}+GFP-\textit{RIM101} strain. The larger band is not due to hyperphosphorylation as this mobility shift was not reversed by treatment with phosphatase. Together, these data indicate that both the cAMP/PKA pathway and the Rim pathway are involved in \textit{C. neoformans} Rim101 processing and cellular localization.

In \textit{Aspergillus} and \textit{Candida}, PacC is activated by two cleavage events, first mediated Rim20 and Rim13 and second by the proteosome (153, 287, 378). We demonstrate that \textit{C. neoformans} Rim101 activation may also occur in response to two protein cleavage events, as the Rim101 protein is further cleaved from the 120kD form to a 70kD form in capsule inducing conditions. This further cleavage was not observed when \textit{PKA1} or \textit{RIM20} were disrupted, suggesting that the initial cleavage to 120 kD is necessary for further processing and activation of Rim101. The multiple smaller bands/laddering observed when \textit{PKA1} or \textit{RIM20} are disrupted may indicate altered proteosome-mediated processing events, suggesting that both Pka1 and Rim20 are necessary to cause appropriate proteosomal involvement and maintain the balance
between processing and degradation. This is consistent with data from *A. nidulans*,
where PacC is first converted by PalB and PalA under alkaline conditions to a 53kD
intermediate which exposes the second processing site to the proteosome (153).
Hervas-Aguilar et al. also demonstrated that phosphorylation can accumulate on the 72
and 53kD PacC intermediates during alkaline conditions and affect processing. This is
consistent with our results that PKA is involved in regulating processing of Rim101 in *C.
neoformans*, although there is large divergence in the C-terminal and in the potential
signaling motifs between these orthologous proteins in these distantly related species.
Interestingly, capsule-inducing conditions are not alkaline and thus are not a traditional
activating condition for Rim101 proteins. Therefore, CnRim101 may have acquired novel
activating conditions in order to respond to the specific host conditions experienced by
cryptococcal cells *in vivo*.

When we examined the targets of Rim101 transcriptional activation, we found
that many Rim101 downstream targets and responses from other pathogenic fungi, such
as *C. albicans*, are conserved in *C. neoformans*. We demonstrated that CnRim101 is
important for growth under alkaline conditions *in vitro*. Using comparative transcriptional
profiling, we determined that *ENA1*, a known downstream target of Rim101 in other
fungal species, showed decreased expression in the *rim101*Δ mutant strain (Table 4).
The promoter of the *ENA1* gene also had a conserved predicted Rim101 binding
sequence, suggesting that it might be a direct target of Rim101 in *C. neoformans*, unlike
in *S. cerevisiae*, where Rim101 regulates *ENA1* through Nrg1 (78). Idnurm et al. showed
that Ena1 is required for *C. neoformans* survival under alkaline conditions, and that
appropriate response to alkaline conditions is necessary for virulence of *C. neoformans*
(164). Therefore, decreased expression of *ENA1* in the *rim101*Δ mutant strain may
explain the defect in alkaline growth of the \textit{rim101}Δ mutant.

Extracellular pH is involved in regulating iron uptake genes through the Rim101 pathway in \textit{C. albicans} and \textit{S. cerevisiae} (12, 13, 27, 84, 85, 201, 202). The relationship between iron homeostasis and Rim101 is also conserved in \textit{C. neoformans}. In order to determine the mechanism for the \textit{rim101}Δ mutant strain sensitivity to low iron, we compared the transcriptional profile between wild-type and the \textit{rim101}Δ mutant strain after incubation in capsule-inducing conditions. Our microarray analysis concluded that a number of iron homeostasis genes are differentially regulated between the \textit{rim101}Δ mutant and the wild-type and we confirmed these alterations in gene expression using quantitative real-time PCR. When we examined the putative promoter regions of the candidate genes, we discovered potential Rim101 consensus binding sequences in \textit{CFT1}, \textit{FET3}, and \textit{SIT1} among others, suggesting these genes are direct targets of Rim101. Similarly, in \textit{C. albicans}, Rim101 binds directly to the promoter region of the ferric reductase genes \textit{FRE1} and \textit{FRP1} to cause increased transcription under iron-limited environments (12).

In \textit{C. neoformans}, iron uptake is regulated by two pathways: PKA and Cir1. Transcriptional profiling showed that many iron genes, such as the iron permease Cft1 and reductase Cfo1 are differentially regulated by PKA (158, 177, 218, 340). We have demonstrated that Rim101 is regulated by PKA, thus providing a mechanism for PKA regulation of these iron genes. However, in our transcriptional profiling, we did not demonstrate any difference in expression of Cir1 in the \textit{rim101}Δ mutant strain, further suggesting that there are two pathways that regulate iron homeostasis. In \textit{C. albicans}, two signaling pathways regulate iron homeostasis in response to different forms of iron limitation. In \textit{C. albicans}, the ferric reductase gene \textit{FRP1} is differentially regulated by
Rim101 and by CBF transcription factors in response to different forms of iron limitation (12). It is possible that C. neoformans has a similar set of transcription factors to regulate the expression of these iron homeostasis genes under different iron-limiting environments, and that the cell uses both Cir1 and Rim101 to regulate the expression of Cft1 under different environmental stimuli and iron source limitations.

Despite the decreased surface capsule observed in the rim101Δ mutant cells when stained with India ink, this strain was still able to secrete glucuronoxylomannan (GXM) at a similar size and concentration as wild type when the cells were grown in capsule inducing conditions. This does not preclude other differences in structure and modifications to the GXM in the mutant strain. In accordance with the amount of secreted polysaccharide from the rim101Δ mutant strain, our transcriptional profiling revealed that few capsule biosynthesis genes are transcriptionally regulated by Rim101. In the rim101Δ mutant strain we observed decreased expression of UDP-glucose dehydrogenase Ugd1, mannosyltransferase Cmt1, and phosphomannomutase (75, 142, 247, 330). Unlike the iron uptake genes, these capsule biosynthesis genes do not have conserved Rim101 binding sites in the promoter regions, suggesting that these are not direct targets of Rim101. Therefore, our data indicate that CnRim101 is required for the transcriptional activation of some genes involved in capsule biosynthesis; however, the most important effects of Rim101 on capsule are likely due to changes in polysaccharide binding to the cell surface. We hypothesize that Rim101 regulates capsule by altering the expression of genes responsible for anchoring capsule to the cell wall, rather than acting as a direct regulator of these capsule biosynthesis genes.

Unexpectedly for an acapsular strain, the rim101Δ mutant displayed no attenuation in virulence in the mouse inhalation model of cryptococcosis. This confirms
prior broad screening experiments of *C. neoformans* mutants to identify genes required for survival within mice (221). In these studies, the *rim101Δ* strain was slightly more virulent than wild-type, as we demonstrated here. Follow-up experiments determining fungal load in the brain, lung, and spleen showed no defects in dissemination. When Rim101 is mutated in *Candida*, the resulting strains are avirulent as Rim101 regulates processes necessary for fungal virulence (27, 31, 354). In a fungal pathogen of plants, *Fusarium oxysporum*, a *rim101Δ* mutant strain is more virulent than wild-type due to the derepression of acid response genes conferring a survival advantage in the acidic host environment of the tomato (10). Similarly, our data indicate that the *C. neoformans rim101Δ* mutant grows better than wild-type within the acidic phagolysosome of the activated macrophage (261, 262). Perhaps the derepression of acid responsive genes in the *rim101Δ* mutant could explain the increased growth within the acidic phagolysosome and thus within the lungs of the infected host. Another explanation for the retained virulence of the *rim101Δ* mutant strain is that the capsular polysaccharide may be shed into the surrounding tissues. This capsular material has well defined immunosuppressant effects. Capsular polysaccharide has even recently been used as an experimental therapy for autoimmune diseases such as rheumatoid arthritis (245). Therefore, the retained virulence may be attributed to the profound immunomodulatory effects of strains that produce and secrete large amounts of capsule. Also, not all capsule-defective *C. neoformans* strains are hypovirulent in model systems. The acapsular *ags1Δ* mutant is fully virulent in the nematode model of cryptococcosis, although sensitive to temperature and thus avirulent in the mouse (305, 306). The virulence of these strains suggests that capsule may be playing an important role in suppressing the immune system, even when not bound to the cell as an anti-phagocytic
mechanism.

It is also possible that the hypcapsular rim101Δ mutant may present an altered cell surface for immune recognition, exposing different antigens resulting in a substantively different immune response than for an encapsulated WT strain. In this model, the increased virulence might result from alterations of the exposed C. neoformans surface antigens leading to over-stimulation of the immune system, such as seen in the response to β-glucan in the C. albicans cell wall (139, 254). In our microarray data, we observed increased expression of MP88 and MP98, two immuno-dominant mannoproteins, in the rim101Δ mutant strain, further supporting a model of an altered antigen surface on the fungus as a result of absent Rim101 activity (159, 213). MP88 has also been documented as having increased expression in a pka1Δ mutant strain, which may be due to decreased Rim101 activity (158). A more detailed evaluation of the nature of this cellular infiltration into the infected lungs will help define the varied immune response to different C. neoformans strains.

In summary, we have demonstrated that the C. neoformans Rim101 transcription factor retains conserved functions with orthologous proteins from other fungal species, such as regulation of pH response, cell wall formation, and iron homeostasis. However, the phenotypic output resulting from a C. neoformans Rim101 mutation supports the hypothesis that this conserved protein has been co-opted for unique, species-specific function. In contrast to other fungal species such as Candida or Aspergillus that have adapted to the neutral/alkaline pH of the host lungs and use Rim101 as an inducing signal for virulence, C. neoformans may be better adapted for acidic microenvironments in the host, such as the macrophage phagolysosome. Moreover, our experiments demonstrating PKA regulation of CnRim101 further suggests that conserved signaling
elements can be regulated in novel ways to allow adaptation of microorganisms to specific niches in the environment of the infected host.

3.5 Methods and Materials

Strains and media

_Cryptococcus neoformans_ strains used in this study are listed in Table 1. All _C. neoformans_ strains were created in the H99 strain background unless otherwise stated. Strains were maintained on YPD (1% yeast extract, 2% peptone, 2% glucose), a standard yeast medium. Selective medium contained nourseothricin (100mg/L Werner BioAgents, Jena-Cospeda, Germany) or neomycin (G418) (200mg/L Clontech, Takara-Bio Inc.). Capsule inducing medium (Dulbecco’s modified Eagle’s medium with 25mM NaHCO₃) was prepared as previously described (140). YNB medium (0.67% yeast nitrogen base without amino acids, 2% glucose) was prepared as previously described (6). Alkaline pH medium was created by buffering YNB with 25mM NaMOPS and adjusting to target pH with NaOH. Resistance to hydrogen peroxide was tested by disc diffusion as described previously. Niger seed agar was prepared from 70g Niger seed extract (Niger seed pulverized and boiled for 15 min and filtered through cheesecloth) and 4% Bacto agar as previously described (6).

Molecular Biology Techniques

Standard techniques for Southern hybridization were performed as described (318). _C. neoformans_ genomic DNA for Southern blot analysis was prepared using CTAB phenol-chloroform extraction as described (293).

Gene disruption

The wild-type _RIM101_ gene (NCBI GeneID CNH0097) was mutated using biolistic transformation and homologous recombination with a _rim101::nat_ mutant allele.
in which the entire \( RIM101 \) coding region was precisely replaced with the nourseothrycin resistance gene (\( nat \)) (232, 344). The \( rim101::nat \) mutant allele was created using PCR overlap extension as described (115). Several \( rim101\Delta \) mutants from independent transformation events displayed identical phenotypes \textit{in vitro}; therefore one strain (TOC2) was chosen as the \( rim101 \) strain for the presented experiments. Putative deletion strains were confirmed by PCR and Southern blot analysis. A second \( rim101\Delta \) strain was made by creating an identical \( rim101\Delta \) disruption construct first created by Liu et. al (221) and biolistic transforming it into the H99 strain background. This strain has a partial deletion of the \( RIM101 \) gene. Putative mutant strains were confirmed by PCR and Southern blot analysis, and phenotypically compared to the full TOC2 deletion strain.

To reconstitute the wild-type allele, the \( RIM101 \) locus was amplified from the H99 wild-type strain using primers (5' CTGTATCCTTCCTTGAGGC 3') and (5' AGCTGTGCGATCCACTTGAGGC 3'). The neomycin resistance allele was also amplified separately using the M13 forward and reverse primers and both alleles were transformed using biolistic transformation into strain TOC2 to make the reconstituted strain TOC4 as described previously (136). The reconstituted strain was tested by PCR for presence of the wild-type allele, and examined for reversion of the mutant phenotypes.

The wild-type \( RIM20 \) allele (NCBI GenelID CNG00250) was similarly mutated with a \( rim20::nat \) mutant allele created by PCR overlap extension. Several \( rim20\Delta \) mutants from independent transformation events displayed identical phenotypes \textit{in vitro}; therefore one strain (TOC14) was chosen as the \( rim20 \) strain for the presented experiments. This strain was confirmed using PCR and Southern analysis.

\textbf{Protein Localization / GFP fusion}
We created a green fluorescent protein (GFP)-Rim101 fusion protein to examine the subcellular localization of Rim101. A histone H3 promoter-GFP fusion (163) was cloned into the neomycin-resistance containing plasmid pJAF, utilizing BamHI and EcoRI to make the resulting plasmid, pCN50. The coding region and the terminator sequence of RIM101 was amplified using primers modified with BamHI sites (5'-AGTTAGGATCCATGGCTTACCCAATTCTCCC-3' and 5'-ACTGATGGATCCGAGGAAAGCGTCAAGGATATG-3'). The RIM101 gene was then cloned into the pCN50 plasmid at the BamHI site to create the plasmid pTO2 in which the GFP-Rim101 fusion protein is constitutively expressed under the His3 promoter. pTO2 was then biolistically transformed into C. neoformans strain TOC2, JKH7, CDC7, and TOC17 as previously described to create strains TOC10, TOC12, TOC13 and TOC21 respectively. pTO2 was mutated into pTO3 using PCR-mediated site-directed mutagenesis to change serine 773 to alanine, using primers 5'-GAGAGTGATGCCGCACGTCGATACTGTCCCTG-3' and 5'-AGTTAGGATCCATGGCTTACCCAATTCTCCC-3'. pTO3 was then biolistically transformed into TOC2, CDC7 and TOC17 to create strains TOC18, TOC20 and TOC22 respectively.

Microscopy

Bright field, differential interference microscopy (DIC) and fluorescent images were captured with a Zeiss Axio Imager.A1 fluorescent microscope equipped with an AxioCam mrM digital camera. Confocal images were captured using a Zeiss LSM inverted confocal microscope with the Argon/2 488 laser at x100 magnification. To visualize capsule, cells were grown in inducing conditions (described above), then stained with India ink on glass slides. Images were collected at x63 magnification. To visualize GFP, cells were washed three times in PBS, and images were collected at x63.
magnification, using 488nm wavelength for fluorescence. The strains exhibited significant artifactual fluorescence signal when fixed and DAPI stained, therefore all fluorescence images were taken without fixing, precluding DAPI staining.

**Capsule blotting**

Estimation of shed capsule polysaccharide size and amount was performed using a technique described by Yoneda and Doering (382). Conditioned medium was made by growing strains in Dulbecco’s modified Eagle’s medium or low iron medium for 1 week at 30 °C with shaking. The cells were incubated at 70°C for 15 minutes to denature enzymes, then pelleted for 3 minutes at 1500 rpm. The resulting supernatant was sterile filtered using a 0.2u filter. 15uL of conditioned medium was mixed with 6x loading dye and run on an agarose gel at 25V for 15 h. The polysaccharides were then transferred to a nylon membrane using Southern blotting techniques. The membrane was air-dried and blocked using Tris-Buffered Saline-Tween-20 (TBS-t) with 5% milk. To detect the polysaccharide, the membrane was incubated with monoclonal antibody 18b7 (1/1000 dilution) (48), washed with TBS-t, then incubated with an anti-mouse peroxidase-conjugated secondary antibody (1/25,000 dilution, Jackson Labs) and detected using SuperSignal West Fempto Maximum Sensitivity Substrate (ThermoScientific).

**Protein Extraction, Immunoprecipitation and Western Blot Analysis**

Protein extracts were obtained using a method previously described (256). Briefly, cells were incubated to an optical density at 600nm of 1 in YPD and capsule-inducing conditions. Twenty-milliliter samples of growing cells were pelleted and resuspended in 0.5 mL of lysis buffer containing 2x protease inhibitors (Complete, Mini, EDTA-free; Roche) and 2x phosphatase inhibitors (PhosStop; Roche). To lyse the cells,
the supernatant was removed and the cells were lysed by bead beating (0.5 mL of 3 uM glass beads in a Mini-BeadBeater-16 (BioSpec), 4 cycles for 30 s each). Following lysis, the samples were immunoprecipitated using 1.6µg anti-GFP antibody (Roche) for 1 hour, then rotated with 80µL protein G Sepharose (Thermo Scientific) for 1 hour. After washing, the samples were eluted by the addition of 40 µL 5x Laemmli sample buffer and boiling. Western blots were performed as described previously, using NuPAGE Tris-Acetate gels or NuPAGE Bis-Tris gels to separate the samples. To detect the GFP-labeled proteins, the blots were incubated with anti-GFP primary antibody (1/5,000 dilution) and an anti-mouse peroxidase-conjugated secondary antibody (1/25,000 dilution, Jackson Labs). As a control for non-specific immunoprecipitation, samples were tested in a mock IP (in which no antibody was added) and probed with the anti-GFP antibody, and no bands were observed in this control experiment.

**RNA and cDNA preparation**

Strains were incubated to mid-logarithmic phase in YPD then washed three times with sterile water before incubation in capsule-inducing medium for 3 hours. Cells were washed three times before centrifugation and freezing on dry ice and lyophilizing. RNA was prepared from lyophilized samples using the RNeasy kit (Qiagen). cDNA for real time-PCR was generated using RETROscript (Ambion) using oligo-dT primer. Quantitative real-time PCR was performed as previously described, using the constitutive GPD1 gene to normalize the samples (78).

**Microarray and Data Analysis**

The microarray used in this study was developed by the Cryptococcus Community Microarray Consortium with financial support from individual researchers and the Burroughs Wellcome Fund
RNA labeling and hybridization were performed by the Duke University Microarray Core Facility according to their established protocols for custom spotted arrays (78, 296). Data was analyzed using JMP genomics (SAS institute, Cary NC) and initial background subtraction was performed. We used ANOVA normalization and FDR analysis to calculate differences between treatment effects for pairs of inducing conditions. Genes were considered for further evaluation if they showed log$_2$-transformed fold changes with a $p$-value <0.02.

**Virulence and Data Analysis**

The virulence of the *C. neoformans* strains was assessed using a murine inhalation model of cryptococcosis as described previously (77). 10 female A/Jcr mice were inoculated intranasally with 5x10$^5$ *C. neoformans* cells of wild-type (H99), rim101Δ mutant (TOC2), or rim101+RIM101 complemented strain (TOC4). Mice were monitored daily for signs of infection and were sacrificed at predetermined clinical endpoints predicting mortality.

The statistical significance between the survival curves of all animals infected with each strain was evaluated using the log-rank test (JMP software, SAS institute, Cary NC). Cell counts were analyzed using Student's $t$-test. All studies were performed in compliance with the institutional guidelines for animal experimentation.

Survival within alveolar macrophage-like J744.1 cells was tested by aliquoting 50ul of 1x10$^5$ macrophage cells/mL into wells in a 96-well plate. The cells were activated by adding LPS and INF-γ and incubating overnight. 50ul of 2x10$^6$ cells/mL of each cryptococcal strain were added to the wells and co-incubated for 1 hour after which the
excess cells were removed and fresh medium was added. The engulfed cells were incubated for 24 hours then disrupted with 0.5% SDS for 5 minutes to lyse the macrophages. The medium was removed, and the well was washed 2x with 100uL PBS. The washes were combined and diluted at 1:100 before plating. Plates were incubated for 2 days before colony counts (77).

Acknowledgements

We thank Elizabeth Ballou for assistance with confocal microscopy. We thank Arturo Casadevall for mAb18b7 antibody. We thank Steve Giles for the GAL file used for analyzing our microarray data. We thank the Confocal and Microarray facilities at Duke University.
4. Cryptococcus neoformans Rim101 is associated with cell wall remodeling and evasion of the host immune responses

Chapter 4 was modified from a manuscript (of the same title) published in mBio 4(1) (2012). The authors were Teresa R. O'Meara, Stephanie M. Holmer, Kyla Selvig, Fred Dietrich, and J. Andrew Alspaugh.

4.1 Summary

Infectious microorganisms often play a role in modulating the immune responses of their infected hosts. We demonstrate that Cryptococcus neoformans signals through the Rim101 transcription factor to regulate cell wall composition and the host-pathogen interface. In the absence of Rim101, C. neoformans exhibits an altered cell surface in response to host signals, generating an excessive and ineffective immune response that results in accelerated host death. This host immune response to the rim101Δ strain is characterized by increased neutrophil influx into the infected lungs and an altered pattern of host cytokine expression compared to the response to wild type cryptococcal infection. To identify causative genes for the observed phenotypes, we performed whole-genome RNA sequencing experiments under capsule-inducing conditions. We defined the downstream regulon of the Rim101 transcription factor and determined potential cell wall processes involved in the capsule attachment defects and altered mechanisms of virulence in the rim101Δ mutant. The cell wall generates structural stability for the cell and allows for attachment of surface molecules such as capsule polysaccharides. In turn, the capsule provides an effective mask for the immunogenic cell wall, shielding it from recognition by the host immune system.
4.2 Importance

Cryptococcus neoformans is an opportunistic human pathogen that is a significant cause of mortality in immunocompromised individuals. There are two major causes of death due to this pathogen: meningitis due to uncontrolled fungal proliferation in the brain in the face of a weakened immune system, and immune reconstitution inflammatory syndrome (IRIS) characterized by an overactive immune response to subclinical levels of the pathogen. In this study, we examined how C. neoformans uses the conserved Rim101 transcription factor to specifically remodel the host-pathogen interface, thus regulating the host immune response. These studies explore the complex ways in which successful microbial pathogens induce phenotypes that ensure their own survival while simultaneously controlling the nature and degree of the associated host response.

4.3 Introduction

Disease due to the opportunistic fungus Cryptococcus neoformans is increasingly important in the face of the expanding HIV/AIDS epidemic and the use of immunosuppressant drugs. Over 600,000 deaths per year can be attributed to cryptococcosis; the majority of these infections occur in patients with AIDS in resource-limited countries (281). C. neoformans is also an excellent model to study the interaction of a eukaryotic pathogen with its infected host.

Outcomes of infections are dependent on complex interactions between the vigor of the host immune response and the intrinsic virulence of the pathogen. Therefore, recent studies in microbial pathogenesis have explored the importance of the immune response to infection as a mediator of host damage in addition to examining direct microbial injury to the host. While many microbial pathogens express toxins or other
virulence factors that directly damage the host, over-activation of the host immune system can similarly cause host damage, such as when immunogenic superantigens result in bacterial toxic shock syndromes (63). Cryptococcal disease can also be understood within this damage-response framework. In the face of severe host immunodeficiency, pathogens such as *C. neoformans* induce host damage and symptomatic disease primarily by microbial proliferation. In fact, one of the primary predictors of poor host outcome in cryptococcal meningitis is a very low number of inflammatory cells present in the cerebrospinal fluid at this time of infection (88). However, in other patients, restoration of a dysfunctional immune system can cause immune hyperactivation against subclinical cryptococcal infections, resulting in progressive symptoms despite effective microbial killing. This immune reconstitution inflammatory syndrome (IRIS) emphasizes the potential role of the host immune system in mediating host damage and disease symptoms (327).

One of the classic markers of *C. neoformans* adaptation to the host is the induction of a polysaccharide capsule. The dominant paradigm is that acapsular *C. neoformans* strains either fail to cause disease or are severely attenuated in disease progression. This has been demonstrated for multiple acapsular strains, including an early series of capsule mutants (*cap59Δ, cap60Δ, cap64Δ*), and in other unencapsulated variants (54, 55, 57, 194). Concordantly, hypercapsular strains can be hypervirulent in the host (81, 251).

Previous work demonstrated multiple roles for capsule in modulating virulence. One role is the prevention of phagocytosis by macrophages, an important environmental niche inside the host (110). Glucuronoxylomannan (GXM), the primary component of capsule, can alter phagocytosis rates (193, 351). After phagocytosis, capsule may
defend against reactive oxygen species (ROS) produced by the macrophages (386). Capsule also has a direct role in suppressing the immune system by titrating out complement components and preventing a more active inflammatory response (125, 389). We propose that capsule also acts as a barrier against immune recognition of cell wall components such as α-glucan, β-glucan, chitin/chitosan, and mannans. These conserved fungal cell wall components can act as potent pathogen-associated molecular patterns (PAMPs), triggering a highly active immune response (255, 304, 370). Thus, capsule may play multiple roles in the modulation of host immune responses. The cell shielding effect of capsule on pathogenesis was previously difficult to examine due to the intrinsically poor virulence of capsule-deficient strains. Therefore, the capsule-deficient but infection-competent rim101Δ mutant strain offers unique insight into the interaction of host cells and the exposed C. neoformans cell wall (266).

Recently, we and others demonstrated the paradoxical observation that the hypocapsular rim101Δ strain was hypervirulent in two animal models of cryptococcosis (221, 266), revealing a discordance between the defects in C. neoformans capsule and the expected subsequent reductions in virulence. To further examine the relationship between Rim101, capsule, and virulence, we examined the inflammatory response to rim101Δ mutants, the downstream Rim101 targets, and the roles of these targets in virulence. In this paper, we demonstrate that a pulmonary infection with the rim101Δ mutant strain results in a fundamentally different disease progression characterized by excessive activation of the host inflammatory system. We suggest a model in which the absence of Rim101 leads to an inability of this fungus to appropriately remodel the cell wall, which has functional consequences for capsule attachment and exposure of previously shielded pathogen-associated molecular patterns (PAMPs). Additionally,
Rim101 controls cellular responses to variations in environmental pH, thus regulating proliferation within macrophages.

Our results demonstrate how *C. neoformans* has adapted conserved transcription factors, such as Rim101, to control fundamental cellular processes that allow for survival in the host. Our results also suggest new models defining the interplay of molecules on the surface of pathogens that can control the level of host immune activation, thus leading to pathogen control, dormancy, or host damage.

**4.4 Results**

**4.4.1 Infection with the rim101Δ strain results in increased host inflammation**

To define the role of Rim101 in pathogenesis, we performed detailed studies on fungal survival *in vivo* and the resulting host immune response. We previously tested the *rim101Δ* strain in an inhalational model of murine cryptococcosis using the A/J mouse strain, which has a defect in complement activation (266). To ensure that this observation was not dependent on the mouse background, we infected female C57BL/6 mice (10 per strain) by intranasal inoculation with 2x10⁴ CFU of the wild type, *rim101Δ* mutant, or acapsular *cap59Δ* mutant strains. Mice were monitored for survival and sacrificed at predetermined clinical endpoints predicting mortality (Figure 12A). Infection of C57BL/6 mice with either the *rim101Δ* or wild type strains resulted in similar kinetics of mortality, with no significant difference in animal survival between the strains (*p* = 0.67).

Even though the absolute rate of mortality was similar between the two groups, mice infected with the *rim101Δ* mutant strain demonstrated strikingly different symptoms of disease. Mice infected with wild type *C. neoformans* typically develop prominent neurological symptoms immediately prior to exhibiting profound weight loss, predicting
imminent mortality. In contrast, the rim101Δ-infected mice developed predominant respiratory symptoms (rapid and labored breathing) prior to weight loss and death. This differential pattern of symptomatic disease progression was similar between both mouse strains. As expected, all mice infected with the acapsular cap59Δ strain cleared the infection without any disease symptoms and survived the duration of the experiment (Figure 12A) (54).

To examine host responses to the rim101Δ strain, we performed histological analysis of infected lungs at various time points after infection in both A/J (Figure 12B) and C57BL/6 mouse backgrounds. In A/J mice infected with the wild type strain, there were numerous fungal cells with large capsular haloes present throughout the lung tissue at both four and fourteen days after infection. The underlying alveolar architecture of the lung parenchyma was clearly visualized, with limited foci of leukocytes associated with encapsulated yeast cells, often adjacent to blood vessels. The inflammatory cells were composed primarily of mononuclear cells, with rare eosinophils and neutrophils (Figure 12C).

Mouse lungs infected with the rim101Δ mutant demonstrated a very different histological pattern at both four and fourteen days after infection. Even at the early time point, the lung tissue was filled with an intense inflammatory cell infiltrate (Figure 12B). By day fourteen, the inflammatory cell infiltrate completely obscured the alveolar spaces, consistent with the clinical symptoms of respiratory distress observed in these animals (Figure 12B). In the rim101Δ-infected lungs, the inflammatory cells consisted predominantly of neutrophils and eosinophils. Small yeast cells with thin capsular haloes were observed amidst the host inflammatory infiltrate (Figure 12C).
To ensure that the altered pattern of inflammation in the rim101Δ-infected mice was not due to an increased fungal burden, we performed quantitative cultures of tissues from these infections. We first cultured bronchoalveolar lavage fluid from infected mice after two and four days of infection (Figure 12D). At both time points, we observed significantly more wild type cells than rim101Δ cells (Day 2: WT: 3.8x10⁴ CFU/mL vs. rim101Δ: 0.6x10⁴ CFU/mL, p < 0.01; Day 4: WT: 8.4x10⁵ CFU/mL vs. rim101Δ: 1.7x10⁵ CFU/mL, p < 0.0001). Using whole lung homogenization at day 9, we similarly observed a 4.9-fold increase in fungal burden in the wild type compared with the mutant strain (p<0.0106). Therefore, the increased inflammatory response cannot be attributed to an increased number of rim101Δ cells within the infected lungs.
Figure 12: Rim101 effects on C. neoformans virulence, immune cell infiltration, and inflammation.

A. Hypocapsular rim101Δ mutants maintain virulence in murine models of cryptococcosis. C57BL/6 mice (10 per strain) were inoculated intranasally with 2x10⁴ cryptococcal cells and monitored for survival.

B. Histopathology demonstrates increased inflammation in rim101Δ-infected mice. A/J mouse lungs were harvested on day 4 and 14 post-infection, and the infected lungs were assessed by histopathological analysis.
C. Histopathology demonstrates increased influx of polymorphonuclear cells in \textit{rim101Δ}-infected mice. A/J mouse lungs were harvested on day 4 and 14 post-infection, and the infected lungs were assessed by histopathological analysis.

D. Virulence of the \textit{rim101Δ} mutant is not due to fungal burden. Lavage fluid from 5 mice per strain was collected at day 2 and 4 to quantify the fungal burden and cell proliferation in vivo.

We also noted a striking difference in fungal morphology \textit{in vivo} between the wild type and \textit{rim101Δ} strains. Wild type cells displayed a marked range of sizes, from small budding yeasts (approximately 5 $\mu$m) to large and highly encapsulated titan cells (15-50 $\mu$m), a newly described \textit{C. neoformans} morphotype characterized by enlarged cells with extensive and tightly linked capsules (270, 388). In contrast, the \textit{rim101Δ} mutant displayed small and minimally encapsulated fungal cells \textit{in vivo}, with limited size variation. To quantify this difference in cell sizes, we directly measured the diameter of 500 cells for each strain recovered by bronchoalveolar lavage at day 4. The wild type strain exhibited a broad range of cell size, with 33.6% titan cells (defined as cells $>$15 $\mu$m in diameter (270)). In contrast, most of the \textit{rim101Δ} mutant cells were present as small yeasts with few titan cells (2.0%), consistent with prior data linking Rim101 and titan cell formation (271) (Figure 13A). Overall, the average diameter of the \textit{rim101Δ} cells was significantly smaller than the non-titan wild type cells (WT = 13.9±6.24 $\mu$m, \textit{rim101Δ} = 6.75±2.76 $\mu$m, $p<0.0001$). When incubated \textit{in vitro}, cell size is not different between the strains, even after incubation in tissue culture conditions (WT = 6.3±1.3 $\mu$m, \textit{rim101Δ} = 6.2±1.0 $\mu$m, $p = 0.27$). Despite their smaller size, the \textit{rim101Δ} cells were equally viable as wild type, as assessed by comparative numbers of colony forming units from visualized cells within the lung lavage fluid (data not shown).
Although titan cells have a noted defect in crossing the blood brain barrier (270), the variation in size between the wild type and rim101Δ cells appeared to have no effect on dissemination from the lungs, with both wild type and rim101Δ cells showing equivalent CFUs in the spleen at day four, consistent with our previous work showing similar fungal burdens by quantitative culture of the brains at days 9 and 14 (266). Therefore, we hypothesize that the altered virulence of the rim101Δ strain is not due to increased dissemination.

Titan cells have also been recently implicated in the regulation of inflammation during cryptococcal lung infections. An increase in the proportion of titan cells is associated with a decrease in the phagocytosis of cryptococcal cells and in overall inflammation (269, 388). To test whether the rim101Δ phenotype can be primarily attributed to a lack of titan cells, we co-infected the lungs with both strains to provide a source of titan cells. A/J mice were infected intranasally with a total of 2x10⁴ cryptococcal cells in a 1:1, 2:1, and 1:2 ratio of wild type to rim101Δ mutant cells. After day four, the lungs were harvested and analyzed via histopathology (Figure 13B). We observed that co-infected lungs had more inflammation than lungs infected with just the wild type strain, despite abundant (minimum 19.2%) titan cells (Figure 13B). The amount of inflammation correlated most closely with the initial dose of rim101Δ cells; however, we observed inflammation even in areas containing both titan cells and the smaller rim101Δ cells, suggesting that titan cells cannot completely protect against rim101Δ-driven inflammation (Figure 13C).
Figure 13: Rim101 regulates *C. neoformans* cell size in vivo

**A. rim101Δ cells are smaller than wild type cells in vivo.** Fungal cells were harvested from mouse lungs by lavage 4 days post-infection and examined microscopically. Diameters were measured using ImageJ.

**B. Co-infected lungs demonstrate rim101Δ-mediated inflammation.** Mice were infected with the indicated ratios of wild type to *rim101Δ* mutant cells for a total of $5 \times 10^5$ cells per mouse. Lungs were harvested at day 4 post-infection and assed by histopathological analysis.

**C. Titan cells are not sufficient to prevent rim101Δ-mediated inflammation.** Mice were infected with an equal number of wild type to *rim101Δ* mutant cells for a total of $5 \times 10^5$ cells per mouse. Lungs were harvested at day 4 post-infection and assed by histopathological analysis. Local foci of inflammation contain both titan cells and smaller *rim101Δ* cells. Arrows indicate the two cell size populations present in each lung.
4.4.2 Altered cytokine and cellular immune response to the rim101Δ mutant

To better quantify the population of inflammatory cells in the mouse lungs after infection with 5x10^5 wild type or rim101Δ cells, we collected bronchoalveolar lavage fluid for cytokine and leukocyte analysis from A/J mice four days after infection. We performed cytospin analysis on the lavage fluid and used hematoxylin and eosin staining to differentiate the various cell populations by morphology, as described previously (135). As apparent from the lung histopathology sections, total lung leukocytes were greatly increased in rim101Δ-infected mice (Figure 12B, C). Moreover, the vast majority of these inflammatory cells were neutrophils (Figure 14A). Mice infected with the rim101Δ strain showed a statistically significant increase in the percentage of neutrophils and eosinophils than mice infected with the wild type strain or the saline control (Figure 14A). As expected, we did not observe significant influx of lymphocytes at this early time point, consistent with a predominantly innate immune response.

We also examined the levels of 20 murine cytokines from the lavage fluid using Luminex multiplex assays. Overall, we saw increased cytokine levels in mice infected with the rim101Δ strain compared with the wild type and PBS controls. In accordance with previous studies, we did not observe significant differences between the cytokine profiles of the wild type and PBS-infected lungs at this time point, suggesting that the wild type is effective at dampening or avoiding the host immune response (182, 183). Of particular note were the levels of interleukin-12 (IL-12), IP-10/CXCL10, MIG/CXCL9, MIP1-α, VEGF, and TNF-α, all of which were significantly higher in rim101Δ-infected mice (Figure 14B). Many of these are pro-inflammatory cytokines that have previously
been implicated in the accumulation of immune cells, clearance of *C. neoformans* from the lung, and eventual Th1-weighting of the adaptive immune response (273).

**Figure 14: Rim101 mediates innate immune cell responses**

**A.** The *rim101Δ*-mediated infection is characterized by polymorphonuclear cell influx. Lavage fluid was collected from mice infected with the wild type (*n* = 10), *rim101Δ* (*n* = 11), or a PBS control (*n* = 10) for cytospin analysis. Slides were stained with hematoxylin and eosin, and the cells were characterized and quantified based on morphological characteristics. Bars indicate means ± SEM.
B. *rim101Δ* cells induce increased cytokine responses. Lavage fluid was collected from mice infected with the wild type (n = 10), *rim101Δ* (n = 11), or a PBS control (n = 10). Cytokine levels were assessed by multiplex ELISAs. Data represent means ± SEM for four independent experiments, each with at least three mice per group. Statistical significance was determined by one-way ANOVA with Bonferroni corrections. ** = p < 0.01, *** = p < 0.001. LLOQ = lower limit of quantification.

4.4.3 Rim101 is involved in cell wall remodeling under host-relevant conditions

The exuberant innate response seen early in the *rim101Δ*-infected mice suggested that the *rim101Δ* cells were aberrantly exposing an antigenic trigger. In other fungi, Rim101 regulates cell wall remodeling under specific environmental stimuli. For example, CaRim101 directs the *C. albicans* yeast-hyphal transition in response to alkaline pH (85, 220, 259). Therefore, we hypothesized that the Rim101 protein in *C. neoformans* is also involved in regulating cell wall structure when activated by host environmental signals.

To test this hypothesis, we examined wild type and *rim101Δ* cells by transmission electron microscopy (TEM) after incubation in either tissue-culture conditions (Dulbecco’s modified Eagle’s medium (DMEM) with 5% CO₂ at 37°C) or in rich medium (YPD). Under rich growth conditions, there was no significant difference in the cell wall thickness between the strains (WT: 152.76 nm, *rim101Δ*: 136.6 nm, *p* = 0.44). However, under tissue-culture conditions, *rim101Δ* cells had extremely thick cell walls compared to wild type (WT: 139.11 nm, *rim101Δ*: 445.08 nm, *p*<0.001) (Figure 15A).
Figure 15: Rim101 regulates cell wall components.

**A. rim101Δ cell walls are significantly thicker than wild type in inducing conditions.** 1. TEM microscopy was performed after incubating in either YPD or DMEM (capsule-inducing conditions). Arrows on the TEM micrographs indicate representative cell wall measurements. 2. ImageJ was used to quantify cell wall thickness. Values are means ± SEM. Two-way ANOVA with Bonferroni corrections were used to determine statistical significance (* = p < 0.001).
B. rim101Δ cells have increased chitin/chitosan in inducing conditions. Cells were stained with FITC-conjugated wheat germ agglutinin (WGA) after induction in CO2-independent medium at 37°C. The percent of cells demonstrating fluorescence around the entire cell were counted. 1. Representative fluorescence patterns at the indicated time points. 2. Quantification of the fluorescence patterns using Image J analysis of multiple microscopic fields.

C. rim101Δ cells have increased α-glucan in inducing conditions compared to wild type. 1. Cells were incubated with the MOPC-104E antibody and the fluorescent anti-IgM secondary antibody after induction in CO2-independent medium at 37°C. The percentages of cells demonstrating fluorescence around the entire cell were counted. 1. Representative fluorescence patterns at the indicated time points. 2. Quantification of the fluorescence patterns using Image J analysis of multiple microscopic fields.

4.4.4 Rim101 transcriptionally regulates cell wall genes

To determine the causative genes for the cell wall changes observed in the rim101Δ strain, we performed comparative transcriptional profiling. We sequenced total RNA extracted from either the wild type or rim101Δ strain after induction in DMEM for three hours at 37°C with 5% CO2. A subset of the Rim101-regulated genes from this dataset has been previously published (271).

The Rim101 transcription factor controls the expression of a large number of genes directly or indirectly, with 1257 genes displaying at least 2-fold differential transcript levels between wild type and rim101Δ cells. Among these, we found many genes involved in cell wall biosynthesis, maintenance, and remodeling (Table 5). In the C. neoformans genome, there are twelve genes with an identified role in the synthesis of chitin and chitosan, the predominant components of the cell wall. Eight of these genes were differentially expressed between the wild type and rim101Δ mutant strain, suggesting that Rim101 regulates their expression under host conditions. Additionally, five of these genes contain a putative Rim101 binding site in the 1000 bp upstream of the start codon (13, 266), consistent with direct Rim101 activation, as opposed to the
altered rim101Δ cell wall merely resulting as a non-specific response to cell stress.

Genes encoding other major elements of fungal cell walls were also differentially transcribed in a Rim101-dependent manner, including the β-glucan synthase genes FKS1, SKN1, and KRE6, and the α-glucan synthase gene AGS1.

Table 5: Differentially regulated cell wall genes between wild type and rim101Δ strains.

<table>
<thead>
<tr>
<th>Genomic ID</th>
<th>Annotation</th>
<th>Fold change (WT/rim101Δ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNAG_00546</td>
<td>Chitin synthase 6</td>
<td>-2.86</td>
</tr>
<tr>
<td>CNAG_00897</td>
<td>SKN1 β-1,6 glucan synthase protein</td>
<td>10.08</td>
</tr>
<tr>
<td>CNAG_00914</td>
<td>KRE6 β-glucan synthesis protein</td>
<td>-2.68</td>
</tr>
<tr>
<td>CNAG_01234</td>
<td>Spore wall assembly-related protein</td>
<td>-3.55</td>
</tr>
<tr>
<td>CNAG_01239</td>
<td>CDA3 chitin deacetylase</td>
<td>-8.83</td>
</tr>
<tr>
<td>CNAG_02298</td>
<td>Glucan 1,4-α-glucosidase putative</td>
<td>-3.13</td>
</tr>
<tr>
<td>CNAG_02850</td>
<td>AGN1 glucan endo-1,3-α-glucosidase</td>
<td>-2.18</td>
</tr>
<tr>
<td>CNAG_02860</td>
<td>Endo-1,3(4)-β-glucanase putative</td>
<td>2.39</td>
</tr>
<tr>
<td>CNAG_03120</td>
<td>AGS1 α-glucan synthase protein</td>
<td>-2.05</td>
</tr>
<tr>
<td>CNAG_04033</td>
<td>α-glucosidase</td>
<td>-2.61</td>
</tr>
<tr>
<td>CNAG_04245</td>
<td>Chitinase CHI22</td>
<td>-6.36</td>
</tr>
<tr>
<td>CNAG_05581</td>
<td>Chitin synthase 4</td>
<td>-2.69</td>
</tr>
<tr>
<td>CNAG_05663</td>
<td>SCW1 cell wall integrity protein</td>
<td>-5.84</td>
</tr>
<tr>
<td>CNAG_05799</td>
<td>CDA1 chitin deacetylase</td>
<td>4.78</td>
</tr>
<tr>
<td>CNAG_05818</td>
<td>CHS5 chitin synthase</td>
<td>-2.44</td>
</tr>
<tr>
<td>CNAG_06508</td>
<td>FKS1 β-1,3 glucan synthase</td>
<td>-5.31</td>
</tr>
<tr>
<td>CNAG_06835</td>
<td>KRE61 β-1,6 glucan synthesis protein</td>
<td>8.26</td>
</tr>
<tr>
<td>CNAG_07499</td>
<td>Chitin synthase 8</td>
<td>-2.70</td>
</tr>
</tbody>
</table>

To examine the regulation of some of these genes, we performed targeted RT-PCR experiments on candidate cell wall genes after the cells were incubated in either rich medium or tissue culture medium for three hours. We were able to recapitulate the overall pattern of gene expression changes observed via RNAseq. For a few genes, we observed divergent regulation under rich medium and tissue culture conditions (CDA3,
KRE6, CHI22), although in some cases the overall fold changes were minimal (data not shown).

4.4.5 Rim101-regulated gene expression is reflected in altered cell wall composition.

To assess the altered composition of the rim101Δ mutant cell wall, and the functional effects of Rim101-mediated cell wall gene expression, we examined α-1,3 glucan, chitin/chitosan, and β-glucan levels in the rim101Δ and wild type strains. Using a biochemical assay for cell wall chitin/chitosan content (211), we confirmed a 1.45-fold increase in the levels of these cell wall polymers in the rim101Δ mutant strain compared to wild type (p < 0.05). We confirmed these results and monitored the dynamic changes in cell wall chitin/chitosan content using FITC-conjugated wheat germ agglutinin (WGA) to stain the oligomeric form of chitin. When either the wild type or rim101Δ strain was incubated in rich medium, we observed fluorescence at only the bud necks, consistent with previous observations of localized chitin deposition at this site (113, 312). After two hours of incubation in tissue-culture medium, we observed increased chitin staining in both the wild type and rim101Δ strains, manifesting as an increasing percentage of cells with diffuse fluorescence/chitin incorporation throughout the cell wall (Figure 15B). In the wild type cells, the percentage of cells with this enhanced pattern of chitin deposition decreased quickly during an eight-hour time course, with the majority of the cells demonstrating chitin localization only at the bud neck by the end of observation (Figure 4B). In contrast, the rim101Δ strain maintained a high percentage of cells with elevated chitin staining, even after eight hours (Figure 15B).

We also examined the role of Rim101 in β-glucan localization and abundance. This cell wall molecule is required for virulence in the fungal pathogen Histoplasma
capsulatum because it prevents host recognition of immunogenic components in the cell wall (303). In C. neoformans, α-glucan is the only known cell wall component required for capsule attachment. Using a labeled antibody against α-glucan (303), we determined that wild type cells show a gradual, time-dependent increase in the levels of this cell wall component during incubation in tissue-culture conditions, consistent with an increasing average capsule diameter around these cells (Figure 15C). We observed similar patterns of increased α-glucan incorporation into the cell walls of the rim101Δ mutant cells, although more of the rim101Δ mutant cells stained for α-(1,3)-glucan than the wild type strain after eight hours of induction (Figure 15C). Because neither strain exhibited α-glucan staining after incubation in rich medium, we hypothesize that α-glucan is specifically induced by C. neoformans under host environmental cues.

Finally, we stained cells with aniline blue to examine β-1,3 glucan levels after induction in DMEM or in YPD (320). Under both conditions, wild type and rim101Δ mutant cells showed similar patterns of cell surface fluorescence (data not shown). Together, these results indicate that Rim101 controls multiple cell wall synthesis proteins under host-relevant conditions. However, the main physiological effect of Rim101 on the cell wall appears to be in controlling chitin/chitosan and α-glucan content.

4.4.6 Altered cell walls affect capsule attachment and phagocytosis

C. neoformans capsule induction has three major stages: biosynthesis within the cell, secretion through the cell wall, and attachment and maintenance at the cell surface. Previously, we demonstrated that the rim101Δ strain was able to synthesize and secrete capsular material despite its defect in surface capsule expression (266). We therefore hypothesized that rim101Δ-mediated changes in cell wall composition would have
functional consequences for capsule attachment, providing the mechanism of the hypocapsular phenotype of the rim101Δ mutant. To better differentiate which of these processes is dependent on Rim101, we first tested the ability of secreted polysaccharides from wild type and rim101Δ mutant cells to bind to the cryptococcal cell surface. Previous work demonstrated that capsule-deficient strains, such as the cap59Δ mutant strain, are able to passively bind exogenous polysaccharide from encapsulated strains (305). We used this strain as a recipient of passive capsule binding to assess the function of secreted capsular material from the wild type and rim101Δ mutant strains using indirect immunofluorescence with an anti-capsular antibody (mAb 18b7). Secreted polysaccharides from conditioned medium from either the rim101Δ or wild type cells were able to passively bind to cap59Δ cells, restoring surface capsule to this acapsular strain. Moreover, the pattern of surface fluorescence was similar between the strains (Figure 16A). When we co-cultured cap59Δ cells with cap59Δ-conditioned medium, we observed no surface capsule staining, as expected for a strain with a known defect in polysaccharide secretion (123, 306). These results are most consistent with a model in which the rim101Δ capsule defect is due to its altered cell wall and aberrant capsule binding rather than intrinsic alterations in polysaccharide synthesis or structure.

Given its intact capsule synthesis and defective capsule binding, we hypothesized that the rim101Δ mutant would shed more polysaccharide into the medium than the wild type strain. We quantified the exopolysaccharide levels using an established protocol of electrophoresis and immunoblotting of conditioned medium from the wild type and rim101Δ strains, using a series of two-fold dilutions to quantify the relative levels of shed GXM (Figure 16B). This analysis revealed that the rim101Δ strain releases approximately 4-fold more GXM into the medium than the wild type strain. The
wild type strain retains significantly more capsule at the cell surface, thus maintaining a
more substantial barrier between the fungal cell and the host immune system.

To further examine the potential physiological consequences of altered surface
capsule on phagocytosis, we assessed Rim101-dependent cell wall changes on
phagocytosis rates. In our previous work, we determined that there was no difference in
phagocytosis between wild type and \textit{rim101Δ} strains (266). In contrast, recent work by
Chun et al. showed that \textit{rim101Δ} cells have a higher rate of association with
macrophage cells than wild type after 24 hours of co-incubation (69). However, both of
these prior experiments were performed under conditions that would not induce capsule
or cell wall changes in the cryptococcal cells. Therefore, we compared the phagocytosis
of wild type and \textit{rim101Δ} cells after either incubation in rich medium (non-
inducing conditions) or tissue culture medium (inducing conditions) for 12 hours (Figure 16C).

Both induced and uninduced cells were opsonized with mAb18b7 before co-
incubation with PMA-activated J774A.1 macrophages in standard phagocytosis assays
(266). After 2 hours, unengulfed cryptococcal cells were removed by gentle washing.
The macrophages were then lysed and plated for CFUs. As expected, induction of
capsule around the wild type cells caused a 1.28-fold decrease in phagocytosis rate
(87.7 vs. 68.1%, \( p < 0.004 \)). Induction in tissue-mimicking conditions caused a 1.24-fold
decrease in phagocytosis in the \textit{rim101Δ} cells (88.1 vs. 71.0%, \( p < 0.02 \)). Under both
conditions, there was no significant difference in phagocytosis of the \textit{rim101Δ} mutant
strain compared with the wild type strain.

Finally, to examine the role of shed polysaccharide in these interactions, we pre-
icubated \textit{cap59Δ} mutant cells with conditioned medium from either the wild type or the
\textit{rim101Δ} mutant strain for one hour before examining phagocytosis. We observed no
statistically significant difference in phagocytosis rates of cap59Δ cells coated with either wild type or rim101Δ shed polysaccharide (87.23 vs. 91.07%, p = 0.12), suggesting that the shed capsular material from both strains is functionally equivalent in this assay (Figure 16 C).

Figure 16: Rim101 regulates capsule attachment.

A. Secreted polysaccharide from rim101Δ cells binds the cell surface similar to wild type. cap59Δ mutant cells were incubated with conditioned medium from cap59Δ, wild type, or rim101Δ strains. Secreted polysaccharide passively bound to the cell surface was visualized using an anti-GXM antibody.

B. The rim101Δ mutant strain secretes more GXM than the wild type. Equivalent cell numbers from the indicated strains were incubated in CO2-independent capsule-inducing medium for 24 hours before pelleting to remove the cells. The supernatant was then serially diluted (2-fold) and subjected to gel electrophoresis and immunoblotting using an anti-GXM antibody.
C. Phagocytosis is regulated by capsule and cell wall components. 1. A representative phagocytosed cryptococcal cell. Arrows indicate the engulfed cell. Phagocytosis rates were measured after coincubation of PMA-activated J774A.1 cells with cryptococcal strains for 2 hours. Unphagocytosed yeasts were removed by gentle washing, and the phagocytic index was determined by quantitative culture. Student’s t-test was performed to determine statistically significant differences (*).

4.4.7 Rim101 induces TNF-α production in macrophages

To directly test whether the altered cell wall of the rim101Δ mutant is able to differentially activate immune cells, we co-incubated wild type and rim101Δ mutant strains with J774A macrophages and assessed TNF-α production after a short period of co-incubation. The fungal cells were incubated overnight in tissue culture medium to induce capsule and cell wall changes and washed to minimize the effect of residual shed capsule on fungal-host cell interactions. When co-cultured with macrophages for 4.5 hours, the rim101Δ strain induced significantly more TNF-α production than the wild type strain (Figure 17).

To address whether the shed capsular components from the different strains result in different host cell responses in this assay, we examined the production of TNF-α after incubating J774A.1 cells with conditioned medium from the wild type or rim101Δ mutant. Conditioned medium from either strain resulted in similar repression of TNF-α production by these macrophage-like cells, suggesting that the well-documented immunosuppressant properties of C. neoformans capsule are not compromised in the rim101Δ strain (Figure 17). Therefore, the altered cell surface of the rim101Δ mutant strain, and not its secreted exopolysaccharide, is likely the primary microbial feature resulting in accelerated activation of the host immune system.
Figure 17: The rim101Δ strain induces different TNF-α responses than the wild type.

1x10⁴ serum-starved macrophages were co-incubated with live wild type, rim101Δ or cap59Δ cells or with conditioned medium from wild type or rim101Δ strains. After 4.5 hours, TNF-α levels in the supernatants were measured by ELISA. Macrophages incubated without cryptococcal cells were used as the control. Student’s t-test was performed to determine statistically significant differences (*). Grey = response to live cells, vertical stripes = response to conditioned medium. The results are from three independent experiments. Unstimulated J774A.1 cells produced 82.66 ± 16.39 pg/mL of TNF-α.

4.5 Discussion

4.5.1 The rim101Δ mutant strain causes a fundamentally different disease

Historically, treatment of infectious diseases has primarily focused on rapid eradication of the pathogen. However, antimicrobial agents are often insufficient to cure infections in the absence of an effective immune system. Moreover, an over-exuberant host immune response can be equally detrimental to patient outcome. This balance of killing the pathogen and modulating the host immune response is exemplified in the
practice of combining antibacterial therapy and corticosteroids to treat some types of bacterial meningitis (348).

Recently, immune reconstitution inflammatory syndrome (IRIS) has emerged as an important aspect in the clinical management of various infectious diseases. In patients with advanced AIDS, antiretroviral treatment (ART) can result in rapid restoration of the immune system. As viral replication is impaired, the patient's CD4+ T-cells can recover, resulting in varying degrees of immune restoration. This immune reconstitution can lead to intense cell-mediated responses to latent or subclinical infections, leading to increased symptoms of disease despite effective clearance of the pathogen (321). Excessive IRIS in cryptococcal disease is associated with high morbidity and mortality. Various studies have reported that between 8 and 50% of AIDS patients develop cryptococcal IRIS after treatment with ART, even when previously treated with antifungal therapies, emphasizing the importance of this phenomenon in the management of cryptococcal meningitis (35, 321). In one study, the patients that developed IRIS had increased CSF levels of several Th1 and innate cytokines during the effector phase of the disease (35).

The excessive virulence of the rim101Δ mutant strain is likely due to aberrant stimulation of immune responses, similar to what is observed in cases of IRIS during infections with wild type, clinical strains. Generally, mortality from cryptococcosis in immunosuppressed patients is due to a combination of proliferation of cryptococcal cells within the brain and lungs of the host, as well as damage from the host immune response to these organisms. However, we observed that mice infected with the rim101Δ strain showed a significantly decreased fungal burden, despite demonstrating similar kinetics of mortality as mice infected with the wild type strain.
Histopathological examination of *rim101Δ*-infected mouse lungs demonstrated an increased influx of inflammatory cells, especially polymorphonuclear (PMN) cells and eosinophils, indicative of a fundamentally different nature of disease progression than infection with wild type strains. Cytospin analysis confirmed the consistent increase in the percentage of PMNs after infection with the *rim101Δ* strain. Previous work on the role of neutrophils in cryptococcal disease revealed that, in contrast to many other fungal infections, early neutrophil influx into the infected lungs could be detrimental to the outcome of cryptococcal infections. Transient antibody-mediated inhibition of neutrophils at an early time point in *C. neoformans* infections resulted in increased survival of the mice (236). In another study, skewing the immune cell infiltrate toward a higher proportion of neutrophils (by removing dendritic cells and alveolar macrophages) also resulted in increased mortality after infection with *C. neoformans* (275). Although resistant SJL/J mice have increased neutrophilia compared to susceptible C57BL/6 mice after infection with *C. neoformans*, the increased neutrophils were associated with more areas of localized inflammation (145). These studies are consistent with our results demonstrating that an early, aggressive innate immune response characterized by neutrophil influx into the lungs can cause disruption of the optimal balance between microbial clearance and host damage.

Additionally, the cytokine profile of the infected lungs confirmed that wild type cells are very effective at actively preventing an excessive inflammatory response. We hypothesize that this process of immune evasion is in part mediated through the Rim101 protein. In the *rim101Δ*-infected lungs, we observed increased levels of a number of pro-inflammatory cytokines compared to the wild type, including TNF-α, IP-10, MIG, MIP-1α, VEGF, and IL-12. In most cryptococcal infections, increased Th1 cytokine levels (TNF-α,
IL-12, and IFN-γ) are associated with decreased lung CFUs and an overall protective Th1 immune response (87, 167, 237). Interestingly, we observed an increase in TNF-α, IL-12, IP-10 and MIG levels without a corresponding increase in IFN-γ in the rim101Δ-infected lungs at the particular time-point used for these studies. Previous work has demonstrated that some of the protective effects of Th1 responses, including repression of eosinophil recruitment, are dependent on IFN-γ, potentially explaining the increased levels of eosinophils in the rim101Δ-infected mice (71, 182). Increased levels of MIG and IP-10 are often used as markers of allergic airway inflammation (119), and increased VEGF levels are also associated with pathological Th2 responses, especially in the context of asthma (206). However, we did not observe an increase in the Th2 cytokines (IL-4, IL-5 and IL-13) that are the major drivers of allergic airway inflammation, suggesting that the inflammation in the rim101Δ-infected lungs was primarily driven by Th1-associated cytokines.

Lymphocytes were not a significant component of the inflammatory cell infiltration in either wild type or rim101Δ infections at these early time points, emphasizing the role of the innate immune system in responding to the exposed rim101Δ cell wall. These results in mice were reinforced by C. neoformans infections in the invertebrate wax moth larvae (Galleria mellonella) model. This organism has an innate immune system, but it does not have adaptive effectors of immunity. Unlike other acapsular mutants, the rim101Δ strain maintained its virulence in G. mellonella, further supporting our hypothesis that Rim101-mediated repression of innate immune responses is important for both host and pathogen survival.
4.5.2 Rim101 regulates remodeling of the cell wall upon entry into the host

To understand the mechanism by which the \textit{rim101}\textsuperscript{Δ} mutant strain provokes this aberrant immune response, we examined the host-pathogen interface, especially in the context of decreased capsule shielding by the \textit{rim101}\textsuperscript{Δ} strain. Exposure of cell wall components, especially those that are conserved PAMPs, can stimulate the host immune response. Recent work in \textit{C. albicans} has established a model by which the innate immune system responds to fungal pathogens, as reviewed in (254). In \textit{C. albicans} and \textit{S. cerevisiae}, increased exposure of β-glucan is sufficient to induce the host inflammatory response via C-type lectin receptors on host cells (128, 255, 369, 370). Additionally, Cross and Bancroft demonstrated that, in acapsular strains of \textit{C. neoformans}, mannose and β-glucan receptors on macrophages are important in binding to cryptococcal cells, and this binding results in increased phagocytosis and cytokine production (79).

We demonstrated that \textit{C. neoformans} Rim101 activation plays a profound role in maintaining and remodeling the cell wall under host conditions. Our RNA sequencing experiments revealed that many cell wall genes were significantly differentially regulated in the \textit{rim101}\textsuperscript{Δ} mutant compared with the wild type strain. Many of these genes had potential Rim101 binding sites in their putative promoter regions. In other fungi, Rim101 is also involved in the regulation and remodeling of cell walls, especially under host or stress conditions. In \textit{S. cerevisiae}, Rim101 participates directly in cell wall assembly and remodeling by acting together with the PKC signaling pathway (50). The yeast-hyphal transition, which is necessary for virulence in \textit{C. albicans}, is dependent on CaRim101.
Additionally, *C. albicans* uses Rim101 to regulate the expression of Phr1 and Phr2, which are β-glycosidases that are involved in cell wall remodeling (85).

We observed that the alterations in the *C. neoformans rim101Δ* mutant cell wall appear to be mostly due to excess chitin oligomers. Although the interactions between the host response and chitin are unclear, especially due to variable sources and processing, chitin has been linked with increased eosinophil recruitment and other allergic responses (212, 307). Interestingly, we also saw a significant increase in α-glucan levels in both strains after induction in host-mimicking conditions. These results are the first indication that α-glucan is induced upon entry into the host. Previous work on α-glucan in *C. neoformans* has demonstrated that this molecule is necessary for capsule attachment to the cell (305, 306). The induction of α-glucan in the wild type strain is correlated with an increase in capsule diameter around the cell. However, the *rim101Δ* mutant exhibited more α-glucan staining than the wild type strain, consistent with the subtle increase in transcription of the α-glucan synthase gene in the *rim101Δ* mutant, despite the defect in capsule attachment. We hypothesize that this moiety is not sufficient for maintaining capsule at the cell surface. It is possible that the overall structure of the *rim101Δ* mutant cell wall is altered to a degree that α-glucan cannot maintain capsule. Further analysis of the *rim101Δ* mutant strain will provide more insight into the components and structures required for capsule attachment.

Additional complexity comes from the documented role of α-glucan in masking other, more immunogenic, molecules from the host. Work in *H. capsulatum* demonstrated that α-glucan can mask the highly immunostimulatory β-glucan from recognition by the dectin-1 receptor (303, 304). In *C. neoformans*, however, α-glucan
may not act as an effective shield because the \textit{rim101}\Delta strain stimulated more inflammation than the wild type. Additionally, we did not observe significant IL-10 production in any of the infected lungs, suggesting that C-type lectins may play a relatively minor role in the response that we observed in this model to \textit{C. neoformans} (128), thus minimizing the effect of increased \(\alpha\)-glucan shielding.

In addition to cell wall changes, alterations in capsule attachment and total levels of capsular polysaccharide may also partially explain the differential immune responses. At a systemic level, GXM has been used as a potent immunosuppressive and anti-inflammatory agent, ameliorating the effects of both collagen and endotoxin-induced arthritis in rat models and LPS-induced sepsis in mice (240, 245, 291, 343). Although the wild type and \textit{rim101}\Delta mutant synthesize similar levels of capsule (266), the increase in wild type cells likely contributes to the increased levels of capsular polysaccharide in the host. This may be effective at dampening the host immune response to the wild type cells. However, many infections using a wide range of inoculum doses of the wild type strain have all failed to produce the type of inflammation triggered by the \textit{rim101}\Delta mutant strain. Additionally, completely acapsular strains, such as the \textit{cap59}\Delta strain, also fail to cause this characteristic lung inflammation phenotype.

Another potential consequence of decreased capsule attachment in the \textit{rim101}\Delta strain is the increased exposure of mannoproteins, which can then induce IL-12 production (159, 213, 294). Increased IL-12 can induce increased MIP-1\(\alpha\), which plays a role the recruitment of leukocytes, especially macrophages and neutrophils (160).

Finally, titan cells, which are a recently described \textit{C. neoformans} morphotype, are characterized by thickened cell walls and altered capsule attachment, in addition to massively increased cell size (270, 388). Titan cells inhibit phagocytosis, and the degree
of inflammation in the lungs is higher when the number of titan cells decreases (388). The \textit{rim}101Δ mutant strain has a demonstrated defect in titan cell formation in our experiments and in previous observations (271). By histology, we observed that only 2.0\% of the \textit{rim}101Δ cells were titans, compared to 33.6.8\% of the wild type. The inability of the \textit{rim}101Δ mutant to appropriately remodel cell walls under host conditions may be responsible for the lack of titan cell formation in this strain. Additionally, the dramatic reduction in the number of titan cells may also be a factor in the increased inflammatory response that we see in \textit{rim}101Δ infections. However, we were unable to completely abrogate the inflammation when adding wild type cells as a source of titan cells to a \textit{rim}101Δ infected mouse, implying that a decrease in titan cell formation is not sufficient to explain the \textit{ rim}101Δ mutant virulence phenotype. Together, these data suggest the hypothesis that CnRim101 directs remodeling of the cell wall when activated by host-specific stresses, thus minimizing the inflammatory immune response. In summary, we previously demonstrated that \textit{C. neoformans} responds to specific host signals by cleaving the Rim101 protein (266). Activated Rim101 induces cell wall changes that favor capsule attachment and minimize PAMP exposure. Both of these Rim101-mediated cell wall events have potentially important implications for pathogen survival in the host. Without Rim101, the cell is unable to mask immunogenic PAMPs, which could then trigger increased and aberrant inflammation in the mouse lungs. The \textit{rim}101Δ cells also have decreased survival in the harsh host environment, potentially reducing the overall levels of immunosuppressive GXM in the host organism. These studies therefore demonstrate fundamental ways in which pathogens induce protective phenotypes to avoid detection and actively modulate the host immune response, thus determining the outcome of the infectious process.
4.6 Methods and Materials

Strains and media:

Cryptococcus neoformans strains used in this study were H99, rim101Δ (TOC2), rim101Δ + RIM101 (TOC4), cap59Δrim101Δ (TOC39) and cap59Δ (54, 81, 266, 290). Capsule inducing medium (Dulbecco’s modified Eagle’s medium with 25 mM NaHCO₃) was prepared as previously described (140).

Virulence and Data Analysis:

Virulence of the C. neoformans strains was assessed using a murine inhalation model of cryptococcosis, as described previously (77). 10 female C57BL/6 mice were inoculated intranasally with 2x10⁴ C. neoformans cells of the wild type, rim101Δ mutant, cap59Δrim101Δ double mutant, or cap59Δ mutant strains. Mice were monitored daily for signs of infection and were sacrificed at predetermined clinical endpoints predicting mortality. Statistical significance between survival curves of all animals infected with each strain was evaluated using the log-rank test, and p-values are reported in the text (JMP software, SAS institute, Cary NC). Cell counts were analyzed using Student’s unpaired t-test. All studies were performed in compliance with institutional guidelines for animal experimentation.

For co-infections, two female A/J mice were inoculated intranasally with 5x10⁵ cryptococcal cells in a 1:1, 2:1, or 1:2 ratio of wild type to rim101Δ mutant cells. For irradiated cell infections, two female A/J mice were inoculated intranasally with 5x10⁵ UV-irradiated wild type or rim101Δ mutant cells. Before irradiation, the cells were incubated overnight in DMEM with 5% CO₂ at 37°C to induce capsule. For cytokine analysis, 13 female A/J mice were inoculated intranasally with 5x10⁵ wild type or rim101Δ mutant cells in a 25 µL volume or with 25 µL of sterile PBS.
Histopathology:

Lungs were inflated and harvested in 10% neutral buffered formalin from A/J or C57BL/6 mice at the indicated days post-infection. All lungs were then embedded in paraffin, cut into 5 µm-thick slices, and stained with hematoxylin and eosin (H&E) by the Duke histopathological core facility. All slides were examined by light microscopy.

Cytospin and cytokine preparations:

The lungs of euthanized mice were lavaged twice with 0.5 mL of lavage buffer (PBS with EDTA) for cytokine collection (1 mL total) and a further 10 times (5 mL total) to obtain cells for cytospin analysis, according to established protocols (145). The supernatant of the first mL of lavage fluid was used for multiplex cytokine analysis. The remaining cells were combined with cells from the last 5 mL and resuspended in a total volume of 1 mL. Cell densities were then normalized to 1x10^5 cells/mL by hemocytometer, and 200 µL were centrifuged onto slides. Slides were stained with H&E and manually inspected to directly quantify the nature of the host cells in the lung cell infiltrate, as described previously (276). At least 300 cells were counted per sample. To quantify the immune cell infiltrate, cryptococcal CFUs were subtracted from the normalized cell counts obtained by hemocytometer. Statistical significance was determined by performing one-way ANOVAs with Bonferroni correction.

To determine cryptococcal cell diameters after infection, 0.5 mL of the lavage fluid was pelleted and resuspended in 0.05% SDS to lyse mammalian cells. The remaining cryptococcal cells were washed three times in water. Cells were obtained from at least three mice per strain. To determine in vitro cell diameters, all strains were incubated overnight in CO_2-independent medium at 37°C. All cell diameters were quantified using ImageJ, and titan cells were defined as cells with a diameter greater
than 15 μm (270). Capsules were not included in diameter measurements. At least 500 cells were counted for each sample.

**Multiplex cytokine analysis:**

Fibroblast growth factor basic, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, IFN-γ-inducible protein 10 (IP-10), KC, MCP-1, MIG, MIP-1α, TNF-α, and vascular endothelial growth factor (VEGF) were analyzed using the Invitrogen Cytokine Mouse 20-plex Panel on a Bio-Plex Luminex-100 machine at the Duke Human Vaccine Institute Shared Resource facility, according to the manufacturer’s instructions.

**ELISA:**

J774A.1 macrophages (1x10⁴ cells/well) were inoculated into 96-well plates for 15 hours in medium with 1% FBS. Macrophages were then co-incubated with 100 μL of either medium or cryptococcal cells (1x10⁴ cells/well) for 4.5 hours at 37°C with 5% CO₂. The supernatant was then removed, and TNF-α levels were assessed by ELISA following the manufacturer’s instructions (Mouse TNF-α ELISA MAX standard Kit, Biolegend, San Diego, CA).

**Phagocytic Index:**

To examine phagocytosis, we directly measured the interactions of macrophages according to previous protocols with some modifications (110, 264). J774A.1 cells (1x10⁵ cells/well) were added to 96-well plates and activated by adding PMA and incubating for 2 hours at 37°C with 5% CO₂. Then, 10⁵ cryptococcal cells/well were added and co-incubated for 2 hours. Unengulfed cells were removed by gentle washing with 2 x 50 μL PBS. The macrophages were then lysed with 100 μL of 0.5% SDS, and the phagocytosed cells were plated for CFUs.
**RNA preparation, sequencing and transcript analysis:**

To induce expression changes, all strains were incubated in YPD to mid-log phase, washed twice, and then incubated in DMEM at 37°C with 5% CO₂ or YPD for three hours. Cells were then washed twice, frozen on dry ice, and lyophilized for three hours. Total RNA was extracted using the Qiagen RNeasy Plant Minikit (Qiagen, Valencia, CA), as previously described (266).

All library preparation and RNA sequencing was performed by the Duke Sequencing Core Facility. Total RNA samples were purified and prepared according to the manufacturer’s protocols (Illumina, San Diego, CA) and as previously described (271). Sequencing was performed on a GAI1 Illumina Genome Analyzer. To achieve sufficient sequence coverage for a reference transcriptome, the wild type sample was sequenced with 72 bp paired-end reads. The rim101Δ sample was sequenced with 36 bp single-end reads.

All reads were mapped to the C. neoformans reference genome provided by the Broad Institute using TopHat v1.3.0 (1, 345). The aligned reads were then analyzed for FPKM (Fragments Per Kilobase of transcript per Million mapped reads) using Cufflinks v.1.0.3. Statistically significant differences between strains was determined by CuffDiff (346). Of the 2.3x10⁷ reads for the wild type sample, only 5.7x10⁴ reads (0.2%) were filtered for poor quality. The rim101Δ sample had less than 0.5% of the reads excluded. Genes were considered significantly differentially expressed if p-values were greater than the false discovery rate after Benjamini-Hochberg corrections for multiple testing and if the fold-change was greater than 2.0. All data were uploaded to the NCBI GEO database under accession number GSE43189.
RNA was converted to cDNA using the Clontech Advantage RT for PCR kit according to the manufacturer’s instructions. Semi-quantitative RT-PCR was performed and fold-change was determined by ImageJ analysis of the resulting amplicons.

**Electron Microscopy:**

Electron microscopy was performed at the Center for Electron Microscopy at North Carolina State University. Cells were incubated at 37°C in DMEM with 5% CO₂ until they reached log-phase growth. Cells were fixed according to the procedures developed by Reese et al. (306).

**Cell Wall Analysis:**

For cell wall staining, strains were washed and then stained with either 0.01% aniline blue (β-glucan) or WGA conjugated to AlexaFluor 488 (chitin) (Molecular Probes, Eugene, OR) (113, 320). After staining, cells were washed twice before observation by fluorescence microscopy. Aniline blue staining was observed using a 350 nm wavelength for fluorescence, and WGA was observed using a 488 nm wavelength for fluorescence. To visualize α-glucan, cells were incubated with the α-(1,3)-glucan MOPC-104E antibody (Sigma, St. Louis, MO) and a fluorescent IgM anti-mouse antibody (Sigma), as previously described (303). Fluorescence was measured using a 494 nm wavelength. Bright field, differential interference microscopy (DIC), and fluorescent images were captured with a Zeiss Axio Imager.A1 fluorescent microscope equipped with an AxioCam mrM digital camera.

Chitin/chitosan levels were determined according to the method developed by Lehmann and White (211), using 20 OD₆₀₀ units per strain to normalize the number of cells for each sample.

**Capsule quantification assays:**
Conditioned medium was made by incubating cells in CO$_2$-independent medium (Gibco) for 24 hours. This tissue culture medium results in capsule induction similar to DMEM. Unlike previous experiments where the cells were heated at 70°C to release capsule, these cells were untreated. To collect the conditioned medium, a 1 ml culture sample was centrifuged at 14,000 rpm to remove cells. The supernatant from each sample was diluted in a series of 2-fold dilutions, and 10 µL of each dilution was subjected to gel electrophoresis and immunoblotting as previously described (266, 305).

**Capsule attachment assays:**

Capsule attachment was performed by incubating cap59Δ cells with conditioned medium, as previously described (305). Capsule was visualized using the mAb 18b7 α-GXM antibody and an AlexaFluor 594-conjugated anti-mouse secondary antibody (Invitrogen).

**Acknowledgements:**

We thank the Duke Sequencing Core Facility, the Duke Human Vaccine Institute Biomarker Analysis Shared Resource facility, the North Carolina State University Electron Microscopy facility, and the Duke Histopathology Core facility for their assistance. We thank Arturo Casadevall for the mAb 18b7 antibody. We thank Gregory D. Sempowski for consultation on multiplexes assays and critical reading of the document. These studies were supported by NIH grants AI050128 and AI074677 (JAA) and an American Heart Association Pre-doctoral Fellowship (TRO).
5. The *Cryptococcus neoformans* Rim101 transcription factor directly regulates genes required for adaptation to the host.

This chapter was modified from a manuscript in preparation. The authors will be Teresa R. O’Meara, Wenjie Xu, Kyla M. Selvig, Matthew J. O’Meara, Aaron Mitchell, and J. Andrew Alspaugh.

### 5.1 Introduction

Microorganisms adapt to the stressful conditions of the infected host by translating stimuli from multiple signal transduction cascades into a coordinated transcriptional response. These signal transduction cascades can be activated by a number of host environmental stimuli, including high temperature, physiological pH, and low nutrients (7). In *C. neoformans*, these stimuli induce the expression of a number of virulence factors that allow it to survive and cause disease. One important virulence factor is the induction of a polysaccharide capsule, which is the primary method of avoiding the host immune response and allowing for disease (54, 57, 96, 193, 263). Encapsulation requires increased polysaccharide biosynthesis, secretion of this polysaccharide across the cell wall, and remodeling of the cell wall to allow for attachment and maintenance around the cell (92, 263). The Rim101 transcription factor appears to regulate encapsulation primarily through cell wall changes in response to signals from the conserved pH response pathway and the cAMP/PKA pathway (266). Without these adaptive cell wall changes, the *rim101Δ* mutant is unable to maintain capsule or antigen masking, thus resulting in massive inflammation in the host (265). Our focus here is to determine the mechanism by which Rim101 regulates the transcriptional responses that allow *C. neoformans* to cause disease in the host.
The Rim101/PacC zinc-finger transcription factor was first identified in *S. cerevisiae* and *A. nidulans* as the major effector of alkaline pH responses (43, 338). Extracellular pH signals are recognized at the plasma membrane and transferred to the endosomal membranes through non-canonical arrestin signaling and the ESCRT machinery (45, 73, 121, 147, 150, 151, 200, 216, 315). At the endosomal membranes, Rim101 is cleaved at the C-terminus and then localized to the nucleus (215, 239). The members of this pH-response pathway are highly conserved and have been identified in most fungal species. The *C. neoformans* genome also contains many elements of this conserved pathway, but there are no obvious homologues of the membrane-associated pH sensors. Instead, *C. neoformans* Rim101 has additional activating signals through the cAMP/PKA pathway (266).

In *C. neoformans*, Rim101 is important in the regulation of a number of responses to host stresses. In addition to altered cell wall composition and defects in capsule, the rim101Δ mutant is sensitive to low iron, high salt concentrations, and alkaline pH (44, 179, 266). Many of these mutant phenotypes are conserved among rim101Δ mutants in other fungi, including in the pathogens *C. albicans*, *A. fumigatus*, and *U. maydis* (10, 27, 83, 84, 103, 286, 342). Extensive work in these fungi has revealed a conserved 5'-GCCAAG binding motif for the Rim101 transcription factor, allowing for identification of direct targets that influence adaptation to external pH signals (12, 13, 201, 302).

Some previously identified direct targets of Rim101 in *C. albicans* include the cell wall glycosidases *PHR1* and *PHR2*, and the ferric reductases *FRE1* and *FRP1* (13, 27, 84, 302). Rim101 induces the expression of both ferric reductases, but represses the expression of *PHR2* (12, 13). In *S. cerevisiae*, however, Rim101 acts primarily as a
negative regulator of gene expression, and a major target is the Nrg1 transcription factor (201). Moreover, binding of Rim101 to promoters may act in an additive manner; the *A. nidulans ipnA* gene requires PacC/Rim101 binding at all three sites in the promoter for full activation of expression (102).

The role of *C. neoformans* Rim101 in transcriptional adaptations to the host had been previously implied by gene expression data. In this work, we demonstrate that the *C. neoformans* Rim101 protein directly interacts with genes that are necessary for adaptation to the host, especially those processes involved in cell wall remodeling and capsule attachment. We hypothesize that the connections between Rim101 and Pka1 allow for a wider range of activating signals for the Rim101 transcription factor. Using chromatin immunoprecipitation, we demonstrate that *C. neoformans* Rim101 can act as both an activator and a repressor, depending on the target gene. Moreover, we used *in vivo* RNA profiling as a way to examine the transcriptional response to the host. This analysis revealed the limitations of *in vitro* growth conditions and emphasized the importance of examining a pathogen in the context of infection to determine biologically relevant phenotypes.

### 5.2 Results

**5.2.1 Pka1 and Rim101 share downstream targets**

In *C. neoformans*, Pka1 regulates Rim101 localization and function (266). To define the overlapping and unique sets of genes with Pka1- and Rim101-dependent transcription, we compared the global transcriptional profiles of the *pka1Δ* and *rim101Δ* mutant strains to the wild type (34, 106). Previously, we performed deep mRNA sequencing of wild type and *rim101Δ* cells after incubation in tissue culture conditions for three hours (265). We subsequently performed identical transcriptional analysis of the
pka1Δ mutant strain incubated under the same conditions. This profiling revealed 1476 genes with at least 2-fold differences in expression between the pka1Δ mutant and the wild type.

Of the 1257 genes with significantly different expression between the rim101Δ mutant strain and the wild type, 1077 were also significantly transcriptionally dependent on Pka1. Moreover, pairwise correlation analysis of the entire transcriptomes of the pka1Δ and rim101Δ mutant strains revealed a strong correlation between Pka1- and Rim101-dependent genes (adjusted r²=0.515, p < 0.001). This correlation increased dramatically when we examined only those genes that were at least 2-fold differentially expressed between the wild type and mutant strains (adjusted r² = 0.923, p < 0.001). The majority of genes with Rim101-dependent expression demonstrated a similar direction and magnitude of transcriptional control by Pka1 (Figure 18). This strong correlation in the transcriptomes of the rim101Δ and pka1Δ strains provides further evidence that C. neoformans Pka1 and Rim101 are in the same pathway.
Figure 18. Pairwise correlation analysis of the entire transcriptomes of the \textit{rim101}\textDelta and \textit{pka1}\textDelta mutant strains.

Gene expression levels were determined in comparison with the wild type strain. All strains were incubated for 3 hours in tissue culture medium to induce expression changes. Genes that were less than two-fold differentially expressed between the wild type and mutant strains are in gray. Adjusted $r^2$ for the entire transcriptomes was 0.515 ($p < 0.001$). Adjusted $r^2$ for when comparing only the significantly differentially expressed genes was 0.923 ($p < 0.001$).

We observed similar differences in expression of multiple kinases, phosphatases and DNA binding proteins, potentially extending the downstream network of the Pka1-Rim101 pathway. The shared targets also included proteins involved in cell wall biosynthesis and remodeling (especially processes related to $\alpha$-1,3 glucan, $\beta$-1,3 glucan, and $\beta$-1,6 glucan synthesis), consistent with previously documented changes in \textit{rim101}\textDelta cell walls (265). Additionally, 5 of the 7 genes involved in chitin and chitosan biosynthesis (20-22, 25) were differentially expressed in the two mutant strains compared with the wild type. Therefore, we used WGA to examine chitin localization after incubation in DMEM and observed similar patterns of staining in the \textit{rim101}\textDelta and \textit{pka1}\textDelta mutant strains.
pka1Δ mutant strains (Figure 19A). Unlike the wild type strain, which shows only staining at the bud necks, the rim101Δ, rim20Δ, and pka1Δ strains show a more diffuse pattern of WGA-associated fluorescence around the entire cell, consistent with similar alterations in cell wall chitin content.

We previously documented that the rim101Δ mutant has increased growth in macrophages, presumably due to resistance to acid pH. To test this in vitro, we performed a growth curve in synthetic complete medium buffered at pH 3 because the pH of alveolar macrophage phagolysosomes ranges from 3 to 5 (262). Under these conditions, the rim101Δ and rim20Δ strains grew better than wild type (Figure 19B). The pka1Δ mutant, however, had intermediate growth compared with the wild type. This suggests that the pka1Δ mutant has further phenotypic alterations that are responsible for decreased growth under these conditions. However, the core gene set responsible for acid resistance are likely to be regulated by both Pka1 and Rim101.

Despite the striking similarities between Pka1 and Rim101-dependent gene expression, there were a number of genes that were transcriptionally regulated by only one of these proteins. This observation may explain some of the phenotypic differences between the pka1Δ and rim101Δ mutant strains. For example, Rim101-specific targets include the sodium transporter ENA1, perhaps the basis for differences in rim101Δ and pka1Δ sensitivity to high pH (Figure 19C) (164). Pka1-specific targets included Ste12a, potentially contributing to the previously documented pka1Δ-specific defect in melanin (58, 266).
Figure 19. The rim101Δ and pka1Δ strain have both overlapping and divergent phenotypes

A. The rim101Δ and pka1Δ mutants demonstrate similar alterations in WGA binding patterns. Cells were incubated for 24 hours in tissue culture medium before staining with FITC-conjugated wheat germ agglutinin (WGA). All micrographs were taken at the same exposure to differentiate fluorescence levels between the strains.

B. The rim101Δ and pka1Δ mutants proliferate better than wild type at pH 3. Strains were incubated in SC buffered to pH 3 and growth was quantified by monitoring the absorbance of the culture at 600 nm.

C. The rim101Δ and rim20Δ mutants have a defect in alkaline pH. 1x10⁶ cells were 10-fold serially diluted onto the indicated plates and incubated for 2 days.
5.2.2 Rim101 binds a conserved motif

The set of genes with Rim101-dependent transcription discovered through comparative transcriptional profiling is composed of both direct and indirect targets of the Rim101 transcription factor. To determine the direct targets of Rim101 action, we first investigated whether a previously established Rim101-binding motif from other fungi is conserved in *C. neoformans*. Rim101 homologues in other species bind to the GCCAAG motif in the promoters of direct target genes (302, 342). Therefore, we used electrophoretic mobility assays to determine whether CnRim101 also binds this motif. For these experiments, we chose 25-mers spanning the GCCAAG motif from the promoter of the *C. neoformans* iron transporter gene *CFT1*, which is highly differentially expressed between the rim101Δ and wild type strains. We observed distinct mobility shifts of the labeled oligomer when incubated with protein extracts from the wild type strain, but not from the rim101Δ mutant (Figure 20A, lane 1 vs. lane 2). To determine the specificity of protein binding, we added excess unlabeled 15-mer oligomers containing this motif, and we observed reversal of the shift (Figure 20A, lanes 3 and 4). Incubation with protein extracts from the rim101Δ + GFP-Rim101 strain resulted in a supershift in electrophoretic mobility (Figure 20B). Together, these data strongly suggest that Rim101 binds directly to the GCCAAG motif in target gene promoters. Additionally, when we incubated protein extracts with a mutated oligomer (GCCAAG -> GAGAAG), we did not observe the shift in mobility, indicating specificity of Rim101 interactions with its DNA binding site (Figure 20C).

Then, we analyzed the promoters of the Rim101-regulated genes for the presence of this motif. Of the 1257 genes that were differentially regulated by Rim101, 564 had this conserved motif in the 1000 bp upstream of their start sites. We
hypothesized that genes that are direct targets of Rim101 would maintain this regulation across the Cryptococcus family, including in the *C. gattii* sister species. Therefore, we examined the promoter regions of homologues of the Rim101-regulated genes in *C. gattii* (strain R265). Of the 564 Rim101-regulated genes with Rim101 sites in *C. neoformans* var. *grubii*, 310 also had Rim101 binding sites in their *C. gattii* homologues (Figure 20D).

The majority of genes (76.7%) with Rim101 sites in both *C. neoformans* var. *grubii* and *C. gattii* were repressed by Rim101, suggesting that Rim101 may act primarily as a negative regulator of gene expression. We then mapped the position of each binding site in these promoters to examine whether a certain position was correlated with positive or negative gene regulation (Figure 20E). This analysis suggested that Rim101 sites less than 100 bp from the ATG position are more likely to be associated with genes that are induced by Rim101 binding. In contrast, Rim101-repressed genes had more Rim101 binding sites that were either 500 bp or 900 bp away from the ATG.
Figure 20. Rim101 binds a conserved motif.

A. A biotin-labeled 25-mer containing the GCCAAG motif was incubated with protein extracts from wild type (lane 1 and 4) or rim101Δ (lane 2 and 3) cells. The mixtures were assessed by alterations in electrophoretic mobility by PAGE and immunoblotting using streptavidin detection. Excess unlabeled 15-mers were added to lanes 3 and 4.

B. A 25-mer containing the GCCAAG motif was incubated with protein extracts from wild type (lane 1), rim101Δ (lane 2), or rim101Δ+GFP-RIM101 (lane 3) strains prior to electrophoresis.
C. A 25-mer containing a mutated Rim101 binding motif (GAGAAG) was incubated with protein extracts from wild type (lane 1), rim101Δ (lane 2), or rim101Δ+GFP-RIM101 (lane 3) strains prior to electrophoresis.

D. The promoter of the ENA1 gene from C. neoformans var. grubii and its homologous sequence in C. gattii were aligned. The conserved Rim101 binding motif is highlighted.

E. Genes with Rim101 binding sites in both C. neoformans and C. gattii were examined for Rim101-dependent transcription and separated based on induction (green) or repression (red). The position of the binding sites were counted and plotted along a representation of the distance from the transcription start site. All sequences and Rim101 site positions were obtained from FungiDB (www.fungidb.org).

5.2.3 Rim101 binds a conserved motif in vivo

To verify that Rim101 binds this conserved motif in the cell, we performed chromatin immunoprecipitation after incubating cells in Rim101-activating conditions for three hours (DMEM tissue culture medium at 37°C). We used an anti-GFP antibody to enrich for sequences that were bound to the GFP-Rim101 protein and examined candidate promoters from the subset of Rim101-regulated genes that had Rim101 sites in all examined species. As a control, we examined enrichment at the actin promoter, which does not contain a Rim101 binding site and is not transcriptionally regulated by Rim101.

After incubation in tissue-culture medium for three hours, we demonstrated enrichment for Rim101 binding at the promoters of three candidate cell wall genes. For CDA1 and KRE61, enrichment for Rim101 binding correlated with an increase in expression of the gene in the wild type strain. In contrast, the AGS1 promoter demonstrated 2.8-fold enrichment for Rim101 binding and higher expression in the rim101Δ mutant strain (Figure 21). These results suggest that C. neoformans Rim101 directly regulates the expression of cell wall genes by binding to their promoters, but that
the consequences of binding may be modulated by other factors, such as interactions with other proteins.

Rim101 has also been implicated in the regulation of iron and copper homeostasis, in both *C. neoformans* and in other fungal species (12, 13, 179, 180). Therefore, we examined Rim101 binding at the promoters of the *HAPX*, *CTR4*, and *CFT1* genes. Although there was enrichment at all three promoters, HapX had increased expression in the *rim101Δ* strain while Ctr4 and Cft1 had increased expression in the wild type strain (Figure 21).

![Figure 21. Chromatin immunoprecipitation to detect GFP-Rim101 DNA binding](image)

Cells were incubated in tissue culture medium for 3 hours before fixation and chromatin immunoprecipitation. PCRs were performed using primers that flanked a presumed Rim101 binding site in target gene promoters. Fold change was determined by comparing enrichment between the immunoprecipitated sample and the no antibody control. Actin, which does not contain a Rim101 binding site, was used as a control. Genes with increased expression in the *rim101Δ* mutant strain are highlighted in gray.

### 5.2.4 nanoString profiling of virulence gene expression *in vitro*

Recently, nanoString RNA profiling has been used to accurately quantify RNA levels in many organisms, including pathogenic fungi like *C. albicans*. This technique
avoids the biases introduced during cDNA library creation for sequencing, and it can be used to directly compare expression across multiple mutant strains and conditions. It has therefore been successfully applied to assess relative transcript levels of target genes in biological samples. Therefore, we used this methodology to complement our RNA sequencing results and allow us to examine gene expression under multiple growth conditions.

Taking a targeted approach, we surveyed the expression of 26 genes that were likely to be involved in infection-related processes, such as capsule or melanin induction, metal acquisition, osmotic stress resistance, cell wall remodeling, and pH responses (263). As a control for RNA extraction in each sample, we examined the expression of 5 genes that did not demonstrate variable expression in multiple previous microarray, RNAseq, and rt-PCR analyses (148, 179, 192, 264, 266). These control genes were chosen to represent a range of absolute expression levels, allowing for normalization for both lowly and highly expressed genes.

To ensure that the nanoString data would correlate with other methods of transcriptional measurement, we first compared the nanoString data to the RNASeq data during in vitro growth conditions for the wild type and rim101Δ mutant strains after three hours of incubation in tissue culture medium. For most genes, we observed a strong concordance with the changes in gene expression that we observed by RNAseq. However, the RNAseq results tended to suggest larger differences in expression.

Using nanoString profiling, we were able to distinguish genes based on Rim101-dependence and environmental conditions (Table 6). For example, Ena1 is strongly induced in the wild type strain by incubation in CO2-independent medium. However, the absolute expression levels of this gene are also strongly Rim101-dependent, with 6.7
and 5.9-fold differences in expression between the wild type and \textit{rim101Δ} mutant strains in rich medium or CO\textsubscript{2}-independent medium, respectively. The expression of \textit{SKN1}, a β-glucan synthase gene, and \textit{CDA1}, a chitin deacetylase, also follows this pattern. In contrast, \textit{KRE6} expression was repressed in CO\textsubscript{2}-independent medium in the wild type background and induced in the \textit{rim101Δ} mutant background. Figure 22A displays the normalized RNA counts between wild type and \textit{rim101Δ} mutant strains under the two growth conditions for genes that were at least two-fold differentially expressed between the strains.

### Table 6. Normalized RNA counts from nanoString analysis

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene ID</th>
<th>WT YPD</th>
<th>WT TC</th>
<th>\textit{rim101Δ} YPD</th>
<th>\textit{rim101Δ} TC</th>
<th>WT lung (n = 5)</th>
<th>\textit{rim101Δ} lung (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ags1</td>
<td>CNAG_03120</td>
<td>1321</td>
<td>1960</td>
<td>1400</td>
<td>2356</td>
<td>1040</td>
<td>1418</td>
</tr>
<tr>
<td>Cck1</td>
<td>CNAG_00556</td>
<td>589</td>
<td>748</td>
<td>582</td>
<td>894</td>
<td>547</td>
<td>728</td>
</tr>
<tr>
<td>Cda1</td>
<td>CNAG_05799</td>
<td>1893</td>
<td>3464</td>
<td>671</td>
<td>1239</td>
<td>8110</td>
<td>3046</td>
</tr>
<tr>
<td>Cda3</td>
<td>CNAG_01239</td>
<td>433</td>
<td>1619</td>
<td>376</td>
<td>1830</td>
<td>675</td>
<td>509</td>
</tr>
<tr>
<td>Cdc24</td>
<td>CNAG_04243</td>
<td>235</td>
<td>199</td>
<td>264</td>
<td>284</td>
<td>146</td>
<td>196</td>
</tr>
<tr>
<td>Cfo1</td>
<td>CNAG_06241</td>
<td>373</td>
<td>911</td>
<td>96</td>
<td>889</td>
<td>5176</td>
<td>5083</td>
</tr>
<tr>
<td>Cft1</td>
<td>CNAG_06242</td>
<td>1203</td>
<td>2295</td>
<td>251</td>
<td>2963</td>
<td>5985</td>
<td>5281</td>
</tr>
<tr>
<td>Chs4</td>
<td>CNAG_05581</td>
<td>556</td>
<td>672</td>
<td>561</td>
<td>878</td>
<td>1117</td>
<td>1457</td>
</tr>
<tr>
<td>Chs4</td>
<td>CNAG_00546</td>
<td>211</td>
<td>378</td>
<td>221</td>
<td>431</td>
<td>1310</td>
<td>1863</td>
</tr>
<tr>
<td>Chs5</td>
<td>CNAG_05818</td>
<td>254</td>
<td>404</td>
<td>239</td>
<td>555</td>
<td>713</td>
<td>1189</td>
</tr>
<tr>
<td>Chs8</td>
<td>CNAG_07499</td>
<td>241</td>
<td>268</td>
<td>229</td>
<td>364</td>
<td>261</td>
<td>425</td>
</tr>
<tr>
<td>Cir1</td>
<td>CNAG_04864</td>
<td>343</td>
<td>364</td>
<td>286</td>
<td>340</td>
<td>799</td>
<td>684</td>
</tr>
<tr>
<td>Ctr4</td>
<td>CNAG_00979</td>
<td>2443</td>
<td>2025</td>
<td>214</td>
<td>1418</td>
<td>3366</td>
<td>8524</td>
</tr>
<tr>
<td>Ena1</td>
<td>CNAG_00531</td>
<td>660</td>
<td>4946</td>
<td>99</td>
<td>841</td>
<td>5282</td>
<td>2702</td>
</tr>
<tr>
<td>Fks1</td>
<td>CNAG_06508</td>
<td>107</td>
<td>153</td>
<td>119</td>
<td>183</td>
<td>623</td>
<td>946</td>
</tr>
<tr>
<td>HapX</td>
<td>CNAG_01242</td>
<td>96</td>
<td>217</td>
<td>76</td>
<td>368</td>
<td>720</td>
<td>1065</td>
</tr>
<tr>
<td>Kre6</td>
<td>CNAG_00914</td>
<td>812</td>
<td>348</td>
<td>791</td>
<td>1147</td>
<td>700</td>
<td>2291</td>
</tr>
<tr>
<td>Lrg1</td>
<td>CNAG_05703</td>
<td>104</td>
<td>102</td>
<td>102</td>
<td>122</td>
<td>144</td>
<td>165</td>
</tr>
<tr>
<td>Ova1</td>
<td>CNAG_02008</td>
<td>890</td>
<td>1191</td>
<td>702</td>
<td>1406</td>
<td>3348</td>
<td>1927</td>
</tr>
<tr>
<td>Pbs2</td>
<td>CNAG_00769</td>
<td>339</td>
<td>348</td>
<td>311</td>
<td>431</td>
<td>392</td>
<td>544</td>
</tr>
<tr>
<td>Rim9</td>
<td>CNAG_05654</td>
<td>496</td>
<td>376</td>
<td>462</td>
<td>658</td>
<td>2543</td>
<td>3712</td>
</tr>
<tr>
<td>Pka1</td>
<td>CNAG_00396</td>
<td>271</td>
<td>323</td>
<td>227</td>
<td>312</td>
<td>235</td>
<td>260</td>
</tr>
<tr>
<td>Rim101</td>
<td>CNAG_05431</td>
<td>36</td>
<td>122</td>
<td>0</td>
<td>1</td>
<td>336</td>
<td>16</td>
</tr>
</tbody>
</table>
Interestingly, we documented 3.4-fold induction of \textit{RIM101} gene expression in the wild type strain under tissue culture conditions. The expression of \textit{RIM101} and \textit{PacC} genes in other fungal species is highly regulated by growth conditions, contributing to the levels of nuclear-localized protein \cite{46}. In \textit{C. neoformans}, the \textit{RIM101} gene contains six Rim101 binding motifs in the promoter, suggesting that its expression is highly autoregulated. The \textit{C. albicans RIM101} promoter contains only two Rim101 binding sites \cite{302}. Therefore, we created a GFP-Rim101 fusion protein expressed under the endogenous promoter (\textit{pRIM101-GFP-Rim101}) to examine the subcellular pattern of protein localization that occurs during growth in host-mimicking conditions under more physiological levels of protein expression.

In contrast to the constitutively nuclear signal of the histone-driven GFP-Rim101 fusion protein, we observed a lack of fluorescent signal in cells expressing the \textit{pRIM101-GFP-RIM101} allele when incubated in rich medium, consistent with the low levels of expression observed using nanoString profiling. However, when these cells were shifted to tissue culture medium at 37°C, we observed clear accumulation of fluorescence in the nucleus by three hours (Figure 22B). This observation is consistent with Rim101 transcriptional induction and protein activation under these physiologically relevant growth conditions.
Figure 22. Variation in gene expression and localization due to induction in tissue culture medium

A. Normalized RNA levels of genes with differential expression between the wild type and rim101Δ mutant strain. Strains were grown in YPD or tissue culture conditions for three hours before RNA extraction. RNA levels were determined by nanoString profiling. RNA levels were normalized to control gene expression, as described in the methods.
B. Localization of GFP-Rim101 in YPD and tissue culture medium. Cells containing the GFP-Rim101 fusion protein expressed under the endogenous promoter were incubated in YPD or tissue culture medium. The pattern of GFP fluorescence was visualized using a Zeiss Axio Imager.A1 fluorescent microscope equipped with an AxioCam mrM digital camera.

5.2.5 Rim101 regulates expression changes in animal models of infection

A major advantage of nanoString profiling is the ability to examine fungal-specific RNA in the context of the host sample. Therefore, in addition to studying the role of Rim101 in regulating gene expression in vitro, we also explored Rim101-dependent expression of a set of physiologically relevant genes in the setting of a lung infection. We used nanoString profiling to examine the expression changes in a set of C. neoformans genes during growth in the lung using a murine inhalational model of C. neoformans infection. This fungus typically initiates infection in this organ. We also recently demonstrated that the rim101Δ mutant causes a dramatically increased lung inflammatory response compared to wild type infections (265).

To examine infection-specific gene expression changes, we extracted total RNA from the lungs of female AJ mice infected with 5x10⁵ cells at 4 days post infection (n = 5) (Table 1). Additionally, we were able to determine the contribution of the Rim101 transcription factor to the infection process by examining RNA levels of the rim101Δ mutant in the lung in identical murine infections.

Overall, we found that genes that were induced by incubation in CO₂-independent tissue-culture medium was also induced in the wild type strain during infection of a mouse lung. However, there were a number of genes that only showed differential expression in the lung, suggesting limitations of in vitro culture conditions in mimicking the host. Some of these infection-specific genes include CHS4, CHS5, and FKS1, which are involved in cell wall biosynthesis. Additionally, we observed that the
expression of the *RIM101* gene increased in the wild type strain during infection and was approximately 9.4-fold induced in the lung by day 4 compared to expression in rich medium, and an additional 2.75-fold increased compared to expression in tissue culture conditions.

When comparing the gene expression profiles of wild type and *rim101Δ* mutant strains in the lungs (n = 5), 13 of the 26 genes were significantly differentially expressed between the strains (p < 0.05) (Figure 23). These included the chitin biosynthesis genes and the *AGS1* α-glucan synthase gene. However, only four (*CDA1, CTR4, ENA1, and KRE6*) showed at least a two-fold difference in expression levels between the strains (Figure 23).

![Figure 23. In vivo profiling of gene expression](image)

RNA was harvested from mouse lungs infected with either the wild type or the *rim101Δ* mutant strain (n = 5). Expression levels were determined by nanoString profiling of candidate genes. This graph includes all genes with significantly different expression between the wild type and *rim101Δ* strains (p < 0.05).
5.3 Discussion

In *C. neoformans*, the Rim101 transcription factor responds to signals from both the pH-responsive Rim signal transduction cascade and the cAMP/PKA pathway (266). So far, this connection has only been described in Cryptococcus; in other fungal species, Rim101 activation has not been demonstrated to require phosphorylation by PKA (200, 215, 287). Integration of these two pathways appears to give Rim101 flexibility in the upstream signals that allow for activation. Instead of acting primarily as a pH response factor, *C. neoformans* Rim101 can respond to multiple host stimuli via the cAMP/PKA pathway, including such diverse signals as low iron or tissue culture medium (300). To confirm the connections between the cAMP/PKA pathway and the Rim101 transcription factor, we performed deep RNA sequencing of the *rim101*Δ and *pka1*Δ mutants in comparison to the wild type strain after incubation in tissue culture conditions. Comparison of the downstream transcriptional responses to mutations in *rim101*Δ and *pka1*Δ revealed a striking degree of coordinated gene regulation, providing further evidence for a functional relationship between these genes.

This analysis also revealed some targets that demonstrated divergent regulation in the two mutant strains, including genes related to salt and pH sensitivity and melanin production (81, 158, 164, 175). Although it is clear that the Pka1 kinase has multiple downstream targets, the Rim101-specific targets raise intriguing questions about Pka1-independent regulation of this transcription factor. As the *rim101*Δ and *rim20*Δ mutants had similar phenotypes under all tested conditions, this suggests that there is specific activation of Rim101 through the pH signal transduction cascade. However, there is a lack of obvious homologues to the canonical pH-sensing transmembrane proteins, so it is still unclear how this cascade is activated.
Despite the divergent Rim101 activation pathway in *C. neoformans*, there is significant conservation in the downstream targets of Rim101. One of the major cellular processes regulated by Rim101 is the remodeling of the cell wall in response to host signals. We previously demonstrated the importance of this remodeling in the wild type strain as a mechanism for evading the host immune responses (265). In many other fungi, Rim101 also regulates cell wall processes; for example, *C. albicans* uses Rim101 to allow for the yeast-hyphal transition in response to neutral/alkaline pH, which is a vital step for tissue invasion and virulence (259, 354). Additionally, the *C. albicans* Rim101 pathway regulates the levels of chitin in the cell (215). The *S. cerevisiae* Rim101 transcription factor is also required for cell wall assembly, and the *U. maydis* Rim101 transcription factor modulates cell wall sensitivity to lytic enzymes (10, 50). Using transcriptional profiling and cell wall staining, we previously demonstrated that *C. neoformans* Rim101 also regulates these genes (265). Here, we show that the cell wall of the *pka1Δ* mutant has similar features as the *rim101Δ* mutant, and many cell wall genes are also differentially regulated between the wild type and *pka1Δ* mutant strain.

A potential mechanism for this conservation in Rim101 functions is the conservation of the binding motif across these fungal species. Using EMSAs and chromatin immunoprecipitation, we demonstrated that the *C. neoformans* Rim101 protein binds the GCCAAG motif that had been previously documented in *Aspergillus*, *Candida* and *Saccharomyces* species (102, 201, 302). Presumably, the upstream rewiring of the Rim101 activating signal allows *C. neoformans* to induce the expression of a conserved suite of Rim101 targets, such as the cell wall, in response to a wider range of activating signals.
In this work, we also observed that Rim101 binding was not completely associated with either induction or repression of the target genes, suggesting that there is interplay between Rim101 and other transcription factors on these promoter regions. In *A. nidulans*, the *gabA* promoter contains overlapping Rim101 and IntA binding sites, which results in competition for binding and transcriptional regulation (101). Both Rim101 and a CBF binding factor control the expression of the *C. albicans FRP1* ferric reductase (12). A *C. neoformans* ferric reductase transmembrane component (CNAG_06821) promoter also contains both a Rim101 binding site and a CCAAT motif, suggesting that this gene is regulated by both Rim101 and a CBF protein.

Finally, we were able to use nanoString profiling to examine the transcriptional responses to the host. This technology allows for direct quantification of fungal RNA levels within the context of the infected tissue—in our case, the mouse lung. We first confirmed that nanoString profiling recapitulates the *in vitro* transcriptional profiling of cells incubated in either rich medium or tissue culture conditions.

We were then able to use nanoString profiling to examine fungal gene expression in the mouse lung. This *in vivo* analysis revealed the shortcomings of *in vitro* growth conditions, as many genes showed a much higher degree of induction in the mouse lung than in tissue culture medium. For example, *HAPX* was 2.2-fold differentially regulated between YPD and tissue culture medium, but 7.5-fold differentially regulated between YPD and the mouse lung. Furthermore, only 7 of the 26 candidate genes were differentially regulated between YPD and tissue culture medium in the wild type strain. When comparing YPD expression to *in vivo* expression, 17 of the 26 genes were differentially expressed. The genes with infection-specific induction or repression
included the cell wall biosynthesis genes *CDA1, CHS4, CHS6, and FKS1*, and the iron genes *CIR1* and *CFT1*.

We were also able to examine the transcriptional profile of the *rim101Δ* mutant and the wild type strain under host conditions. The major differences in expression between the strains were in the *CDA1, KRE6, CTR4, and ENA1* genes. The *CDA1* gene regulates chitosan levels in the cell, and *KRE6* is involved in β-glucan synthesis, consistent with the role of Rim101 in regulation of cell wall remodeling. *ENA1* is also required for full proliferation within the CSF, although we have not observed a difference in *rim101Δ* neurotropism or proliferation in the CSF when compared with the wild type (205). Interestingly, the wild type strain expressed more *CTR4* transcripts that the *rim101Δ* strain during both *in vitro* growth conditions but 2.5-fold less in the context of the host lung. Induction of *CTR4* transcripts during lung infections was also recently demonstrated by luciferase assays (90). These results emphasize the importance of using animal models to accurately assess the disease processes, as the *in vitro* assays may not be able to fully replicate the host conditions.

### 5.4 Methods:

**Strains and media:**

*Cryptococcus neoformans* strains used in this study were H99, *rim101Δ* (TOC2), *pka1Δ, rim101Δ + pHis-GFP-RIM101* and *rim101Δ + pRim101-GFP-RIM101*. CO$_2$-independent tissue-culture medium was obtained from Gibco (Invitrogen). Alkaline pH medium was created by buffering YPD with 25 mM HEPES and adjusting to target pH with NaOH. Salt medium was prepared by adding 1.5 M NaCl to YPD. Acid pH medium was created by buffering SC with 50 mM glycine and adjusting to target pH with HCl.
**RNA sequencing and transcript analysis:**

To induce expression changes, the \( pka1\Delta \) mutant was incubated in YPD to mid-log phase, washed twice, and then incubated in DMEM at 37°C with 5% CO\(_2\) or YPD for three hours. Cells were then washed twice, frozen on dry ice, and lyophilized for three hours. Total RNA was extracted using the Qiagen RNeasy Plant Minikit (Qiagen, Valencia, CA), as previously described (265, 266).

All library preparation and RNA sequencing was performed by the Duke Sequencing Core Facility, as described previously (265). The \( pka1\Delta \) mutant was sequenced with 36 bp single-end reads. All reads were mapped to the *C. neoformans* reference genome provided by the Broad Institute using TopHat v1.3.0 as previously described (265, 345). All data were uploaded to the NCBI GEO database. Genes were considered significantly differentially expressed if \( p \)-values were greater than the false discovery rate after Benjamini-Hochberg corrections for multiple testing and if the fold-change was greater than 2.0.

**Gel Shift Assay:**

DNA probes for EMSA assays were double stranded synthetic oligonucleotides corresponding to either 15 or 25 nucleotides spanning the putative Rim101 binding site in the *CFT1* promoter. The sequences are included in Supplemental Table 3. The mutated probe contained a GAGAAG motif instead of the GCCAAG motif. Biotin labeling and EMSAs were performed according to the manufacturer’s instructions (Pierce Lightshift Chemiluminescent EMSA kit; Thermoscientific, Rockford, IL, USA). For each lane, 6 \( \mu \)L of protein extract was used.

**Chromatin immunoprecipitation:**
Strains were incubated in CO\textsubscript{2}-independent medium and then cross-linked for two hours in 1% formaldehyde before quenching with glycine. Chromatin immunoprecipitation was performed as previously described (67), with minor modifications. An anti-GFP antibody was used to immunoprecipitate the GFP-Rim101 fusion protein and the associated DNA. A mock-antibody treated strain and the wild type strain (no GFP protein) were used as controls. Enrichment was determined by real-time PCR of candidate promoters, comparing the immunoprecipitated sample to the mock antibody control with the ΔΔ\textsuperscript{ct} method.

**Microscopy:**

Images were captured with a Zeiss Axio Imager.A1 fluorescent microscope equipped with an AxioCam mrM digital camera. To visualize GFP, cells were washed gently in PBS, and images were collected at 63x magnification, using 488 nm wavelength for fluorescence.

**Cell Wall Analysis:**

For cell wall staining, strains were washed and then stained WGA conjugated to AlexaFluor 488 (chitin) (Molecular Probes, Eugene, OR) (113, 320). After staining, cells were washed twice before observation by fluorescence microscopy. WGA was observed using a 488 nm wavelength for fluorescence. Images were captured with a Zeiss Axio Imager.A1 fluorescent microscope equipped with an AxioCam mrM digital camera.

**In vivo RNA extraction:**

Immediately after harvesting, infected lungs were cut into the separate lobes and flash frozen in dry ice. The frozen lungs were then homogenized using three rounds of 30 s beating on a mini bead beater using 0.2 µ acid-washed glass beads. RNA was then...
extracted from the homogenate using the Qiagen RNeasy Plant Minikit according to the manufacturer's instructions (Qiagen, Valencia, CA).

**NanoString:**

NanoString analysis of *in vivo* fungal gene expression was performed as described previously (107). Briefly, 10 µg of *C. neoformans*-infected mouse tissue RNA was mixed with a custom designed probe set and processed according to the manufacturer's instructions. 600 fields per sample were scanned on the nanoString digital analyzer. The raw counts were adjusted for technical variability using irrelevant RNA sequences included in the code set. The adjusted counts were then normalized to the expression of five housekeeping genes (aldose reductase CNAG_02722, coflin CNAG_02991, microtubule binding protein CNAG_00816, mitochondrial protein CNAG_00279, phosphoglycerate kinase CNAG_03358).
6. Cryptococcus neoformans capsule: a sword and a shield

This chapter was modified from a manuscript (of the same title) published in Clinical Microbiological Reviews 25(3) 2012. The authors were Teresa R. O’Meara and J. Andrew Alspaugh.

6.1 Introduction

Prior to the widespread emergence of human immunodeficiency virus (HIV) infection, disease due to the opportunistic fungus Cryptococcus neoformans was uncommon. However, over the past several decades, this fungal pathogen has caused life-threatening disease in millions of patients worldwide. Recent epidemiological data from the World Health Organization suggests that over one million cases of cryptococcal infection occur each year in Sub-Saharan Africa among HIV-infected patients, resulting in more than 600,000 annual deaths (281). Additionally, Cryptococcus species have caused recent infectious disease outbreaks in the Pacific Northwest regions of Canada and the United States. These trends emphasize the importance of understanding the basic biology of this fungus, especially the ways in which it has adapted to cause human disease.

C. neoformans lives primarily in the environment in a yeast-like form. Spores or small yeast cells are inhaled, resulting in a primary pulmonary infection. Sero-epidemiology studies indicate that the majority of people in endemic areas are exposed to this fungus at a young age; however, in immunocompetent hosts, C. neoformans infections are minimally symptomatic and rapidly cleared (137). Serious disease occurs in the absence of intact cell-mediated immunity, such as in patients with advanced AIDS or organ transplant recipients receiving immunosuppressive therapies. In these
immunocompromised hosts, *C. neoformans* can disseminate from the lungs and cross the blood brain barrier, frequently resulting in meningoencephalitis, a central nervous system infection that is fatal if not treated.

In both the environment and in the infected host, *C. neoformans* produces a characteristic polysaccharide capsule. Investigators have speculated that this capsule may protect the fungus from environmental desiccation and/or natural predators, such as nematodes or amoebae (66, 124, 251, 334, 335, 388). In the host, the capsule serves many protective functions, including reducing host immune responses by down-regulating inflammatory cytokines, depleting complement components, and inhibiting the antigen presenting capacity of monocytes (308, 351, 352). The capsule can also act as a shield on the cell wall to regulate phagocytosis by macrophages (79, 280). Once inside macrophages, capsule serves as a sink for reactive oxygen species generated by the host, thus providing effective antioxidant defenses (386).

The *C. neoformans* capsule is also familiar to clinicians. Its characteristic appearance around the yeast cell is the basis for rapid microbiological identification in clinical samples, such as cerebrospinal fluid (CSF). Recognition of encapsulated yeast cells in histopathological material, clearly visualized by mucicarmine staining, is sufficient to diagnose *C. neoformans* infections, even in the absence of culture data. Additionally, the capsular polysaccharide is the basis for very sensitive and specific diagnostic assays for cryptococcal infections.

There is considerable evidence that the capsule plays a central role in allowing *C. neoformans* to survive within the host and to cause disease. Unencapsulated *C. neoformans* cells are rarely observed in clinical samples. Moreover, specific mutations resulting in capsule defects typically result in the dramatic attenuation of *C. neoformans*
virulence. Therefore, similar to bacterial capsules, the *C. neoformans* capsule is considered to be the most important virulence-associated phenotype in this organism. However, the chemical structure and organization of this fungal capsule are quite distinct from bacterial capsules.

In addition to having a unique chemical composition, the *C. neoformans* capsule is highly regulated in terms of its relative size and complexity. This regulation is important for the survival of *C. neoformans* in the host. When incubated in rich and permissive laboratory growth conditions, this fungus produces a small ring of capsule on the cell surface. However, *C. neoformans* dramatically induces capsule in response to host-specific conditions. In fact, many *in vitro* approximations of human host conditions have been used to induce capsule, including tissue culture medium, 5% CO$_2$, low iron, and human physiological pH (pH 7) (17, 350, 387).

Some aspects of *C. neoformans* capsule regulation occur at the level of transcription. For example, incubation in the presence of the transcriptional inhibitor actinomycin D completely inhibits encapsulation without immediately affecting viability (140). However, many interacting and complementary signaling pathways likely regulate the complex biology of the capsule. The *C. neoformans* transcriptional programs triggered by these host environmental conditions have been investigated in an effort to understand the networks involved in the induction of capsule in response to host conditions. This review will focus on the different regulation programs that respond to specific host environmental cues to induce encapsulation. We will attempt to critically review and synthesize the current information on the regulation of *C. neoformans* capsule synthesis, export, and assembly. Additionally, we suggest that fungal cell wall remodeling is an underexplored component of appropriate encapsulation within the host.
6.2 The biology of cryptococcal capsule

6.2.1 Capsule Structure

*C. neoformans* capsule is composed of complex polysaccharides that are synthesized within the cell, transported across the cell wall through vesicles, and then non-covalently attached to the cell surface where they can assemble into long polymers. Biochemical analyses of capsule using various chromatography techniques and mass spectrometry demonstrated that it is primarily composed of glucuronoxylomannan (GXM) and glucuronoxylomannogalactan (GXMGal). Nuclear magnetic resonance was used to examine the precise structures of these components. GXM is composed of O-acetylated α-1,3 linked mannose residues with xylosyl and glucuronyl side groups (195). The approximate weight-averaged mass of GXM is between 1,700 and 7,000 kDa, and it makes up approximately 90% of the *C. neoformans* polysaccharide capsule (234). In contrast, GXMGal is an α-1,6-linked galactose polymer with mannose, xylose, and glucuronic acid modifications (149).

Dynamic changes in capsule were initially demonstrated by alterations in antibody binding and later by more detailed and direct biophysical measurements of capsule structure (126, 127, 333). For example, the number and order of each of the modified residues in the capsule polymers can vary, leading to the antigenic heterogeneity used in diagnostics and serotyping (235). Analysis of the radius of gyration of the polysaccharide fibrils demonstrated complex branching of the polysaccharide polymer, which can result in further structural heterogeneity (72). Mass spectrometry and NMR analysis demonstrated that some of the structural differences detected by variable antibody binding can be caused by glucuronic acid positional effects (235). Importantly, the overall structure of the capsule can also vary in the
different host environments (61, 86, 195). For example, *C. neoformans* recovered from different organs during murine infections demonstrates variable binding to anti-capsule antibodies (109). Additionally, experimental infection of *Galleria mellonella* wax-moth larvae results in capsules with increased density compared to identical strains grown in *vitro*, as measured by the penetrance of antibody binding (124). The change in capsule structure, size, and density is potentially a mechanism for escape or evasion from the immune system, demonstrating that capsule is a dynamic structure that is highly regulated by the cell in response to specific environmental cues.

### 6.2.2 Capsule Synthesis

The capsular polysaccharide is made from simple sugars that are modified and assembled into more complex structures. Investigators have studied the initial biochemical processes involved in the synthesis of the capsular monomers and the addition of the subunits to the elongating capsule polymer. Using bacterial capsule synthesis as a model, the Doering lab was able to determine via homology some of the enzymes required for capsule synthesis in *C. neoformans*. This work was complemented and supported by genetic screens for capsule mutants performed by the Janbon lab. Although some of the genes and biochemical intermediates of capsule are known, there are many steps that have not been completely elucidated.

#### 6.2.2.1 Capsule monomers:

The capsular polysaccharide is made by polymerization of simple sugars into an elongating carbohydrate backbone. These initial steps depend upon carbohydrate metabolism to allow for a sufficient supply of the starting sugars. Moreover, the addition of differing carbon sources to the growth medium can result in alterations in capsule composition (146). The base components of the capsule are UDP-glucuronic acid, UDP-
galactose, UDP-xylose, and GDP-mannose. UDP-glucuronic acid is made from the conversion from UDP-glucose to UDP-glucuronic acid via the membrane-localized Ugd1 UDP-glucose dehydrogenase (142, 166, 247). The Uxs1 decarboxylase then converts UDP-glucuronic acid to UDP-xylose (26). UDP-Galactose, which is required for GXMGal, is created from UDP-glucose by the Uge1 epimerase (249). GDP-mannose is synthesized via a phosphomannose isomerase, a phosphomannomutase, and a GDP-mannose pyrophosphorylase. Currently, only the phosphomannose isomerase, Man1, has been examined in C. neoformans (372). Potential phosphomannomutases have been putatively identified in the genome, but their direct action on the production of GDP-mannose has not been defined in detail.

6.2.2.2 Modification of capsule monomers:

The base monomers of both GXM and GXMGal are then combined and modified with specific side chain moieties that are important for the assembly, branching, and overall structure of the fibrils. One modification of the GXM and GXMGal monomers is xylosylation. This process is mediated by the Cxt1 β-1,2 xylosyltransferase (49, 188, 189). This enzyme transfers xylose to α-1,3 dimannoside to create Xyl-β-1,2-Man-α-1,3-Man. In the cxt1Δ mutant strain, the cell has reduced xylose on GXM monomers and a complete lack of xylose on the GXMGal monomers; this strain is subsequently attenuated for virulence (188). The Cap10, Cap1, Cap4, and Cap5 proteins have homology to Cxt1, and these enzymes may be involved in the addition of β-1,3 linked xylose to capsule (189). Due to the amount of branching and the observed phenotypic switching of strains, it is likely that these proteins may be regulated specifically to alter the overall capsular structure.
Another modification is the addition of activated mannose groups to the carbohydrate backbone. This addition occurs within an organelle, and transport of GDP-mannose is mediated by the Gmt1 GDP-mannose transporter (75). Mannosylation of the backbone is performed by α-1,3 mannosyltransferases, most likely Cmt1 and Cap59 (93, 330).

Further modification of GXM and GXMGal comes through O-acetylation, and this is performed by the Cas1 glycosyltransferase (169). The O-acetylation occurs on the mannose and glucuronylated mannose residues, and the antigenicity of the capsule in cas1Δ mutant strains is drastically altered (169, 195). The Cap64-like proteins, Cas3, Cas31, Cas32, Cas33, Cas34, and Cas35, may be involved in assembling the monomers or adding modifiers. These proteins were identified in a screen for mutants that are involved in capsule structure (246). However, only Cap64 is required for the production of visible capsule around the cell (57).

Pbx1 and Pbx2 are parallel β-helix genes that potentially act as a complex to regulate the incorporation of glucose residues into the backbone (222). Mutations in these proteins do not prevent encapsulation, but this mutant capsule is easily detached from the cell by sonication. This fragile capsule contains GXM with aberrant glucose molecules. However, the role of normal glucose incorporation in GXM is still unclear.

Finally, the capsule contains hyaluronic acid (HA), which is important for crossing the blood-brain barrier (174). Cells lacking HA have slightly decreased capsule diameter and a defect in cell wall ultrastructure, although the cause and effect relationship is not clear (53, 174). Recent work revealed that the Cps1 protein is responsible for the synthesis of HA, although the timing, amount, and induction of HA is still under examination (174). Most interestingly, the presence of HA on the cell surface may
actually facilitate fungal cell entry into the CNS by facilitated transport across the blood brain barrier (173, 174). The genes involved in capsule biosynthesis are presented in Table 6.

**Table 7. Genes involved in capsule synthesis**

<table>
<thead>
<tr>
<th>CNAG_ID</th>
<th>Gene annotation</th>
<th>Capsule phenotype of mutant</th>
<th>Domains</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNAG_00124</td>
<td>Cas32</td>
<td>Alteration in carbohydrate ratios, hypocapsular when combined with cas3Δ.</td>
<td>Signal peptide, transmembrane domain.</td>
<td>(246)</td>
</tr>
<tr>
<td>CNAG_00596</td>
<td>Utr2</td>
<td></td>
<td>Signal peptide, transmembrane domain.</td>
<td></td>
</tr>
<tr>
<td>CNAG_00600</td>
<td>Capsular associated protein</td>
<td></td>
<td>Homology to chitin transglycolase</td>
<td></td>
</tr>
<tr>
<td>CNAG_00697</td>
<td>Uge1</td>
<td>Larger capsule but no GXMGal.</td>
<td>Transmembrane domain.</td>
<td>(249, 250)</td>
</tr>
<tr>
<td>CNAG_00701</td>
<td>Cas31</td>
<td>Decreased capsule, alteration in carbohydrate ratios.</td>
<td>Signal peptide, transmembrane domain.</td>
<td>(246)</td>
</tr>
<tr>
<td>CNAG_00721</td>
<td>Cap59</td>
<td>Decreased capsule</td>
<td>Signal peptide domain</td>
<td>(54, 92, 123)</td>
</tr>
<tr>
<td>CNAG_00744</td>
<td>α-1,6-mannosyltransferase</td>
<td></td>
<td>Transmembrane domain</td>
<td></td>
</tr>
<tr>
<td>CNAG_00746</td>
<td>Cas35</td>
<td>Decreased capsule</td>
<td>SGNH superfamily</td>
<td>(246)</td>
</tr>
<tr>
<td>CNAG_00926</td>
<td>Glycolipid mannosyltransferase</td>
<td></td>
<td>Homology to mannosyltransferases</td>
<td></td>
</tr>
<tr>
<td>CNAG_00996</td>
<td>Pmt4</td>
<td>Decreased capsule size</td>
<td>11 transmembrane domains</td>
<td>(371)</td>
</tr>
<tr>
<td>CNAG_01156</td>
<td>Cap2</td>
<td></td>
<td>Transmembrane domain</td>
<td></td>
</tr>
<tr>
<td>CNAG_01172</td>
<td>Pbx1</td>
<td>Dry colony morphology, defect in capsule integrity</td>
<td>Signal peptide domain</td>
<td>(222)</td>
</tr>
<tr>
<td>CNAG_01283</td>
<td>Cap5</td>
<td></td>
<td>Transmembrane domain</td>
<td></td>
</tr>
<tr>
<td>CNAG_01654</td>
<td>Cas34</td>
<td>Decreased capsule size</td>
<td>Signal peptide, transmembrane domain</td>
<td>(246)</td>
</tr>
<tr>
<td>CNAG_02036</td>
<td>Cas4</td>
<td>Altered reactivity against GXM antibodies</td>
<td>9 transmembrane domains, transporter domains</td>
<td>(248)</td>
</tr>
<tr>
<td>CNAG_02581</td>
<td>Cas33</td>
<td>Decreased capsule size</td>
<td>Transmembrane domain, SGNH superfamily</td>
<td>(246)</td>
</tr>
<tr>
<td>CNAG_02797</td>
<td>Cpl1</td>
<td>Decreased capsule</td>
<td>Signal peptide, transmembrane domain</td>
<td>(221)</td>
</tr>
<tr>
<td>CNAG_02885</td>
<td>Capsular associated protein</td>
<td></td>
<td>Transmembrane domain</td>
<td></td>
</tr>
<tr>
<td>Strain</td>
<td>Gene</td>
<td>Effect</td>
<td>Domain/Function</td>
<td>References</td>
</tr>
<tr>
<td>----------</td>
<td>------</td>
<td>------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>CNAG_03096</td>
<td>Uge1</td>
<td>Defective GalXM production, larger capsule size</td>
<td>glucose epimerase</td>
<td>(249, 250)</td>
</tr>
<tr>
<td>CNAG_03158</td>
<td>Cmt1</td>
<td>Decreased capsule size</td>
<td>Transmembrane domain</td>
<td>(330)</td>
</tr>
<tr>
<td>CNAG_03322</td>
<td>Uxs1</td>
<td>Capsule is missing xylose</td>
<td>Epimerase domain</td>
<td>(195, 248)</td>
</tr>
<tr>
<td>CNAG_03438</td>
<td>Hxt1</td>
<td>Increased capsule size</td>
<td>Signal peptide, 10 transmembrane domains, sugar transporter</td>
<td>(65)</td>
</tr>
<tr>
<td>CNAG_03644</td>
<td>Cas3</td>
<td>Decreased capsule when combined with cas31Δ, cas32Δ, or cas33Δ mutants</td>
<td>Transmembrane domain, signal peptide</td>
<td>(246)</td>
</tr>
<tr>
<td>CNAG_03695</td>
<td>Cas41</td>
<td>Increased capsule size</td>
<td>8 transmembrane domains, transporter domains</td>
<td></td>
</tr>
<tr>
<td>CNAG_03735</td>
<td>Cap4</td>
<td>Increased capsule size</td>
<td>Transmembrane domain, signal peptide</td>
<td></td>
</tr>
<tr>
<td>CNAG_04312</td>
<td>Man1</td>
<td>Defect in capsule production</td>
<td>Phosphomannose isomerase</td>
<td>(372)</td>
</tr>
<tr>
<td>CNAG_04320</td>
<td>Cps1</td>
<td>Slight defect in capsule</td>
<td>Glycosyl-transferase, 3 transmembrane domains</td>
<td>(53, 174)</td>
</tr>
<tr>
<td>CNAG_04969</td>
<td>Ugd1</td>
<td>Increased capsule size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNAG_05139</td>
<td>Ugt1</td>
<td>Increased capsule size</td>
<td></td>
<td>(250)</td>
</tr>
<tr>
<td>CNAG_05148</td>
<td>Cap3</td>
<td>Dry colony morphology, defect in capsule integrity</td>
<td>Transmembrane domain, xylosyl-transferase</td>
<td></td>
</tr>
<tr>
<td>CNAG_05562</td>
<td>Pbx2</td>
<td>Dry colony morphology, defect in capsule integrity</td>
<td>Pectin lyase-like domain</td>
<td>(222)</td>
</tr>
<tr>
<td>CNAG_06016</td>
<td>Cap6</td>
<td>Dry colony morphology, defect in capsule integrity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNAG_06813</td>
<td>Cap1</td>
<td>Dry colony morphology, defect in capsule integrity</td>
<td>Signal peptide, transmembrane domain</td>
<td></td>
</tr>
<tr>
<td>CNAG_07554</td>
<td>Cap3</td>
<td>Dry colony morphology, defect in capsule integrity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNAG_07937</td>
<td>Cas1</td>
<td>Defect in capsule O-acetylation, reactivity to GXM antibodies</td>
<td></td>
<td>(248)</td>
</tr>
</tbody>
</table>

### 6.2.2.3 Location of capsule synthesis:

Capsule nucleotide sugar donors are synthesized in the cytoplasm, and the backbone and modifiers are assembled near the cell wall in organelles before transport across the cell wall (381). After transport across the cell wall, the polymers grow in
length when the cells are placed in inducing conditions (258, 292, 382). Currently, the mechanism by which the polymers extend is unknown, although there is consensus that the size is mediated at the level of individual polysaccharide molecules (117, 382). One hypothesis is that the capsular fibrils have inherent properties that promote self-assembly via divalent cations (234). Recent work demonstrated that the new capsular material can be incorporated at the edge of the capsule, distally from the cell, with some intercalation of new material throughout the entire structure (390). The long fibrils can then act as a scaffold, allowing for the formation of a dense capsule structure near the cell (117). However, both antibody and complement binding, which were used to determine the position of the newly incorporated capsule, can affect the capsular structure, making it difficult to determine the normal process of capsular enlargement (92, 111, 231). Identifying the position of the new capsule has implications for the processes involved in extending the length of the polymer.

The polysaccharide capsule is most easily visualized when it is maintained at the cell surface. However, it is clear that some polysaccharide is secreted and not maintained around the cell. Recently, there has been interest in exploring the differences between this exopolysaccharide and the surface-attached polysaccharides (116, 143). Analysis of capsular material, either shed into the medium or removed from the cell by various chemical treatments, demonstrated that, although the composition was consistent between the two preparations, the ratios of the components significantly varied between the soluble and the attached polysaccharides (116). It is currently unclear whether different biosynthesis processes create these two types of polysaccharide.
6.2.3 Capsule Secretion

Due to the large size of the capsular polysaccharide, this polymer needs to be actively transported across the cell wall. Initial reports demonstrated the presence of vesicles potentially carrying capsular polysaccharides after the cryptococcal cells were ingested by macrophages. (314, 347, 381). In the past few years, several microscopic, biochemical, and genetic studies have verified the vesicular transport of capsule. Analysis of excreted vesicles demonstrated the presence of virulence-associated components, including capsule (272, 313). Quick-freeze deep etching revealed the accumulation of particles/vesicles in the outer region of the cell wall. The number of particles was greater in vivo than in vitro, which Sakaguchi et al. attributed to an increase in the secretion of vesicles containing capsular precursors (317). Treatment with inhibitors for vesicle transport, such as brefeldin A, nocodazole, monesin, and N-ethylmaleimide decreased capsule (158). Mutations in the secretory pathway (Sec4/Sav1 and Sec6) also resulted in decreased capsule on the cell surface (279, 381). Because Sec4 is involved in post-Golgi secretion events, the Golgi apparatus was implicated in capsule secretion (381). Additionally, Arf1, an ADP-ribosylating factor involved in vesicle formation and intracellular trafficking with the Golgi, is involved in capsule (357). Recently, a Golgi reassembly and stack protein (GRASP) was shown to be required for capsule secretion (190). These graspΔ mutants had defects in capsule size and consequent increases in phagocytosis rates and decreases in virulence. Kmetzsch et al. suggest that the defect in capsule size in the graspΔ mutant may be a product of decreased polysaccharide secretion.

Appropriate vesicle physiology is also required for capsule induction around the cell. Vph1, a V-type ATP-ase that is required for vesicle acidification, is important for
capsule transport. Without Vph1, cells demonstrate a dramatically reduced capsule. Treatment with bafilomycin A1, which prevents vesicle acidification, also represses capsule (100).

However, the mechanism by which the capsule is packaged and released from the vesicles to then attach to the surface of the cell is currently unknown. It is also possible that there is a difference in the secretion of exopolysaccharide and attached polysaccharide (116).

6.2.4 Attachment

After secretion, the capsule must be maintained around the cell. The cell wall appears to be the major determinant of capsular attachment, whether it is through direct linkages between wall components and capsular material, or through providing a scaffold for proteins that then mediate the attachment. The cell wall is a dynamic material, with continuous remodeling required for budding, growth, and mating. Investigators studying other fungal species have demonstrated changes in cell wall composition in response to the host, and this process is being explored in C. neoformans as well (22, 33, 244, 270, 388). Additionally, the cell wall has been of particular interest due to the resistance of C. neoformans to the echinocandin class of antifungal agents that inhibits cell wall β-glucan synthesis (228). The effects of cell wall composition and remodeling on capsule attachment have not been fully explored, but there are hints from transcriptional profiling that changes in the cell wall are required for encapsulation within the host.

The C. neoformans cell wall is composed of β-1,3 and β-1,6 glucan, α-1,3 glucan, chitin and chitosan, in addition to mannoproteins and other GPI-anchored proteins (3, 20-22, 25, 132, 213). Although these components are extensively cross-
linked, there are still overall striations or layers that can be visualized through electron microscopy and quick-freeze deep etching (25, 306, 317). The inner layer is primarily composed of β-glucans and chitin, and the outer layer contains α-glucan and β-glucan (306). Unlike other fungi, the C. neoformans cell wall has more β-1,6 glucan than β-1,3 glucan; however, the β-1,3 glucan synthase, Fks1, is essential, indicating the importance of this conserved cell wall component (132, 341). Table 7 includes all cell wall genes that have a demonstrated effect on capsule attachment and some genes putatively involved in cell wall biogenesis.

Table 8. Genes involved in cell wall biogenesis and capsule attachment

<table>
<thead>
<tr>
<th>CNAG_ID</th>
<th>Gene annotation</th>
<th>Capsule phenotype of mutant</th>
<th>Domains</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNAG_00373</td>
<td>Glucan 1,3-β-glucosidase</td>
<td></td>
<td>Transglycosidase family</td>
<td></td>
</tr>
<tr>
<td>CNAG_00546</td>
<td>Chs4</td>
<td></td>
<td>5 transmembrane domains</td>
<td>(25)</td>
</tr>
<tr>
<td>CNAG_00897</td>
<td>Skn1</td>
<td>Increased capsule diameter and altered appearance when combined with kre6Δ</td>
<td></td>
<td>(132)</td>
</tr>
<tr>
<td>CNAG_00914</td>
<td>Kre6</td>
<td>Increased capsule diameter and altered appearance when combined with skn1Δ</td>
<td></td>
<td>(132)</td>
</tr>
<tr>
<td>CNAG_00939</td>
<td>Glucan 1,3-β-glucosidase putative</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNAG_01230</td>
<td>Cda2</td>
<td>Increased capsule when combined with cda1Δ</td>
<td></td>
<td>(20)</td>
</tr>
<tr>
<td>CNAG_01239</td>
<td>Cda3</td>
<td>Increased capsule when combined with cda1Δ</td>
<td></td>
<td>(20)</td>
</tr>
<tr>
<td>CNAG_01941</td>
<td>β-1,3 glucan biosynthesis-related protein putative</td>
<td></td>
<td>Homology to glucan synthesis and regulation proteins</td>
<td></td>
</tr>
<tr>
<td>CNAG_02217</td>
<td>Chs7</td>
<td></td>
<td>7 transmembrane domains</td>
<td>(25)</td>
</tr>
<tr>
<td>CNAG_02225</td>
<td>Cellulase</td>
<td></td>
<td>Signal peptide</td>
<td></td>
</tr>
<tr>
<td>CNAG_02283</td>
<td>Glucan 1,4-α-glucosidase</td>
<td></td>
<td>Signal peptide</td>
<td></td>
</tr>
<tr>
<td>CNAG_02351</td>
<td>Chi4</td>
<td>No change</td>
<td></td>
<td>(22)</td>
</tr>
<tr>
<td>CNAG_02598</td>
<td>Chi21</td>
<td>No change</td>
<td></td>
<td>(22)</td>
</tr>
<tr>
<td>CNAG_02850</td>
<td>α-1,3 glucosidase</td>
<td></td>
<td>Homology to Agn1</td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_02860</strong></td>
<td>Endo-1,3(4) β-glucanase</td>
<td>Signal peptide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------</td>
<td>-------------------------</td>
<td>----------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_03099</strong></td>
<td>Chs1</td>
<td>6 transmembrane domains (25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_03120</strong></td>
<td>Ags1</td>
<td>Decrease in capsule attachment (305, 306)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_03326</strong></td>
<td>Chs2</td>
<td>7 transmembrane domains (25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_03412</strong></td>
<td>Chi2</td>
<td>No change (22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_03648</strong></td>
<td>Kre5</td>
<td>Increased capsule diameter, altered appearance (132)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_04033</strong></td>
<td>α-1,4-glucosidase</td>
<td>Signal peptide, transmembrane domain</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_04245</strong></td>
<td>Chi22</td>
<td>No change (22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_05581</strong></td>
<td>Chs3</td>
<td>6 transmembrane domains (25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_05663</strong></td>
<td>Scw1</td>
<td>RNA binding domain</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_05799</strong></td>
<td>Cda1</td>
<td>Increased capsule when combined with cda2Δ and cda3Δ (20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_05815</strong></td>
<td>Kre64</td>
<td>Transmembrane domain (132)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_05818</strong></td>
<td>Chs5</td>
<td>6 transmembrane domains (25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_06031</strong></td>
<td>Kre63</td>
<td>Homology to Skn1 (132)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_06336</strong></td>
<td>Glucan 1,3 β-glucosidase protein</td>
<td>Transmembrane domain</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_06411</strong></td>
<td>α-1,3 glucanase</td>
<td>Signal peptide</td>
<td>Homology to Agn1</td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_06487</strong></td>
<td>Chs6</td>
<td>5 transmembrane domains (25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_06508</strong></td>
<td>Fks1</td>
<td>16 transmembrane domains (341)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_06509</strong></td>
<td>Hex1</td>
<td>Signal peptide (22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_06678</strong></td>
<td>Csr1</td>
<td>Sel1 repeats (25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_06726</strong></td>
<td>Csr3</td>
<td>Sel1 repeats (25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_06832</strong></td>
<td>Kre62</td>
<td>Transmembrane domain (132)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_06835</strong></td>
<td>Kre61</td>
<td>Transmembrane domain (132)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_07499</strong></td>
<td>Chs8</td>
<td>6 transmembrane domains (25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_07636</strong></td>
<td>Csr2</td>
<td>Sel1 repeats (25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CNAG_07736</strong></td>
<td>Glucan endo-1,3-α-glucosidase</td>
<td>WSC domains</td>
<td>Homology to Agn1</td>
<td></td>
</tr>
</tbody>
</table>
6.2.4.1 Cell wall glucans

Recently, the Skn1/Kre6 family of potential β-1,6 glucan synthases was examined in detail, and Gilbert et al. demonstrated that Kre5 and both Kre6 and Skn1 are required for maintenance of normal capsular architecture, as determined by dextran penetrance and India ink staining (132). However, a more dramatic phenotype was observed when the gene encoding the α-1,3 glucan synthase, AGS1, was mutated. In the ags1Δ strain, there was no capsular attachment, but apparently normal capsular material was shed into the medium where it could attach to other acapsular cells (305, 306, 328). Our recent work demonstrates that α-glucan is induced on the cell wall in capsule-inducing conditions (unpublished data). H. capsulatum, another opportunistic pathogen, induces α-1,3 glucan to hide immunogenic cell wall components from recognition by the host (229, 303). Therefore, the α-glucan in C. neoformans may be involved in avoiding immune recognition in two ways. First, it is required for attaching capsule, and second, it may shield the immunogenic β-glucans and chitin molecules from the host immune system.

6.2.4.2 Chitin/Chitosan

Chitin and chitosan make up approximately 10% of the C. neoformans cell wall in the cap67Δ mutant strain (130, 168). In the genome, there are 8 genes for chitin synthesis, 3 chitin synthase regulators, 4 chitin deacetylases, and 5 chitinases, making the role of a single gene difficult to determine (20, 22, 25). However, substantial work by the Lodge lab has elucidated the role of many of these components.

In S. cerevisiae, a chitin synthase gene is transported to the membrane through the Golgi secretory pathway. During cell stress, chitin accumulates in the cell wall, and the overall increase in chitin can also be regulated by increases in the levels of chitin.
precursors (UDP-GlcNAc) (40). The regulation of chitin accumulation in *C. neoformans* is similar, with accumulation during cell stress. Unlike *S. cerevisiae*, the levels of chitosan in *C. neoformans* are three to five times higher than the levels of chitin, and the ratio of chitin to chitosan changes with cell density (25). Banks et al. also determined that, during vegetative growth, the Chs3 protein produces chitin that is subsequently converted to chitosan. Additionally, they demonstrated that Chs3 activity is regulated by Csr2. Accordingly, in the chs3Δ and csr2Δ mutant strains, the levels of chitin are increased, and the levels of chitosan are decreased.

To further examine the regulation and synthesis of chitosan, Baker et al. created triple and quadruple mutants of the four chitin deacetylase genes (20). In cda1Δcda2Δ double mutants and the chs3Δ single mutant, decreased chitosan levels correlated with increased chitin levels and increased capsule size (20). One hypothesis is that chitosan normally masks capsule attachment sites, preventing encapsulation of the cell. Chitooligomers can interfere with capsular assembly *in vitro*, so decreased chitosan may allow for better capsule assembly (112). However, chitin-like structures can be incorporated into the capsular material, and this can result in increased shear resistance and crosslinking (388). Additionally, the chitosan-deficient strains grow slowly, especially under *in vivo* conditions. This slower growth may allow for increased capsule size, as suggested by Zaragoza et al. (21, 385).

### 6.2.4.3 Cell Wall Proteins

Proteins that are embedded in the cell wall carbohydrates are also likely to be important for capsule attachment, potentially acting as anchors for the polysaccharide fibrils. The two most highly studied mannoproteins in *C. neoformans* are MP98 and MP88, both of which have GPI-anchors (159, 213). These proteins were first identified
as highly immunogenic molecules, capable of stimulating a robust T-cell response. The MP98 protein is a chitin deacetylase, and it may play a role in chitosan levels in the cell wall (20).

Phospholipase B1 (Plb1) is another GPI-anchored protein in C. neoformans. Plb1 is covalently bound to β-1,6 glucan and is involved in the maintenance of cell wall integrity (325). Although the diameter of the capsule of plb1Δ mutants was similar to the wild type, TEM established that the capsule density was decreased in the mutant. Recent work has suggested an association between Plb1 activity and titan cell formation, and this will be discussed below. Plb1 may be necessary to cleave certain host phospholipids to allow for activation of specific signaling pathways (66). Plb1 is secreted using the same vesicle-dependent pathway as the capsule monomers.

6.3 Regulation of capsule induction in specific environments

An important facet of C. neoformans surface capsule is that it is induced upon entry into the host. The cell must be able to sense the external environment and respond appropriately, especially as the capsule is an important factor in survival within the host. The degree of encapsulation corresponds with survival under many host-specific conditions.

There are a number of external signals that are able to induce capsule in C. neoformans. Each condition can induce specific capsule phenotypes, ranging from the size of the induced capsule to the antigenic variability of the capsule. The variable capsule phenotypes in different organs suggest that the ability of C. neoformans to dynamically alter capsule is physiologically relevant (235). Figure 24 demonstrates that the production of capsule in a wild type strain under commonly used in vitro capsule-inducing conditions, including low iron medium (LIM), Dulbecco’s modified Eagle’s
medium at 37°C with 5% CO₂ (DMEM), and 10% Sabouraud’s medium buffered to pH 7.3. However, the induction of capsule around the cell does not appear to be due solely to the induction of the various biosynthetic genes. The next sections will discuss the signaling pathways that regulate capsule and the transcriptional outputs that result in capsule induction.

Figure 24. Different inducing conditions result in varying degrees of encapsulation in the wild type strain.

Cells were incubated for 48 hours in the specified medium. Capsule is visualized by counterstaining with India ink.

6.4 Signal transduction pathways that induce capsule

6.4.1 Low Iron

Iron binding and sequestration of iron is one of the most basic mechanisms of protection against invading microorganisms (162, 177, 349, 366-368). The host sequesters iron in hemoglobin, transferrin, lactoferrin, and ferritin, preventing microbes from accessing this essential nutrient (367). To adapt to this low-iron environment, many
microorganisms use iron transporters and siderophores to facilitate the uptake and scavenging of iron from the environment (162, 299, 340). Some fungi are unable to synthesize their own siderophores, but they may also acquire iron via siderophores produced from other species. As an opportunistic human pathogen, C. neoformans must also adapt to these low iron conditions. In addition to increasing active transport of iron and uptake of siderophores, C. neoformans responds to low iron by inducing a large amount of surface capsule (350). Low iron alone is able to induce larger capsules than most of the other known in vitro capsule-inducing conditions, see Figure 24 (207, 350). Currently, there are two main signaling pathways that regulate adaptation to low iron, although the specific sensors and downstream outputs are still being defined. Figure 25 demonstrates the current knowledge of the iron-regulated transcriptional network.

One of the major regulators of low iron adaptation is the Cir1 transcription factor. Cir1 is a repressive GATA-type transcription factor with homology to the iron-regulating Fep1, Sfu1, and Urbs1 transcription factors in other fungi (284, 285, 356). Additional levels of iron regulation come from the HapX, Hap5, and Hap3 CCAAT-binding factors that act in concert with the Cir1 transcription factor in C. neoformans and with other iron-regulating factors in other fungi (179). The CCAAT-binding complex is able to repress iron-dependent processes in low iron conditions, and in C. neoformans, this complex also induces processes that increase iron uptake (12, 171, 179). Interestingly, only the hap3Δ and hap5Δ mutants have a defect in capsule; the hapXΔ mutant, despite having a larger effect on iron-related transcription, does not exhibit a capsule defect (179). Upstream of Cir1 is the Gat201 transcription factor, which directly transcriptionally regulates the expression of Cir1 (67).
Sensing or maintaining iron homeostasis is important for multiple host-specific phenotypes. The importance of iron regulation in adaptation to the host is exemplified by the phenotypes of the cir1Δ mutant strain. cir1Δ mutants have a defect in capsule induction, along with temperature sensitivity and dysregulation of melanin production, all of which are important adaptations to the host (181). The abundance of the Cir1 protein increases in high iron conditions, as expected for a transcription factor that responds to high iron by increasing iron uptake by the cell (178).
Figure 25. *C. neoformans* signal transduction networks that respond to iron, glucose, physiological CO₂, and host pH signals.

The dashed lines indicate connections that are established primarily by transcriptional data or homology; these processes require further study to determine the nature of the interaction.
Further evidence for low-iron induction of capsule comes from two mutant strains. Cig1 is involved in iron uptake, and mutations in this gene result in increased surface capsule in iron-replete conditions (181). In the JEC21 strain, mutation of the Cft1 iron uptake gene also results in increased capsule (218). In these mutants, it is likely that cells cannot accurately maintain iron homeostasis because they are unable to import iron under normal conditions. By mutating these iron uptake processes, the cells are effectively experiencing a low iron state, which results in capsule induction.

To determine the processes induced by low iron that are involved in regulating capsule, the Kronstad group performed SAGE and microarray analysis to assess global transcription patterns in low iron and high iron conditions. Surprisingly, in response to incubation in low iron medium (LIM), most of the known capsule biosynthesis genes discussed previously were not significantly differentially regulated. Although the transcription of Cap60 is increased twofold in LIM at 6 hours in the B3501 strain, Cap59, Cap10, and Cap64 were not differentially expressed (218). In the H99 strain after 6 hours in LIM, the only capsule biosynthesis genes that were differentially transcribed were a Cap64-like gene product and UX51 (181). The major biological process induced by low iron appeared to be cell wall and membrane synthesis, as revealed by both microarray and SAGE analysis of expression (179). These transcriptional profiles suggest that cell wall attachment may be the main capsule-associated phenotype regulated in low iron conditions.

To determine how the Cir1 transcription factor regulates these processes, Jung et al. examined the transcriptional profile of the cir1Δ mutant in both high and low iron conditions. From these experiments, they determined that Cir1 also regulates many cell wall integrity processes in low iron conditions, in addition to regulating the expression of
the capsule biosynthetic enzymes (181). These results were supported by the data from Chun et al., who examined the transcriptional profile of the gat201Δ strain in tissue culture conditions (67).

Another important pathway for responding to low iron is the Pka1/cAMP pathway; mutants in this conserved signaling cascade are unable to induce capsule in response to low iron conditions (6-8, 81, 158, 218, 300). Figure 25 illustrates the known elements of the highly conserved cAMP/PKA pathway. External signals are sensed by G-protein coupled receptors that then activate a heterotrimeric G-protein and cause dissociation of the Gα subunit (Gpa1) from the Gβγ subunits (Gib2, Gpg1 and Gpg2) (6, 214, 277, 379). Activated Gpa1 then signals through the adenylyl cyclase Cac1, which acts to convert ATP to cAMP (8). Although the cAMP and Ras pathways are separate in C. neoformans, the C. neoformans Cac1 protein still interacts with a CAP/Srv2 homologue (Aca1) to regulate cAMP levels (15). Cac1 also directly responds to intracellular carbon dioxide, a process mediated by the Can2 carbonic anhydrase (see below) (17, 243). Production of cAMP causes release of the two regulatory subunits (Pkr1) from the protein kinase A active subunits (Pka1) (81). Pka1 is then free to phosphorylate a number of downstream targets, including the Rim101 transcription factor, to allow for cellular adaptation to the environmental conditions that initially activated the cascade (81, 157, 266). The Ova1 mannoprotein is negatively regulated by Pka1, and the ova1Δ mutant has increased capsule (158). Due to its homology with phosphatidylethanolamine-binding proteins (PEBP), Ova1 was implicated in the regulation of capsule trafficking. PEBPs have also been implicated in MAPK signaling, potentially connecting Ova1 with other signaling cascades.
The pathway also has a number of negative feedback elements, including the Crg2 regulator of G-protein signaling, which interacts directly with Gpa1 to limit cAMP production (322, 380). Additionally, the Pde1 phosphodiesterase negatively regulates the pathway by degrading intracellular cAMP (156). The basic structure of this signaling cascade is highly conserved among eukaryotes. However, the specific activating stimuli and downstream effectors in *C. neoformans* allow this pathogenic fungus to use the cAMP/PKA pathway to respond to numerous host-relevant conditions.

The connections between *C. neoformans* cAMP signaling and low iron were first examined in the context of the *gpa1Δ* mutant. This mutant displays a striking defect in capsule in low iron conditions, and this defect is rescued with the addition of exogenous cAMP (6). In contrast, addition of exogenous cAMP to a *cir1Δ* mutant strain is not sufficient to restore capsule (181). Despite this separation, there is evidence of crosstalk between the Cir1 transcription factor and elements of the cAMP pathway. Cir1 transcriptionally regulates Gpr4, which can associate with Gpa1 to activate cAMP signaling (181). Downstream of Pka1, there are further connections to iron homeostasis. The Rim101 transcription factor is directly activated by Pka1 phosphorylation and transcriptionally regulated by both Cir1 and HapX. Cir1 induces Rim101 transcripts in both low and high iron conditions, and HapX induces both Cir1 and Rim101 in LIM (179). Finally, Pka1, Cir1 and HapX are all involved in the transcriptional regulation of many iron transporters and siderophore uptake genes (Cft1, Cfo1, Sit1), and this is likely mediated through Rim101 activation (158, 179-181, 266, 340). However, *rim101Δ* cells do not have a defect in capsule production under low iron conditions. Currently, the direct connections between these transcription factors and the elements that regulate surface capsule induction are still unknown.
To further examine this relationship, it is possible to compare the downstream targets of each of these pathways. Similar to the downstream responses regulated by Cir1 and HapX, Pka1 does not appear to induce the transcription of known capsule biosynthesis genes in low iron conditions. In a \textit{pka1\Delta} strain incubated in low iron medium, \textit{CAS35} and \textit{CAP10} transcripts are decreased (158). The level of \textit{UGD1} transcripts was not significantly differentially regulated between the wild type and mutant strains. Interestingly, \textit{UXS1} transcripts were increased in both the \textit{pka1\Delta} and \textit{pkp1\Delta} strains (158). Overall, the majority of the other capsule synthesis genes were not differentially expressed in the \textit{pka1\Delta} mutant. Instead, multiple genes potentially involved in secretion and cell wall remodeling demonstrated significantly different expression (158). The \textit{Ags1} \textit{\alpha}-glucan synthase, \textit{Fks1} \textit{\beta}-glucan synthase, and other glucan modifying enzymes were differentially regulated in the \textit{pka1\Delta} mutant under low iron conditions (158). RNAseq experiments performed on the \textit{pka1\Delta} mutant after incubation in DMEM confirmed the differential regulation of the \textit{Fks1}, \textit{Ags1}, \textit{Agn1}, \textit{Kre6}, \textit{Kre61}, and \textit{Skn1} glucan synthesis related genes. The cell wall remodeling processes are presented in Table 7. These results suggest that Pka1 is primarily involved in regulating the secretion and attachment of capsule in low iron conditions instead of the expression of capsule biosynthesis genes.

6.4.2 Host CO$_2$ levels

Increased carbon dioxide is a strong host-specific signal that is used by many fungal pathogens to trigger phenotypes that allow for invasion and disease. In \textit{C. albicans}, 5\% CO$_2$ triggers invasive hyphal formation and disease (187, 326). \textit{C. neoformans} also responds strongly to host levels of carbon dioxide—in this pathogen, the important phenotype is the induction of the polysaccharide capsule (140, 187, 350,
Both *C. albicans* and *C. neoformans* use the cAMP pathway to respond to CO$_2$, and the response bypasses the membrane-bound G-proteins (17, 187). Instead, the dissolved bicarbonate can directly stimulate adenylyl cyclase to induce cAMP synthesis (187, 395). See Figure 25 for the detailed structure of the cAMP/PKA pathway.

Two parallel studies on the Can2 carbonic anhydrase protein demonstrated that *C. neoformans* uses Can2 to convert CO$_2$ to HCO$_3^-$ (17, 243). Can2 is required for growth in the environment but is dispensable for growth in the host, ostensibly due to the high levels of carbon dioxide in the host environment (17). The natural conversion between CO$_2$ to HCO$_3^-$ at physiological pH with 5% environmental CO$_2$ provides sufficient bicarbonate to stimulate cAMP production (187). This direct activation of cAMP production most likely acts in concert with the other activating signals of the cAMP/PKA pathway to induce a robust downstream response leading to the production of encapsulated yeast in the host.

Interestingly, a study by Zaragoza et al. demonstrated that a *pka1Δ* mutant incubated in DME with 10% CO$_2$ was able to produce capsule (387). Although this concentration is higher than human physiological concentrations of CO$_2$, it is possible that there are other signals that respond either to cAMP levels or directly to bicarbonate that are involved in inducing capsule. By examining the differential transcriptional response of cells incubated in DME with or without additional bicarbonate, it will be possible to define the CO$_2$-responsive regulon. It is likely that this will overlap significantly with the genes that are regulated by the cAMP/PKA pathway. Further examination of the specific transcriptional response to 10% vs. 5% external CO$_2$ is necessary to determine how the cell bypasses Pka1 phosphorylation. These experiments will give clues to the additional pathways involved in CO$_2$ response.
6.4.3 Ambient pH

There is a strong physiological connection between iron availability, CO$_2$, and pH. At human physiological pH (pH 7), iron is often found in insoluble compounds as ferric iron, thus creating a low-iron signal inside the host in addition to the pH signal (350). The CO$_2$-HCO$_3^-$ equilibrium is also vital for maintaining the pH of the host, which then influences both the available CO$_2$ levels and the environmental pH sensed by the fungus (309).

Most fungi use a conserved pH-sensing pathway to respond to environmental pH, and this response is required for virulence in the host (31). In other fungi, this Pal/Rim signal cascade is initiated through activation of a seven transmembrane-domain protein (Rim21) that senses pH (11). The Rim9 three transmembrane-domain protein may assist Rim21 localization to the cell membrane (32, 45, 74). Under neutral to alkaline pH, Rim21 is activated, and this activation can trigger the Rim8 arrestin-like protein to be phosphorylated and ubiquitinated, although other fungi, such as S. cerevisiae, have constitutively monoubiquitinated Rim8 proteins that may be regulated by localization (150, 152). The entire Rim21/Rim8 complex can then transported via the ESCRT system to the endosomes (73, 151, 153). Rim20 then associates with this endosomal structure and forms a scaffold on the Rim101 transcription factor by direct interaction with the C-terminal region of the Rim101 protein (355, 378). This Rim20 scaffold mediates cleavage of Rim101 by the Rim13 calpain-like protease by bringing the protease into association with Rim101 (215, 216, 378). Dissociation of the ESCRT complex is required for removal of the proteolytic Rim20/Rim13 scaffold (147). Proteolytic cleavage is necessary for Rim101 activation (27, 239, 274, 287). In some species, the transcription factor undergoes a second cleavage event, which may be
mediated by the proteasome or by other proteases, depending on the species (13, 89, 355). The Rim101 transcription factor then is able to induce the responses necessary for adaptation to host pH, which allows for fungi to cause disease (13, 27, 84, 200).

In contrast to many model fungi, *C. neoformans* has integrated the conserved pH-sensing pathway with the cAMP pathway. The CnRim101 transcription factor requires phosphorylation by Pka1 in addition to Rim20-mediated cleavage for localization and activation (266). Vps25 is part of the ESCRTII complex, and vps25Δ mutants have similar capsule size to the rim101Δ mutant, consistent with activation of Rim101 through the ESCRT pathway (69). Although other members of the pathway, such as Rim13 and Rim8 have putative homologues in the genome, the homologue to the Rim21 pH-sensing receptor has not been identified. Additionally, the regulation of the Rim13 and Rim8 proteins, including the role of ubiquitination and localization, has not been fully explored. Figure 25 displays the putative elements of the pH-responsive pathway in *C. neoformans*. This model provides a platform to further explore the link between pH and cAMP levels. Because physiological pH is regulated in part by CO$_2$ levels and because the cell can sense CO$_2$ levels using Cac1, this may also serve as a signal for neutral pH (8, 17, 187, 243). Therefore, the Rim101 transcription factor may act by synergizing the pH-related inputs from both the conserved pH-sensing pathway and the cAMP pathway.

To determine the Rim101-dependent processes, O’Meara et al. performed comparative transcriptional analysis comparing the wild type and rim101Δ mutant strain after incubation in tissue-culture conditions. Downstream of Rim101, there are a number of cell wall integrity proteins, but not many capsule biosynthesis proteins. Of the 12 chitin-related processes, 8 are differentially regulated in the rim101Δ strain.
Correspondingly, the rim101Δ mutant has a defect in maintaining capsule at the cell wall, but the strain can secrete capsule similar to the wild type strain (266). The rim101Δ strain also has a growth defect in alkaline pH, confirming its role in neutral/alkaline pH responses. Rim101 transcriptionally regulates the Ena1 sodium transporter, and this protein is required for growth in alkaline pH and the CSF (164, 205, 266).

To look specifically at capsule biosynthesis genes induced by physiological pH, Zaragoza et al. examined the expression of the CAP10, CAP59, CAP60, and CAP64 after incubation in 10% Sabouraud’s medium buffered to pH 7.3 with MOPS. Similar to what was observed in the rim101Δ mutant strain, there was no significant change in expression for any of these genes (390). This suggests that other processes, such as cell wall remodeling or capsule structure alterations, must be responsible for the dramatic encapsulation of cells under this inducing condition.

Despite the potential activation of both the low iron and cAMP pathways, rich medium buffered to physiological pH is insufficient as a signal to trigger capsule induction (140). The cells must also receive input from another signaling pathway, such as the response to nutrient limitation. Multiple studies have demonstrated that incubation of cells in nutrient-poor conditions at physiological pH results in a strong induction of capsule around the cell (96, 385). The role of limited nutrients will be discussed below.

### 6.4.4 Low glucose and low nitrogen

The upstream elements of the C. neoformans cAMP/PKA pathway also respond to low glucose and low nitrogen. G-proteins and their G-protein coupled receptors sense the environmental signals of low glucose and low nitrogen and activate the pathway by inducing the production of cAMP (6, 277, 322, 379, 380). Interestingly, nutrient poor signals do not consistently result in the induction of capsule around the cell. Granger
determined that incubation of cells in DME with glucose concentrations between 5 and 50 mM had no effect on capsule (140). However, incubation in Sabouraud’s medium with approximately 50 mM of glucose at pH 7 resulted in induced capsule (96). It is likely that multiple inputs are necessary for capsule induction, even within a single signaling cascade.

The requirement for multiple inputs is clearly demonstrated by the role of Gpr4 on capsule. Under low nitrogen conditions, Gpr4 interacts with Gpa1 to activate the signaling cascade (379). However, the gpr4Δ mutant has no melanin defect despite the clearly defined role for Gpa1 in melanin production (379). Additionally, low nitrogen transcriptionally induces both Gpr4 and Gpa1, and methionine triggers Gpr4 internalization, but low glucose only induces Gpa1 expression. By adding both the nitrogen and the glucose signals, it is possible to induce higher cAMP levels than by adding each signal alone (379).

Another example of specificity in downstream responses to the cAMP/PKA cascade is the Nrg1 transcription factor. The nrg1Δ mutants have a defect in capsule induction similar to the pka1Δ mutants, and the Pka1 phosphorylation consensus sequence is important for Nrg1 activation. However, comparison of the Nrg1-dependent targets under low glucose and tissue-culture conditions revealed very little overlap, despite both conditions acting as activators of the cAMP/PKA pathway ([78] and our unpublished data). Under tissue culture conditions, the Nrg1 transcription factor did not appear to be regulated by Pka1, as determined by the examining the correlation in downstream targets (our unpublished data).

Additionally, C. neoformans appears to repress capsule formation in low glucose conditions unless other inducing signals are also present. This is exemplified by the
repression of capsule by Ssa1, which is a member of the Hsp70 heat shock family of transcriptional co-activators. The ssa1Δ mutant has increased capsule diameter after incubation in malt agar without glucose; a starvation condition. However, this condition does not induce encapsulation in the wild type strain (391). The exact mechanism by which Ssa1 represses capsule in response to specific glucose starvation signals has not been fully explored.

For both low nitrogen and low glucose, these signals are not sufficient to induce capsule without an additional host environmental cue. This can be demonstrated by examining the pkr1Δ mutant. This mutant strain causes constitutive activation of Pka1 and results in the production of a capsule even in rich medium. However, the capsule is even larger in the pkr1Δ strain when it is incubated in tissue culture conditions with CO2 (81), see Figure 25. These results demonstrate that a single signal transduction cascade can respond to multiple inputs and regulate multiple downstream outputs, presumably by coordination with parallel signaling cascades.

6.4.5 Stress

Certain stresses on the cell also play a role in repressing capsule induction, potentially by altering cell wall integrity. For example, high osmolarity can repress capsule formation, even when the cells are incubated with the otherwise inducing conditions of low glucose at pH 7 (96). To understand the role of cell stress on capsule formation, it is important to examine two conserved osmotic stress response pathways—the Hog1 pathway and the PKC pathway.

The Hog1 signaling pathway responds to a number of cell stresses, and mutations in this pathway lead to alterations in the ability of C. neoformans to regulate capsule under normal conditions. The Bahn lab has determined many of the elements of
the Hog1 pathway in *C. neoformans* and their roles in responding to environmental conditions. The known elements of these signal transduction cascades are presented in Figure 26.
Figure 26. Elements of MAPK cascades in *C. neoformans* and their roles in capsule regulation.
The role of Hog1 in capsule regulation was first documented by the observation of a hypercapsular phenotype of the \textit{hog1}\textDelta\ mutant strain (16). The two-component sensor kinases, Tco1 and Tco2, respond to environmental conditions and signal through the Ypd1 histidine kinase relay protein to phosphorylate the Ssk1 response regulator (19, 68, 184, 209). Ssk1 then phosphorylates Pbs2, which phosphorylates Hog1 (16, 19). Under stress conditions, Hog1 is rapidly dephosphorylated. However, phosphorylated Hog1, which is present under normal conditions, acts to repress capsule and melanin (16, 18, 19). The phosphorylation status and localization of Hog1 under various capsule-inducing conditions, such as DMEM with 5% CO\textsubscript{2} or low iron, has not been established, but it is likely that Hog1 is dephosphorylated under these conditions to allow for induction of capsule. Examination of a constitutively dephosphorylated Hog1 strain placed under capsule-inducing conditions would shed light on the processes necessary to repress capsule.

Downstream of Hog1 are a number of kinases and transcription factors, and the interaction and regulation of these proteins is still being explored. The Sch9 kinase is likely regulated by Hog1; however, it is also likely controlled by additional inputs because Sch9 regulates only a subset of the Hog1 phenotypes. Additionally, Sch9 is only transcriptionally induced under oxidative stress (192). Similar to the \textit{hog1}\textDelta\ mutant, the \textit{sch9}\textDelta\ mutant has increased capsule, suggesting that Sch9 also normally represses capsule (192, 362). In contrast, the Hrk1 protein kinase does not regulate capsule and plays only a minor role in melanin, despite being downstream of Hog1 (185).

There are currently two known transcription factors that are regulated by Hog1. The \textit{Atf1} transcription factor was first connected to Hog1 because the \textit{atf1}\textDelta\ mutant has increased capsule and melanin production and increased sensitivity to osmotic stresses. 
The connection was confirmed by microarrays demonstrating that Hog1 regulates the expression of Atf1 (184, 192). However, the atf1Δ mutant has some drug sensitivities that are not shared with the upstream Hog pathway mutants, which suggests that Atf1 is also regulated by other elements. Because Atf1 is also transcriptionally regulated by Can2, Pka1 and Rim101, it is likely that the cAMP pathway is involved in the regulation of this transcription factor (184).

The Mbs1 transcription factor is repressed by Hog1, and mbs1Δ mutants have a minor defect in encapsulation (331). These data imply that Hog1 represses capsule under normal conditions by repressing the Mbs1 activator of encapsulation. To determine the differentially regulated processes responsible for repressing capsule under normal conditions, Ko et al. examined the transcriptional profile of the hog1Δ mutant strain (192). The capsule-associated genes CAP59, CAP60, and CAP64 demonstrated between 1.5 to 1.9-fold increased expression in the hog1Δ mutant strain, and the CAP10 gene was induced 1.8-2.2-fold in this strain background. This modest increase in expression of four capsule genes suggests that increased capsule biosynthesis may not be the major biological process responsible for increased encapsulation in the hog1Δ mutant (192). In contrast, Hog1 may have its main effect on capsule by regulating various cell wall components. Microarray studies indicate that several cell wall modifiers display Hog1-dependent transcription, including five chitin and chitosan proteins, the Agn1 glucosidase, and two Kre glucan synthase genes. Confirmation of the Mbs1 downstream targets by transcriptional profiling and examination of Mbs1 binding sites will provide further insight into how Hog1 is able to specifically repress capsule production.
Crosstalk between the Hog1 pathway and other capsule-inducing pathways was examined by comparative transcriptional profiling. Interestingly, many of the iron transporter genes (\textit{SIT1, CFO1, CFO2, and CFT1}) were highly induced in the \textit{hog1Δ} mutant strain (192). These microarray studies demonstrated parallel downstream regulation of ergosterol biosynthesis by the cAMP pathway and the Hog1 pathway. Additionally, the arrays revealed transcriptional regulation of Tco2 by cAMP pathway components. However, these experiments were performed under rich medium conditions where the cAMP pathway is not necessarily activated (227). The relationship between the cAMP and Hog1 pathways needs to be explored further, especially with the potentially coordinated regulation of the Mbs1 and Atf1 transcription factors.

The Protein Kinase C (PKC) pathway is the other major cell stress-responsive pathway, and it is responsible for maintaining cell wall integrity and chitin distribution in the cell. The structure of the Pkc1 signaling cascade is illustrated in Figure 26. Environmental stresses, such as osmotic or cell wall stresses, are sensed by an unknown cellular component. Inositol-phosphorylceramide synthase (Ipc1) regulates the levels of phytoceramide and diglycerol (DAG), which act as intracellular signaling molecules that are able to activate the Pkc1 protein kinase (155). Pkc1 can also be activated by the Rho1 GTPase, which is itself activated by the Rom2 protein. Activated Pkc1 then initiates the MAPK cascade, which activates the Bck1 MAPKKK, the Mkk2 MAPKK, and finally the Mpk1 MAPK kinase (130). Separate from the MAPK cascade, Pkc1 can also regulate the Sp1 transcription factor (4).

Mutations in Pkc1 cause overproduction of capsular polysaccharides that are not maintained at the cell surface. Deletion of the entire coding region of the \textit{PKC1} gene results in dramatically increased capsule production, as measured by packed cell
volume and the mucoid appearance of the cells on plates. However, examination of the cells using India ink did not reveal capsule around the cell, demonstrating that this strain has a defect in capsule attachment. In a strain missing just the C1 domain of the Pkc1 protein, preventing activation by DAG, capsule diameter around the cell was decreased 42% compared to the wild type, and the density of the remaining fibrils was also decreased (154). Additionally, this strain has significant growth defects (129). In \( pkc1\Delta \) mutants, chitin and chitosan levels are similar to wild type, but the distribution of these components in the cell is altered. Additional regulation of the PKC pathway comes from the Lrg1 and Ppg1 proteins, which were discovered by comparing the \( C.\ neoformans \) pathway to its \( S.\ cerevisiae \) counterpart. In both the \( ppg1\Delta \) and \( lrg1\Delta \) mutant strains, capsule production was decreased (130).

Of the downstream targets of Pkc1, the Sp1 transcription factor may be the main negative regulator of capsule in this pathway (4). The \( sp1\Delta \) mutant strain exhibits large amounts of surface capsule even under non-inducing conditions, such as YPD with sorbitol. The increased surface capsule was greater than in the \( pkc1\Delta \) mutant, potentially due to basal levels of Sp1 in the \( pkc1\Delta \) mutant (4). Analysis of the downstream targets of the Sp1 transcription factor implicated carbohydrate metabolism and cell wall integrity defects. Additionally, the Fks1 \( \beta \)-glucan synthase was reduced in the \( sp1\Delta pkc1\Delta \) double mutant strain and in the \( mpk1\Delta \) mutant strain; this decrease may result in fewer attachment sites for the secreted polysaccharide.

Recent work has shown that the Hog1 and PKC pathways are intimately connected. In the \( hog1\Delta \) mutant, the PKC/MAPK pathway is constitutively activated (16). Dephosphorylation of Hog1 under stress conditions is regulated by the Pkc1-dependent Cck1 casein kinase I protein (364). Although \( cck1\Delta \) mutants have no defect in capsule,
Cck1 also regulates the phosphorylation of Mpk1, further connecting these two cell wall integrity pathways.

The third MAPK cascade in *C. neoformans*, the pheromone response pathway, is also involved in regulating capsule production, although it does not appear to be one of the primary mediators of stress response. The Ste12α transcription factor induces the expression of capsule biosynthesis genes in glucose medium, and both *ste12αΔ* and *ste12aΔ* mutants have decreased capsule *in vivo* (58, 59). Ste12 can activate the Cpk1 MAPK cascade, which regulates mating in *C. neoformans* (82). Downstream of the Cpk1 MAPK cascade is the Cwc1/Cwc2 complex, and this complex negatively regulates Ssn801/Ssn8 (CNAG_00440). Ssn8 is a homologue of the cyclin subunit of the Mediator complex, and the *ssn8Δ* mutant has increased capsule (221, 361). Additionally, *ssn8Δ* mutants show a dramatic alteration in morphology that can be attributed to a defect in cell wall construction and integrity. Chitin and chitosan localization are disrupted in this strain, and there is increased β-1,3 glucan on the *ssn8Δ* cells (361). Wang et al. identified this cell wall defect as being similar to the phenotypes seen in the *rom2Δ* mutant, potentially connecting Ssn8 to the Pkc1 pathway as well (361).

### 6.4.6 Hypoxic stress

In the microenvironment of the human lung, *C. neoformans* cells are frequently exposed to hypoxic stress. Under this condition, the fungus uses the conserved SREBP pathway to regulate adaptations to this stress. In *C. neoformans*, the Scp1 protein (CNAG_01580) processes the Sre1 transcription factor (CNAG_04804) (51, 68). Processed Sre1 is then imported into the nucleus by the Kap123 protein (CNAG_05884) and phosphorylated by the Gsk3 kinase (CNAG_06730) (52). The Dam1 protein regulates the turnover of Sre1, thus regulating the transcriptional response to low
oxygen. Although there are more elements in the mammalian hypoxic response pathway that regulate the processing, trafficking, and degradation of the SREBP complex, these proteins are still being identified and examined in *C. neoformans* for their role in low oxygen response and capsule induction.

In the H99 background, *sre1Δ* mutants have slight capsule defects in 10% Sabouraud’s medium at pH 7.3, although this is not a low oxygen environment (68). In the B3501 background, *sre1Δ* mutants have defects in capsule *in vivo* (51). Although Tco1 is also involved in hypoxic response, there is evidence that the Hog1 and Sre1 pathways act in parallel. For example, the *tco1Δ sre1Δ* double mutant is more sensitive to low oxygen than either single mutant (68). Additionally, *sre1Δ* mutants have decreased melanin instead of the increased melanin of the Hog1 pathway mutants.

To understand the biological processes that are regulated by the Sre1 transcription factor, Chun et al. performed comparative transcriptional profiling on the *sre1Δ* mutant strain. These experiments revealed that neither capsule nor cell wall biogenesis proteins were differentially regulated in the *sre1Δ* mutant strain in response to hypoxia (68). However, Sre1 did play an important role in the regulation of ergosterol synthesis. The connection between ergosterol, a membrane component, and capsule has not been fully explored.

Downstream of Sre1 is the Gat1 GATA type transcription factor (51, 208). In both RPMI and DMEM, the *gat1Δ* mutant has a decreased capsule (208), similar to the phenotype of the *sre1Δ* mutant. Interestingly, this protein negatively regulates the secretion of exopolysaccharide in non-inducing conditions (191). In minimal medium, Gat1 represses the expression of genes involved in capsule biosynthesis, including *UGD1* and *UXS1* (191). However, the size of the capsule surrounding the *gat1Δ* mutant
cell under these conditions was similar to wild type, supporting the hypothesis that secretion of exopolysaccharide is separate from encapsulation. It is possible that the Sre1 transcription factor also regulates secreted, as opposed to attached capsule, but this has not yet been established.

6.4.7 Unconnected Genes and Conditions

Some genes that are required for proper capsule formation are not obviously connected to one of the known capsule-inducing pathways. In the literature, there are also genes and environmental conditions identified as regulating capsule that have not been investigated further. This section will highlight some of these genes and environmental conditions, proposing connections that should be addressed in future experiments.

6.4.7.1 TUP1

The C. neoformans tup1Δ mutant has increased capsule compared to isogenic wild-type strains, and this capsule difference was maximized by incubation in RPMI medium (207). In S. cerevisiae, Tup1 is a transcriptional repressor that acts by establishing repressive chromatin in response to Hog1 regulation of the Sko1 protein (297). However, C. neoformans does not have an obvious homologue of the Sko1 protein, and the Tup1 protein does not appear to be downstream of Hog1 (192, 207). Currently, the upstream regulator of Tup1 is unknown, although the tup1Δ strain is sensitive to cell wall stressors. Unlike many other capsule regulators, the expression of specific, known capsule genes (CAP10, CAP64, and CAS35) were at least 3-fold higher in the tup1Δ mutant than in the wild type (207).

Although certain genes involved in iron transport (SIT2, CTR4, FRT1, CIG1) had decreased expression in the tup1Δ strain, Tup1 did not transcriptionally regulate the
CIR1 transcription factor or the CFT1 and CFT2 iron uptake genes (207). Additionally, the induction of capsule in LIM appears to be separate from Tup1 because the capsule of the *tup1Δ* strain could be induced further in LIM (207). Tup1 is also distinct from the cAMP pathway because addition of exogenous cAMP does not alter capsule production in the *tup1Δ* strain, and Tup1 does not transcriptionally regulate elements of the cAMP pathway (207). In *S. cerevisiae*, Tup1, Sko1 and Hog1 are involved in the recruitment of the SAGA chromatin-remodeling complex, and this remodeling is important for transcriptional regulation (224, 230, 297, 298, 384). However, due to the distinction between Hog1- and Tup1-dependent phenotypes as well as the lack of a Sko1 protein in the *C. neoformans* genome, the role of CnTup1 in the regulation of chromatin remodeling is unclear.

6.4.7.2 *Gcn5, Ada2*, and chromatin remodeling.

The Gcn5 protein is a conserved acetyltransferase in the SAGA complex, controlling chromatin structure and the associated expression of many genes. The *gcn5Δ* mutant displayed markedly decreased capsule attachment. However, distinct from mutations in other stress responsive elements such as Hog1, the *gcn5Δ* mutant displayed no change in susceptibility to osmotic stresses (264). Interestingly, the expression of Gcn5 and Ada2, another component of the SAGA complex, is altered in the *hog1Δ* mutant strain, demonstrating repression of these two under normal growth conditions (148, 192). The *ada2Δ* mutant strain has a similar capsule defect to the *gcn5Δ* mutant strain, but Ada2 plays a role in mating where Gcn5 does not (148).

To determine the connection between Gcn5, Ada2 and Hog1, we examined the downstream targets that are shared by these transcriptional regulators. This analysis revealed 79 genes that are coordinately regulated by Gcn5 and Hog1; however, the
strains were incubated in different conditions before microarray profiling, limiting this type of analysis. Therefore, there may still be further connections between the pathways (192, 264). One of the genes that is transcriptionally induced by Gcn5 is Tco2, but whether that is sufficient to activate the Hog1 kinase is unclear (264). However, in the *hog1Δ* mutant, the transcription of the *ADA2* gene increases, although the levels of GCN5 are not altered. Currently, the SAGA complex seems to be regulated by the Hog1 MAPK cascade, but the connections between dephosphorylated Hog1 and SAGA activity are still being examined.

Further analysis of the downstream targets of Gcn5 did not reveal significant differences in expression of the capsule biosynthesis genes, complementing the experiment demonstrating wild type levels and electrophoretic mobility of secreted capsule in the *gcn5Δ* mutant strain (264). The *ada2Δ* mutant, however, showed differential regulation of the *CAS3, CAS32, CAS1*, and *MAN1* genes. Therefore, the SAGA complex may still be involved in the differential regulation of some polysaccharide biosynthetic processes. Overall, the mechanism by which the SAGA chromatin-modifying complex regulates capsule has not been completely elucidated, especially in the separation of Gcn5 and Ada2 targets.

Other members of a histone deacetylase complex are also involved in capsule regulation. Mutation of Set301 and Hos2 results in increased capsule (221). However, in *S. cerevisiae*, Hos2 is associated with highly-expressed genes, acting as an activator instead of a repressor (359). Currently, these proteins and the their function in a histone deacetylase complex have not been confirmed in *C. neoformans*. 
6.4.7.3 ZDS3

The Zds3 protein was identified through insertional mutagenesis, and this protein negatively regulates the production of capsular polysaccharides (217). Zds proteins in S. cerevisiae regulate protein phosphatase activity, and Zds1 and Zds2 are involved in cell polarity and cell cycle. Interestingly, the overproduction of capsule in the C. neoformans zds3Δ mutant is tightly linked with pH, with the most capsule produced at pH 4. However, limiting glucose, which presumably limits the pool of available carbohydrate precursors, can prevent the overproduction of capsule. Similar to the pkc1Δ mutant strain, increased production of capsule does not correlate with an increased diameter of encapsulation around the cell. Additionally, the zds3Δ mutant is also sensitive to cell wall stresses (129, 130, 217). However, the phenotypes of the zds3Δ mutant cannot be rescued by sorbitol, making it more severe than the pkc1Δ pathway and implying basal activity of the unphosphorylated Zds3 protein. Currently, the elements downstream of the Zds3 protein are unknown.

6.4.7.4 Copper

In addition to low iron response, the low-copper regulon has also been implicated in capsule regulation. In the C. neoformans genome there are two copper transporters, Ctr2 (CNAG_01872) and Ctr1 (CNAG_07701) (69, 91). Chun et al. determined that the ctr1Δ mutant strain has a defect in encapsulation and in growth in low copper medium. However, the capsule-deficient phenotype of the ctr1Δ mutant was not replicated in further experiments by Ding et al (91). The Ccc2 protein is a copper transporter that is involved in negatively regulating capsule (176, 358). However, Ccc2 may be required for the assembly of the Fet3/Cft1 iron transporter, and the altered iron homeostasis may be the primary capsule-inducing signal. Additionally, the Hxt1 protein negatively regulates
capsule production (65). The hxt1Δ mutant has increased capsule compared to wild type after incubation in malt agar. Although Hxt1 is a copper chaperone in other species, C. neoformans Hxt1 is not involved in copper resistance (65).

6.4.7.5 IRE1

The Ire1 kinase is involved in the cellular response to unfolded proteins (64). Activated Ire1 removes an unconventional intron from a downstream transcription factor, either Hxl1 in C. neoformans or Hac1 in ascomycetous fungi. The spliced transcription factor can then induce genes necessary for responding to cell stress. Cheon et al. determined that both ire1Δ and hxl1Δ mutants are sensitive to cell walls stressors, but only Ire1 plays a role in inducing encapsulation (64). By examining the levels of Hxl1 splicing in the cac1Δ, cna1Δ, cpk1Δ, hog1Δ and mpk1Δ mutant strains, Cheon et al. were able to determine that Hxl1 regulation is independent of these signaling pathways (64). However, because Ire1 regulation of capsule is separate from Hxl1 splicing, there may still be some crosstalk between known capsule-regulating pathways and the Ire1-mediated response.

In C. albicans, the SAGA complex demonstrated direct binding to the Ire1 promoter to increase expression of the Ire1 protein. However, analysis of the downstream targets of the SAGA complex revealed no change in Ire1 expression in either the gcn5Δ or the ada2Δ mutant strain (148, 264). Interestingly, expression of Hxl1 was 2-fold decreased in the rim101Δ mutant, potentially linking this pathway with Rim101. Further experiments are necessary to determine the activator of Ire1 and whether it is connected with known signaling cascades.
6.4.7.6 CPL1

Cpl1 is a putative secreted protein that was discovered to have a capsule defect in a systematic deletion screen (221). It is transcriptionally induced in response to oxidative stress (192), but its regulation and function are currently unknown.

6.4.7.7 CIN1

Cin1 is a putative intersectin homologue that has a demonstrated role in vesicle transport, resulting in alterations in chitin deposition, cell morphology, and surface capsule in the mutant strain (323). Although the Cin1 protein interacts in vitro with Cdc420, the interaction appeared to be dispensable for Cin1 action, which is consistent with the wild type capsule of the cdc420Δ mutant strain (24). Cin1 also interacts with the Wsp1 WASP protein, and the wsp1Δ mutant strain also has a defect in capsule and chitin deposition (324). Additionally, the localization of the Sav1/Sec4 vesicle transporters was disrupted in this mutant strain, suggesting a potential role in the regulation of capsule secretion (324). The regulation of the Cin1/Wsp1 complex is still being examined.

6.4.7.8 CLCA

In addition to the Rim and cAMP pathways, the ClcA chloride channel protein is required for growth at alkaline pH. The clcAΔ mutant also has a significant defect in encapsulation compared to the wild type strain after incubation on malt extract agar (394). However, this inducing medium is acidic, and so the effect of pH on capsule production in the clcAΔ mutant has not been fully examined. Due to the similarity in phenotypes between the clcAΔ and vph1Δ mutant strains, it is possible that the clcAΔ mutant also has a defect in capsule secretion (100). Further analysis of the capsule
defect in the \( \textit{clcA} \Delta \) mutant strain will provide more insight in the role of this chloride channel in capsule regulation.

6.4.7.9 Serum

In addition to genes that have not been connected to known signaling cascades, there are some environmental cues that alter \textit{C. neoformans} capsule production where the genes responsible are unknown. For example, the signaling pathways that respond specifically to mammalian serum is unclear. Although serum contains iron-binding components, it is unlikely that the cell is using low-iron responsive signaling pathways to induce capsule in response to serum because supplementation with iron does not repress capsule formation in this condition (387). Difficulties in understanding the signaling response to serum derive in part from the complexity of serum. Recently, Chrisman et al. determined that the serum lipids might be responsible for the induction of capsule, and the induction may depend on interactions with cryptococcal phospholipase proteins (66).

6.4.7.10 Carbohydrate source

Another cue that regulates capsule is supplementation with mannitol. Mannitol is able to cause different presentation of capsular antigens, and growth in mannitol causes larger capsule than growth in glucose (75, 146). Furthermore, initial reports indicated that high glucose is able to induce the production of exopolysaccharide, if not encapsulation (70, 146). However, other studies indicated that high glucose represses both encapsulation and exopolysaccharide production, potentially by preventing activation of the cAMP cascade (66).
6.4.7.11 Nitrogen source

In addition to the methionine-regulated activation of Gpr4, nitrogen source can influence capsule regulation. Early work on the induction of capsule ascertained that ammonium sulfate ((NH₄)₂SO₄) can suppress capsule (140), but the mechanism is unknown. However, recent work has shown that other nitrogen sources can induce large amounts of surface capsule, as long as ammonium sources are not present (208). The repression of capsule in the presence of ammonium is likely due to Gat1-mediated nitrogen catabolite repression (208).

Amino acid transport is also regulated by nitrogen source, as demonstrated by the auxotrophies of the ilv2Δ mutant strain (186). The ilv2Δ strain has a significantly smaller capsule diameter than the wild type strain after growth in DMEM. However, the ilv2Δ mutant also has a growth defect under these conditions, even when supplemented with isoleucine and valine, making the mechanism for the decreased capsule unclear (186). The capsule defect of the met6Δ mutant is also associated with poor growth under capsule inducing conditions (282). The growth defect may be due to the toxic accumulation of homocysteine, which cannot be converted to methionine in the met6Δ mutant (282). However, it is also possible that the altered amino acid transport in these mutant strains interferes with the normal nitrogen regulation of capsule.

6.5 Titan cells-A special case

The titan cell is a newly described C. neoformans morphological form observed primarily in the lungs of the infected host. In addition to its very large size (12-50 microns), titan cells have a very different capsule structure than normal yeast cells. The titan cell capsule appears to be especially effective at dampening the host immune response. Although wild type capsule in vitro can suppress the host inflammatory
response, titan cell capsules appear to be more efficient at this process, as evidenced by the negative correlation between the degree of inflammation and the number of titan cells in the fungal population (352, 388).

One of the differences in titan cell capsule is the increased size and number of chitin-like structures in the capsule (124). These structures may be related to the increased resistance of titan cell capsule to radiation, organic solvents, and DMSO (388). It is possible that these structures increase the crosslinking and density of the capsule. Additionally, the titan cells have extremely thickened cell walls, reaching between 2 and 3 \( \mu m \) instead of the normal size of 50-100 nm for cells grown in vitro (388). Another important feature of titan cells is that they are polyploid (270, 388). In titan cells, the nuclear content, cell wall, and capsule crosslinking must be specifically regulated.

The inducing factors for this unique morphology have been the subject of recent investigations. Host conditions appear to be extremely important in the formation of titan cells. Previously, the titan cells could only be induced during infection of mammalian lungs (270, 388). Recently, Chrisman et al. demonstrated that increased cell and capsule diameter can be induced by amoeba membrane lipid components, especially phosphatidylcholine (PC), in a dose-dependent manner (66). Under these conditions, the cells also induce twice as much surface capsule and six times more exopolysaccharide, consistent with separate pathways regulating the production of these two types of capsule. The response appears to be mediated through the action of the Plb1 phospholipase. Phospholipase B mutants (plb1\( \Delta \)) produce capsule in response to serum, but not to amoeba extracts, showing that Plb1 is required for responding to amoeba (66). Additionally, infection of Galleria mellonella wax moth larvae with C.
*C. neoformans* can also induce titan cell formation (124). However, incubation of cells with whole *Galleria* extract is sufficient to trigger enlargement, presumably due to the lipid components and not the process of colonization (124).

The cryptococcal signaling pathways responsive to these environmental cues are also being examined. The initial reports on titan cells demonstrated that the mating and pheromone pathway is important in the induction of titan cells, with co-infections of “a” and “α” cells producing the most titan cells. However, only “a” cells undergo increased titan cell formation, suggesting that there is a specific response to the “α” pheromone (270). Additionally, deletion of the Ste3a pheromone receptor did not abolish basal levels of titan cell formation in the “a” cells, implying that multiple signals are required for titan cell induction (270).

Screening through the seven G protein-coupled receptor (GPCR) mutants in *C. neoformans* revealed that only Gpr5 is required for titan cell formation (271). In the *gpr5Δ* mutant strain, there is a decreased rate of titan cell formation, and the cells that do undergo an increase in size are not as large as the wild type titan cells. Gpr5 likely signals through Gpa1 and Ste3a, with Ste3a interacting directly with Gpa1. The Ste3α protein, which plays no role in titan cell formation, interacts with Gpa2 and Gpa3 to regulate mating (271). The other members of the Gpa1 complex (Gib2, Gpg1, Gpg2) have not been investigated for their role in titan cell formation. Downstream of Gpa1, the Rim101 transcription factor also plays a major role in titan cell formation (271). The *rim101Δ* mutant strain has a more severe defect in titan cells than the *ste3αΔ* mutant, suggesting that uninduced Rim101 activity is sufficient for basal levels of titan cell formation (271).
Other factors have also been implicated in the regulation of titan cells and their concomitant increase in capsule formation. Mbs1 is required for titan cell formation, but the impact of the upstream regulators of Mbs1 on titan cell formation has not been investigated (331). The *cdc420* and *gap1Δ* mutants have defects in titan cell formation, and the Cnc1560, Pcl103, and Rho104 proteins act to repress titan cell formation (271, 388). However, the upstream signals for these elements and the downstream biological process that they regulate to influence titan cell formation are unknown. Currently, there are many unanswered questions about this process of host-induced morphological change, but the known elements that control the titan cell transition are presented in Figure 27. Future experiments to better understand titan cell biology, including transcriptional profiling of titan cells, are now more feasible due to the ability to induce the morphotype *in vitro.*
6.6 Clinical considerations of *C. neoformans* capsule and its regulation

The biology of the polysaccharide capsule has been intimately linked to our clinical understanding of *C. neoformans* disease. As mentioned previously, the surface capsule is a distinctive cellular phenotype that clinicians and microbiologists intimately
associate with this fungal pathogen. The recognition of encapsulated yeasts in CSF or other clinical samples allows these clinicians to rapidly diagnose the presence of *C. neoformans* at the site of disease.

The capsule has also served as a clinical epidemiological tool. Exposure to this fungus results in the production of specific antibodies to various capsule epitopes. Most individuals in an endemic region develop anti-cryptococcal serum antibodies by the age of five years (137). Because related strains tend to react *in vitro* to similar antibodies in human serum, investigators were able to group *C. neoformans* strains into “serotypes” long before detailed analysis of the fungal cell surface was possible. In this way, *C. neoformans* strains were historically divided into four major serotypes: A, B, C, and D (28, 310). The serotype distinctions were based upon conserved structural features in the capsule that are shared among related strains (95, 98).

Subsequent molecular epidemiology based on DNA sequence has supported many of the strain classifications derived initially by these capsule-based serological studies (376, 377). However, the recent combination of DNA and phenotypic analysis has resulted in a rigorous reclassification of “*Cryptococcus neoformans*” strains into two different species (*C. neoformans* and *C. gattii*), as well as two varieties within the species *C. neoformans* (var. *neoformans* and var. *grubii*) (199). This renaming of *Cryptococcus* strains has important clinical relevance. For example, most cryptococcal disease occurs in highly immunocompromised patients. However, *C. gattii* strains are able to cause disease in apparently immunocompetent individuals. Moreover, *C. gattii* infections often result in small, focal brain abscesses (cryptococcomas), as opposed to diffuse meningoencephalitis (332). These distinctive clinical features were first noted in subtropical regions of the world, such as Australia, in which *C. gattii* is routinely isolated.
However, new outbreaks of *C. gattii* infections have recently occurred in more temperate regions, such as British Columbia and the U.S. Pacific Northwest (42, 336). Therefore, it is important for clinicians to be aware of the potentially varying presentations of infection caused by the different cryptococcal species.

The polysaccharide capsule is also the basis for the very sensitive and specific cryptococcal antigen tests used widely in clinical practice. More sensitive than routine culture, this assay documents the presence of *C. neoformans* in clinical specimens, especially CSF and serum. In highly immunocompromised patients, such as patients with late-stage AIDS, this test has greater than 95% sensitivity for systemic *C. neoformans* infections (108).

Prospective testing for *C. neoformans* capsular antigen is also being studied in certain HIV-infected populations to prevent excessive immune reconstitution inflammatory syndrome (IRIS). Patients with AIDS who have clinically asymptomatic cryptococcal infections can develop very serious symptoms mimicking progressive *C. neoformans* infections after being initiated on antiretroviral therapy (ART) (327). Investigators therefore hope to identify patients with early, asymptomatic cryptococcal antigenemia or CNS infections prior to beginning antiviral therapy. In this way, clinicians may be able to co-administer antifungal therapy and ART, hopefully preventing or minimizing cryptococcal-based IRIS during the period of rapid improvement in immune function. While IRIS can be medically managed, this syndrome may also be life threatening, especially in patients with CNS symptoms and limited medical access. The availability of an inexpensive cryptococcal antigen test, suitable for urine or serum samples, would allow the application of this type of pre-emptive screening for clinically
inapparent cryptococcal infections in resource-limited settings where the confluence of AIDS and cryptococcosis is most striking (170).

6.7 Conclusions

Induction of the *C. neoformans* capsule is a complex biological process. As such, there are many ways that the cell can fail to produce capsule, and many redundant pathways that allow for robust encapsulation around the cell. Despite these complications, significant progress has been made in the illumination of the signal transduction networks that regulate capsule. In future research on this important virulence factor, it will be necessary to define the specific aspect of capsule that has failed in a particular mutant strain. Does the cell produce GXM? Are these GXM molecules structurally sound? Are alternative moieties incorporated into the capsular material? Is the capsule secreted across the cell wall? Is it maintained at the cell surface appropriately? What aspect of the mutant cell surface prevents capsular attachment? By defining the capsular defect of a mutant strain more precisely, we will be better able to dissect out the mechanism by which the cell is able to regulate the induction of capsule.

In conjunction, the importance of transcriptional profiling cannot be debated. By examining the elements downstream of a transcription factor, it is possible to determine the biological processes that are the primary target of a signaling cascade. Using this approach to dissect the capsule phenotype, we can determine that many signaling pathways that control the appearance of capsule on the cell surface do not transcriptionally regulate many of the classical capsule biosynthetic genes. Instead, many of the differentially regulated genes in the current array of capsule-defective mutants are involved in cell wall structure and integrity. The direct connections between cell wall phenotypes and capsule attachment are still being examined.
Finally, there is mounting evidence that the specific inducing condition plays a significant role in regulating the amount and the structure of the resulting capsule. This is exemplified by the variability in antibody binding to the capsules of cells collected from different organs in a single infected host. This plasticity of capsule structure in response to varying host conditions underscores the complex relationship between host and parasite. Not only do infectious microorganisms need to evade host defenses, they also often shape their microenvironment for better survival. By precisely regulating the composition of its cell surface and its secreted polysaccharides, the pathogenic fungus *C. neoformans* has developed intricate ways to establish chronic infection and dormancy in the human host. Recent events, including new outbreaks of cryptococcal infections and the staggering prevalence of *C. neoformans* disease in untreated AIDS patients, underscore the success of this encapsulated pathogen. Understanding capsule biosynthesis, assembly, and regulation will allow us to develop new strategies to better diagnose and treat these serious infections.

Acknowledgements:

This work was supported by NIH grants R56-AI074677 and R01-AI074677 to JAA. TRO was supported by a pre-doctoral fellowship from the American Heart Association. All gene annotation was facilitated by the FungiDB website [http://FungiDB.org](http://FungiDB.org) created by Jason Stajich.
7. Thesis conclusions

7.1 Summary

In this thesis, my goal was to understand how a fungal pathogen adapts a conserved signaling pathway to allow for disease in a human host. The core elements of the cAMP/PKA pathway are highly conserved among eukaryotes. However, the upstream activating signals and downstream transcriptional outputs appear to be specific to each organism, creating an enormous range of potential responses to the environment. By examining the network in C. neoformans, we can gain a better understanding of how this opportunistic pathogen co-ordinates multiple signaling pathways to adapt to survival within the host.

I discovered that the cAMP/PKA pathway in C. neoformans has integrated with a conserved pH-sensing pathway to activate the Rim101 transcription factor (Chapter 3). Rim101 and alkaline pH sensing have been studied in many other fungi, but this was the first report of connections between Rim101 and the cAMP pathway. The data showing that Pka1 phosphorylation is required for Rim101 cleavage and activation was bolstered by the high degree of correlation between the downstream targets of Pka1 and Rim101 (Chapter 5).

I also demonstrated that cell wall remodeling is one of the major phenotypic changes that result from activating the Rim101 transcription factor in response to host stimulus (Chapter 4). These cell wall changes were predicted from Rim101 function in other species and from the large number of cell wall biosynthesis genes that demonstrated Rim101-dependent expression. I then demonstrated that this cell wall remodeling is necessary for capsule attachment and avoidance of host responses. In collaboration with the Nielsen lab, we demonstrated that the rim101Δ mutant has a
defect in titan cell production, and this may also be due to a defect in cell wall remodeling (271). The lack of cell wall remodeling had important implications for virulence; mice infected with \textit{rim101Δ} mutant cells produced a highly damaging inflammatory immune response that was ultimately fatal.

Finally, we were also able to perform analysis of fungal gene expression within the host lung (Chapter 5). We examined a set of candidate genes that included potential targets of Rim101 and genes previously documented to play a role in pathogenesis. I was also able to use gel shift assays and chromatin immunoprecipitation to demonstrate direct interactions between Rim101 and target gene promoters, allowing me to distinguish between direct and indirect targets of Rim101 signaling. The direct targets included several genes involved in cell wall biosynthesis and remodeling. I hypothesize that these direct targets are important in altering the host pathogen interface in a way that promotes fungal proliferation.

\textbf{7.2 Future directions}

\textbf{7.2.1. Examining of the layers of crosstalk and integration in the upstream activators of Rim101.}

The integration of the pH and cAMP pathways allows \textit{C. neoformans} to include human physiological pH as a stimulus for activating a suite of transcriptional responses to the host. However, homologs to the most upstream members of the conserved pH response pathway are not present in the \textit{C. neoformans} genome, suggesting that further adaptations are controlling the response to neutral/alkaline pH. Unlike many other fungi, \textit{C. neoformans} is unable to grow well at pH above 7.6 (human physiological pH). Additionally, the \textit{rim101Δ} mutant has a more severe defect in growth on alkaline pH than the \textit{pka1Δ} mutant, suggesting some Pka1-independent responses to pH.
The lack of upstream pH response elements may prevent *C. neoformans* from responding to these highly alkaline environments. Additionally, the canonical pH response pathway may utilize a different environmental sensor that responds to stimuli other than pH. By cataloging the environmental conditions that trigger activation of Rim101 and carefully defining each Rim101 cleavage product, we should be able to define the stimuli for this pathway.

Although we have data demonstrating that Rim20 and more upstream members of the pH pathway are involved in Rim101 activation and capsule formation, it is possible that Pka1 phosphorylation occurs on multiple members of this pathway (Figure 25). For example, Rim13, which is the protease responsible for Rim101 cleavage, contains a potential PKA phosphorylation consensus sequence. Therefore, I would examine the phosphorylation status of Rim13 and other upstream members of the pH response pathway.

Additionally, pH levels likely modulate the PKA pathway. The levels of bicarbonate are dependent on pH, thus potentially regulating Can2 activation of the cAMP pathway and allowing for neutral-alkaline pH activation of PKA. Finally, deep RNA sequencing revealed a subset of genes that were dependent on Rim101 but not Pka1. This demonstrates there are additional levels of complexity in the crosstalk between the cAMP and pH signaling cascades, and these connections may be important in regulating virulence.

**7.2.2. Extending the cAMP/PKA pathway by identifying downstream targets of Rim101**

To identify a larger set of Rim101 targets, we can use a number of approaches. In this thesis, I performed RNA sequencing, which gave us both direct and indirect
targets of Rim101 after incubation for three hours in tissue-culture conditions. I was then able to use gel shift analysis and chromatin immunoprecipitation experiments to define the Rim101 binding site in *C. neoformans*. Bioinformatic analysis of the promoters of Rim101 regulated genes revealed potential direct targets, but these have not been confirmed. However, ChIP-seq would give the full set of Rim101 direct targets. Potentially, one or more of these genes is responsible for the aberrant exposure of PAMPs on the cell surface of the *rim101Δ* mutant strain or for the defect in titan cell production.

It is likely that Rim101 will have different targets under different conditions because its binding to promoters will be influenced by interactions with other transcription factors. For example, the *C. albicans* Rim101 protein interacts with a CAAT transcription factor on the promoters of genes involved in iron uptake (12). I have also observed Rim101-dependent expression of iron uptake genes, and microarray experiments demonstrated that the *C. neoformans* HapX and Cir1 proteins share a number of targets with Rim101 (177, 179). I hypothesize that Rim101 and these other transcription factors compete for binding, thus causing differential regulation of iron uptake genes under high or low iron conditions. Further exploration of these interactions would allow us to make better predictions about Rim101 targets and potentially connect the cAMP pathway with low iron responses.

To find additional downstream targets of Rim101, we can also use an unbiased forward genetics screen for *rim101Δ*-like phenotypes. The *rim101Δ* mutant has a striking defect in growth in alkaline pH and high salt conditions, both of which are easily assessed using *in vitro* assays. Using the deletion collection or a series of transposon insertion mutants, we should be able to find mutations that phenocopy the *rim101Δ*
mutant. These targets may play a role in the important virulence phenotypes of titan cell production or increased PAMP exposure, and examining these mutants in animal models may increase our understanding of the cryptococcal disease process.

7.2.3. Examining the interaction between cryptococcal cell wall components and host immune responses

A major finding of this work is that assaying for death in animal models of infectious diseases gives a very incomplete picture of microbial virulence. The kinetics of mortality between the wild type and \( \text{rim101}\Delta \) mutant strain do not reveal the dramatically different disease process and immune response triggered by the \( \text{rim101}\Delta \) mutant strain. Instead, performing histology and quantifying CFUs most easily demonstrate the major differences in host inflammation and \( \text{rim101}\Delta \) mutant cell morphology. Although I demonstrated that the \( \text{rim101}\Delta \) mutant cell wall was dramatically thicker and contained more chitin/chitosan and \( \alpha \)-glucan, we have not yet defined which fungal cell moiety is triggering the host immune response. However, we know that the \( \text{rim101}\Delta \) cell is able to induce the production of TNF-\( \alpha \) from macrophages. Therefore, in future experiments, we can examine the TNF-\( \alpha \) induction of macrophages after incubation with a series of candidate cell wall mutants and the targets identified in the previous section.

From the host side, it is important to identify which pattern recognition receptors (PRRs) are responding to the fungal cell moieties. Our initial data on the immune response to \( \text{rim101}\Delta \) cells emphasized the importance of the early innate immune response. To test the role of a particular receptor, we can assess the induction of TNF-\( \alpha \) from macrophages collected from mice deficient in that receptors, such as macrophages collected from \( tlr2^{-/-} \) mice. We can also use a set of pharmacological agents against
specific signaling cascades activated by various PRRs, using rapamycin and
cyclosporine A to show initial involvement of PRR signaling.

Currently, one recommendation for AIDS patients with cryptococcal infections is
to complete a course of antifungal therapies before starting antiretroviral treatments
(289). Otherwise, the patients are at a higher risk for cryptococcal IRIS. Treatment of
IRIS includes corticosteroids or dexamethasone for severe cases (289). The infections
driven by the rim101Δ mutant share a number of similarities to cases of IRIS, potentially
allowing us to use it as a model for IRIS. Identification of the exact host-pathogen
interactions could allow for directed therapies, such as an anti-TLR2 agent, to prevent
inflammatory responses to the remaining cryptococcal cells. This targeted approach may
allow for earlier treatment of AIDS patients with antiretrovirals and would prevent the
side effects of using systemic corticosteroids.

7.2.4. Identification of additional Pka1 targets

The pka1Δ mutant has a more severe capsule defect than the rim101Δ mutant,
in addition to defects in melanin and mating (81). Additionally, RNA sequencing showed
that a greater number of genes were dependent on Pka1 than on Rim101. Therefore,
there are likely a number of other transcription factors that are regulated by Pka1
phosphorylation. At the time of our initial screen, the incomplete annotation of the
genome meant that we only identified 34 genes as likely candidates. Using the new
genome sequence and more current annotation, I identified 252 potential transcriptional
regulators with at least one putative PKA phosphorylation consensus motif. These are
intriguing targets for future analysis, and one of these may serve as a promising drug
target.
References


86. **De Jesus, M., S.-K. Chow, R. J. B. Cordero, S. Frases, and A. Casadevall.** 2010. Galactoxylomannans from *Cryptococcus neoformans* varieties *neoformans* and *grubii* are structurally and antigenically variable. Eukaryotic Cell 9:1018-1028.

100. **Erickson, T., L. Liu, A. Gueyikian, X. Zhu, J. Gibbons, and P. R. Williamson.** 2001. Multiple virulence factors of *Cryptococcus neoformans* are dependent on VPH1. Molecular Microbiology 42:1121-1131.


susceptibility to pulmonary cryptococcosis. European Journal of Immunology 33:1744-1753.


Biography

Teresa Rodgers O’Meara was born in Seoul, South Korea on January 27th, 1986. She received an AB with Honors in Biology from the University of Chicago. In August, 2007, she began her graduate work in the University Program in Genetics and Genomics at Duke University where she joined the Alspaugh lab.

In the Alspaugh lab, Teresa was the first author of the publications “Cryptococcus neoformans Rim101 is associated with cell wall remodeling and evasion of host immune responses” in mBio, “The Cryptococcus neoformans capsule: a sword and a shield” in Clinical Microbiology Reviews, “Interaction of Cryptococcus neoformans Rim101 and protein kinase A regulates capsule” in PLoS Pathogens, and “Cryptococcus neoformans histone acetyltransferase Gcn5 regulates fungal adaptation to the host” in Eukaryotic Cell. She also collaborated on the publication “Cryptococcal titan cell formation is regulated by G-protein signaling in response to multiple stimuli” in Eukaryotic Cell.

Teresa received two years of research funding from the American Heart Association in 2009, and was supported in her travels to conferences and the 2010 Woods Hole course in Molecular Mycology by the DeLill Nasser Award for Professional Development (2011), the Chairman’s Travel Award (2010), and the DUMRU Meritorious Research Travel Award (2009). At these conferences, she received the Eukaryotic Cell Outstanding Young Investigator Award (2008) and a Keystone symposium Poster Book Award (2012).