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UNIVERSITY OF CALIFORNIA,
IRVINE

Expanding the *Candida albicans* response to acidic pH

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Sciences

by

Ohimai Kennedy Unoje

Dissertation Committee:
Professor Haoping Liu, Chair
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2017

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DEDICATION

To

My family, both immediate and extended, who always inspired and motivated me to achieve my best and never settle for anything less.

To my Lord and Savior Jesus Christ who called me on this journey, sustained me through the ups and downs, and gives me inspiration for what is to come.

"Those who trust in the LORD will renew their strength; they will soar on wings like eagles; the will run and not grow weary; they will walk and not faint"
Isaiah 40:31

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ABSTRACT OF THE DISSERTATION

Expanding the *Candida albicans* response to acidic pH

By

Ohimai Unoje

Doctor of Philosophy in Biomedical Sciences

University of California, Irvine, 2017

Professor Haoping Liu, Chair

The aim of the studies presented in this dissertation is to further our knowledge on the pathways responsible for responding to the effects of external pH in *Candida albicans.* For the majority of this work, we focused on identifying novel genetic pathways responsible for acidic pH repression of the initiation of hyphal morphogenesis, and provided some preliminary studies on pH effects on the *C. albicans* cell wall.

Chapter 1 presents an introductory overview on the current knowledge of hyphal growth, and how it is regulated by a variety of environmental conditions encountered by the fungus. In Chapter 2, we showed that translation is important for effective hyphal growth, and inhibition of translation genetically or chemically delays hyphal initiation.

The study in Chapter 3 identified several genes that are responsible for acidic pH repression of hyphal initiation. We focused on the Hog1 stress response pathway and identified Hog1 activity as a requirement for suppressing hyphal initiation in acidic pH and in the presence of stress. We observed that acidic pH (~4) regulates Hog1 by stabilizing Hog1 phosphorylation, different from stress stimulation of Hog1, by suppressing the expression of the tyrosine phosphatase ptp3.

Furthermore, stresses that activate Hog1 also reveal an impaired ability to initiate hyphal growth, supporting the role of Hog1 in repressing hyphal initiation. This study also identified Sfl1 as a key hyphal repressor that functions down-stream of the Hog1 pathway. The Hog1-Sfl1 pathway is activated in response to stress parallel to the Nrg1 repressor. Overall, we have identified a new genetic interaction and regulation that's necessary for hyphal regulation.

In Chapter 4, we present preliminary data showing that β-Glucans are exposed in acidic pH but live cells are able to protect themselves from that exposure. We have experimental results that exclude the Hog1 and the Cek1MAPK pathways in mediating this protection. Therefore, we propose a genetic screen to identify mutants that are defective in their protection of β-Glucans

CHAPTER 1

INTRODUCTION

Medical Relevance: Why study *Candida Albicans?*

The human body is a complex network of relationships interacting to ensure the general good and survival of the human itself, from the smallest molecular interactions to the human-human connections we make. These relationships also occur between the human body and the trillions of unseen microorganisms that live within our bodies, with some beneficial to our survival, some harmful, and some commensal. The fungus *Candida albicans* is one of the trillions of organisms that are housed within the human body from our birth to death(F. C. Odds 1988). Within a healthy human host, *C. albicans* colonizes its niches without presenting any infections or symptoms. However, when the host protective mechanisms are compromised, *C. albicans* colonization can rapidly expand resulting in superficial or a potentially fatal systemic infection. *Candida* species are the most common cause for hospital-related fungal infections, estimated at 8-10% of all hospital related bloodstream infections, with *C. albicans* leading the way(Montagna et al. 2014; Yapar 2014), and systemic *Candida* infections present a mortality of 30-40% of cases (Gudlaugsson et al. 2003).

The success of *C. albicans* as a human-specific pathogen is in no small part due to its ability to colonize a wide variety of niches within the host, including the skin, mouth, internal organs, gastrointestinal tract, the urinary tract, and the colorectal cavity (Kumamoto 2008). Each different site presents a unique and dynamic combination of environmental conditions for the fungus to adapt to and thrive in. *C. albicans* must therefore actively detect the environmental changes within its niche and mount an appropriate response to that environment, resulting in its

survival and success (Dalle et al. 2010; Miramón, Kasper, and Hube 2013). From the host perspective, the body has a multitude of mechanisms that can detect a variety of fungal signals and adequately respond to ensure the destruction of the pathogen and the survival of the host(Duggan et al. 2015). Our understanding of *C. albicans* biology indicates that several of the conditions found in the body are often stressful for the fungus, limiting its virulence and promoting commensalism in the host(Miramón, Kasper, and Hube 2013). In addition, host phagocytic cells can engulf and destroy fungal cells they interact with. Therefore, survival depends on the ability of both the host and the pathogen to enact an appropriate response to each other. Overall, the ultimate outcome is a peaceful coexistence where the fungus is allowed to live within the host without any trouble, resulting in a commensal relationship between host and fungus(Prieto et al. 2016). In certain conditions, however, where the host's protective mechanisms are compromised, the risk for *C. albicans* infection is increased(Mavor, Thewes, and Hube 2005). For example, individuals with a compromised immune system or damage to the epithelial barriers often present increased frequency and mortality of *C. albicans* infections. Therefore, an understanding of the different mechanisms that influence this host-pathogen interaction in different conditions is vital to addressing the issue of candida related infection. Increasing the knowledge of how these mechanisms work will contribute to the identification of newer therapeutic approaches. One of the most well characterized virulence factors is *C. albicans* yeast to hyphal transition (Gow et al. 2012) and, in this chapter, we will review several environmental conditions that regulate hyphal growth and the pathways through which they enact these regulation.

Yeast to hyphal transition in *C. albicans*

C. albicans has been shown to undergo a very dynamic morphological transition within the mammalian host. Studies in *C. albicans* virulence have characterized the morphological switch between round yeast form and filamentous hyphae form as a critical virulence factor(Gow et al. 2012). Traditionally, the yeast forms were considered as a commensal morphology and the filamentous forms considered the pathogenic morphology. However, later studies in candidiasis models disproved this notion as *Candida* infected tissue samples presented a combination of yeast and hyphal morphology(Chin et al. 2014). Moreover, certain strains that are locked in both yeast and hyphal morphology showed reduced virulence(Braun 1997; Lo et al. 1997). Taken together, the revised model suggests that it is *C. albicans'* ability to reversibly switch between morphologies that is the virulence factor, rather the presence of one particular morphology. Hyphal cells appear as segments of elongated cells attached end to end. Upon stimulation of hyphal growth, the cells switch from axial round growth to polarized growth, resulting in the germination of a tube-like structure called the germtube which become hyphae as a result of multiple rounds of nuclear and cell division without cytokinesis. Hyphal morphogenesis is sensitive to the cell cycle status of the cell and cell cycle mutants often present a deregulation of hyphal growth; however these two processes are regulated independent of each other to contribute to the formation of a mature hyphal morphology(Sudbery 2011).

In addition to the morphological changes during hyphal growth, a change in the transcriptional profile of *C. albicans* also occurs during this process(D. Kadosh and Johnson 2005). Of important notice is that induction into hyphal conditions (37°C + 10% Serum) results in the down-regulation of *NRG1,* a key hyphal repressor which results in the up-regulation of the 2 transcription factors *UME6* and *BRG1*, which are repressed by Nrg1. Activation of this pathway spurs the hyphal transcriptome and promotes the expression of Hyphal Specific Genes (HSGs),

such as GPI-anchored Hyphal Wall Protein 1 (*HWP1),* a class of Secreted Aspartic Proteases (SAPs), the cell wall adhesion Agglutin-Like Sequence 3 (*ALS3),* and the cell elongation gene *(ECE1).* The hyphal specific expression of these genes has rendered their transcription as valuable reporters for hyphal growth in *C. albicans* genetic studies. While morphogenesis and transcription have been shown to occur simultaneously during hyphal growth, newer evidence suggests that these processes are regulated independently to contribute to hyphal formation. For example, during hyphal initiation, the G1-cyclin gene *HGC1* is up regulated during hyphal growth in a Tup1-Nrg1 dependent manner and is required for the formation of mature hyphae but not for the expression of hyphal genes (Zheng, Wang, and Wang 2004).The *C. albicans* yeast-hyphal transition is also a cellular response to a variety of environmental cues, particularly those found in the host. *In-vitro* studies have shown hyphal formation is facilitated by growth in the presence of a temperature switch to 37°C(Shapiro et al. 2009), neutral pH(D. Davis, Wilson, and Mitchell 2000), 5% CO_2(Mock, Pollack, and Hashimoto 1990), hypoxia(Lu et al. 2013), N-Acetyl-D-Glucosamine (GlcNAc)(Mattia et al. 1982), and serum(TASCHDJIAN, BURCHALL, and KOZINN 1960). Conversely, hyphal formation is inhibited by growth in acidic pH condition(Buffo, Herman, and Soll 1984) and in the presence of secreted quorum sensing molecules such as Farnesol and Dodecanol (Mitchell and Soll 1979; Hall et al. 2011). Hyphal formation has also been shown to occur upon macrophage phagocytosis, a process that is important for survival and escape from within the macrophages as mutants that are unable to filament are easily killed after phagocytosis(Lo et al. 1997).

The dynamic range of hyphal stimuli within the host suggests the presence of a very robust hyphal regulatory mechanism within the fungal biology. Therefore, the ability to monitor both morphological changes and transcriptional changes within hyphal growth simultaneously

provides a clearer understanding of how these processes are co-regulated and where these regulations are distinct, providing a greater understanding of hyphal formation in general.

Environmental Regulation of Hyphal growth.

Temperature: It has been well characterized that hyphal growth requires the environmental temperature at 37°C, reflecting the adaptation to physiological conditions, and the heat shock protein Hsp90 has emerged as a central player in *C. albicans* temperature regulation of filamentation (Shapiro et al. 2009). In reduced temperature (30°C), the heat shock molecular chaperone Hsp90 is active and carries out its repressive function. Upon switching the temperature from 30°C to 37°C, a strong hyphal inducing condition, its repressive function is compromised and results in activation of hyphal growth. Hyphal growth in temperature requires the transcription factor Hms1(Shapiro et al. 2012), which is defective only in temperature induction but shows no phenotype in other hyphal inducing conditions at 30°C. In addition to Hms1, several pathways mutants have been identified that mediate hyphal initiation after Hsp90 inactivation/compromise since these mutants can prevent hyphal formation when Hsp90 in inhibited via the Hsp90 inhibitor Geldanamycin. These pathways include, but are not limited to, the cAMP/PKA pathway, iron homeostasis pathway, amino acid sensing Stp2 pathway, and the Cph2-Tec1 hyphal transcription pathway(Shapiro et al. 2012; Lane et al. 2015). Activation of the PKA has been shown to represses the transcription of *NRG1* gene, supporting hyphal formation. Furthermore, the transcription factors Efg1 and Flo8, which are believed to function downstream of the cAMP/PKA pathway, are required for the down-regulation of Nrg1 during hyphal induction (Lu et al. 2011). Temperature also appears to regulate hyphal maintenance since cells that were grown to initiate hyphae at 37°C and then switched to 30°C soon revert back to yeast

growth, but addition of the Hsp90 inhibitor Geldanamycin helped to stabilize the hyphal form(Lane et al. 2015). Hyphal maintenance from Hsp90 inhibition requires the transcription factor Cph2, which binds and promotes expression of Hms1, since their mutants can prevent the maintenance of hyphal growth (Lane et al. 2015).

Quorom Sensing Molecules: When cells are grown to saturation, the Quorum sensing molecule Farnesol is secreted by *C. albicans,* and is a potent inhibitor of filamentous growth(Hornby et al. 2001; B. Enjalbert and Whiteway 2005). Farnesol in saturated cultures promotes the stability of the transcriptional repressor Cup9, resulting in repression of the kinase gene *SOK1* (Lu et al. 2014). Upon release from Farnesol inhibition, when the cells are shifted to fresh medium, the absence of Farnesol results in the degradation of Cup9 via the E3 ligase Ubr1. Loss of Cup1 de-represses expression of the kinase gene *SOK1,* which is essential for the protein degradation of Nrg1. Deletion of *NRG1* in a *sok1* mutant background completely restores its ability to filament, confirming that the main function of Sok1 is to degrade Nrg1. In addition to transcriptional down-regulation of *NRG1*, Nrg1 protein levels are also down-regulated during hyphal initiation, as the cells are released from the effects of the secreted quorum sensing molecule Farnesol when shifted to fresh medium. It was previously reported that Farnesol functions via the PKA pathway(Davis-Hanna et al. 2007; Hall et al. 2011; Lindsay et al. 2012), however this regulation is negligible for Nrg1 protein down-regulation as the *cyr1* and *tpk2* mutants fail to prevent Nrg1 protein degradation l, and Farnesol fails to prevent *NRG1* transcriptional down-regulation. Farnesol has also been shown to regulate the various MAP kinase pathways in *C. albicans.* The Hog1 stress response kinase and the Mkc1 cell wall integrity kinase are inactivated and the Cek1 filamentation kinase is activated when removed from farnesol inhibition upon switching to fresh

media(Román et al. 2009; Smith et al. 2004), however how the influence of these regulations on hyphal initiation is unknown.

Oxygen and Hypoxia: The aerobic condition used for hyphal growth also influences the mechanism of hyphal growth. Hyphal induction in physiological concentrations of CO_2 activates the adenyl cyclase Cyr1 and activates the transcription factor Flo8 to promote hyphal growth (Klengel et al. 2005; Du et al. 2012) . On the other hand, CO_2 signaling, in hypoxic conditions, was also shown to repress hyphal growth via the AGC kinase Sch9(Stichternoth et al. 2011). In addition to CO_2, O_2 levels also regulate hyphal growth (Lu et al. 2013). In hypoxic conditions with 5% CO_2, which is similar to *in-vivo* conditions, hyphal growth is maintained by the stabilization of Ume6 protein. In aerated condition, the oxygen sensor Ofd1senses O_2 and promotes the degradation of Ume6. In the absence of O_2, Ofd1 is inactivated and increases the levels of cellular Ume6. Ume6 can bind to its own promoter to increase expression and also repress *NRG1* transcription, maintaining hyphal growth. In contrast, hyphal initiation in air recruits the deacetylase Hda1 to the promoters of hyphal gene to remodel the chromatin region around those promoters and prevent Nrg1 re-association and repression of hyphal genes, including *UME6*.

Environmental pH: The host site presents a range of pH from the acidic stomach to the alkaline GI tract and *C. albicans* has adapted to respond and thrive in these diverse conditions with hyphal growth is permitted by neutral-alkaline pH but restricted at acidic pH(Buffo, Herman, and Soll 1984). *C. albicans* response to neutral-alkaline pH is primarily regulated by the Rim101 pathway(D. Davis, Wilson, and Mitchell 2000; D. A. Davis 2009). A change in the external pH to neutral-alkaline is hypothesized to be sensed by the membrane proteins Rim21 and Dfg16, which promotes the formation of a protein complex that includes the calpain-like protease Rim13

and Rim20. Rim20 binds with the C-terminal domain of Rim101, brings it in contact with Rim13, and the C-terminal is cleaved off and results in a truncated Rim101 that localizes into the nucleus to promote pH dependent responses, including transcription of hyphal genes. As such, mutants of *rim101* and its upstream members show reduced filamentation in neutral-alkaline pH, and their defects can be rescued by ectopic expression of a truncated Rim101 fragment. Rim101 is also hypothesized to be a repressor in acidic pH where it undergoes a different processing event; however the function of this processing has yet to be confirmed (M. Li et al. 2004).

In addition to Rim101, the cAMP signaling pathway has been shown to regulate the pseudohyphal-to-hyphal transition in acidic pH supplemented with GlcNAc(Hollomon et al., n.d.). In *C. albicans* yeast cells, Ras undergoes a cleavage event at its Asn212 residue, deleting a 76 residue fragment from its C-terminus and reducing Ras1 activity(Piispanen et al. 2013). This process is repressed by the Adenyl cyclase, Cyr1, although the responsible protease is still unidentified. Upon hyphal growth, Ras1 cleavage in inhibited and Ras1 activity is increased as a consequence of increased cellular cAMP, therefore Ras1 cleavage can be indicative of cAMP levels and Cyr1 activity. Hyphal induction in acidic pH results in reduced cAMP levels, as reported by increased Ras1 cleavage, resulting in pseudohyphal growth rather than mature hyphae, and reduced expression of hyphal genes(Hollomon et al., n.d.).

N-Acetyl-D-Glucosamine (GlcNAc): GlcNAc has been shown to be a potent inducer of hyphal formation for decades, but it is not until recently that mechanisms of regulations were identified(Mattia et al. 1982). GlcNAc in the environment is transported into the cells via the membrane transporter Ngt1(Alvarez and Konopka 2007), and intracellular GlcNAc is sensed by the GCN5-related N-Acetyltransferase Ngs1 upon binding to the protein's N-terminal domain (Su, Lu, and Liu 2016). Hyphal formation and expression of hyphal genes with GlcNAc requires

Ngs1 sensing the presence of GlcNAc to stimulate downstream functions as the *ngs1* mutant is unable to form hyphae and express hyphal genes in GlcNAc media but is able to in the presence of serum. Studies into the mechanism of hyphal growth with GlcNAc showed, using a mutant that binds but doesn't metabolize GlcNAc, that its binding and signal transduction induced morphological changes without inducing expression of hyphal genes (Naseem, Araya, and Konopka 2015). Restoring GlcNAc metabolism restored hyphal gene expression, showing that these two processes can be regulated independent of one another. It is proposed that hyphal gene expression after GlcNAc metabolism is dependent on cAMP/PKA pathway, as *cyr1* and *efg1* mutants of the cAMP/PKA pathway fail to induce hyphae or hyphal gene expression in the presence of GlcNAc and morphogenesis occurs via GlcNAc induction of the hyphal cyclin Hgc1.

Amino Acids: *In-vitro* studies to assess amino acids effect of hyphal growth are carried out in acidic medium and supplemented with amino acids to evaluate hyphal formation. Two particular models have been put forward to explain how amino acids induce hyphal growth in the macrophages. In one case, extracellular amino acids are sensed by the SPS sensor complex, consisting of an amino acid transporter Ssy1, a membrane associated protein Ptr3, and the endoprotease Ssy5, and are taken up as a carbon source in the absence of preferred carbon sources, such as glucose(Miramón and Lorenz 2016). Sensing of amino acids by Ssy1 promotes recruitment of Casein Kinase I to the complex to phosphorylate an inhibitory domain on Ssy5, activating it. A key target of Ssy5 is the transcription factor Stp2, which regulates the expression of amino acid uptake genes. Activation of Stp2 results in its translocation to the nucleus to initiate the expression of the ATO class of acetate and ammonia transporters(Miramón and Lorenz 2016). A byproduct of amino acid metabolism is ammonia, which is secreted outside of the cell by the activities of ATO transporters(Vylkova et al. 2011). A consequence of ammonia

secretion is the alkalinization of the acidic environment, making it favorable for hyphal growth. As such, ATO mutants that are deficient in ammonia secretion, and are unable to alkalinize the medium, fail to induce hyphal growth(Danhof and Lorenz 2015). In parallel, amino acid catabolism also generates carboxylic acid to be used as a carbon source in the absence of glucose. Subsequent studies also showed that carboxylic acid metabolism also alkalinizes the medium in an ammonia- and Stp2-independent process, with the alkalizing agent in place of ammonia yet to be identified(Danhof et al. 2016). In the second model, Arginine metabolism within the macrophage generates urea which is degraded, releasing CO_2 as a byproduct which can serve as a hyphal inducer *in-vivo*. CO_2 can function as a signaling molecule as activates adenyl cyclase Cyr1 and its down-stream transcription factor Efg1 to stimulate hyphal growth(Ghosh et al. 2009).

Conclusion: *C. albicans'* ability to sense and respond to the host environment employs a remarkably dynamic system, often interweaving different pathways to adapt to multiple signals, which result in the regulation of its dimorphic growth between yeast and hyphae. Continued studies on the fungus, and its related species, will contribute to our understanding and targeting the pathogenic activities of this fungus.

CHAPTER 2

Translation is essential for hyphal initiation

Introduction

Candida albicans is a commensal fungus found on epithelial surfaces in the human body like the oral and vaginal tissues, the gut, the skin, and, in some cases, blood(F. Odds 1988). While considered a part of the normal host microbiota, it can readily infect tissues in certain conditions, a trait that has led it to be considered an opportunistic pathogen. *Candida* is the 4th leading cause of hospital-related infections in the United States, accounting for about 8% to 10% of cases. Among *Candida* species, *C. albicans* accounts for about 50% of cases studied(Montagna et al. 2014; Yapar 2014). Within healthy individuals, it is a harmless organism and is suppressed by the immune system but in certain conditions, *C. albicans* can disseminate, colonize, and infect its various niches(Kirkpatrick 1994).

The human body presents a variety of stresses for *C. albicans* that the fungus has to respond to in order to survive in the body. Some of these are active stresses, such as immune cells that target and engulf foreign bodies. Upon engulfing by immune cells, *C. albicans* cells are exposed to a combination of cationic, oxidative, and acidic stresses within the macrophages, ultimately resulting in death of the fungal cells (Miramón, Kasper, and Hube 2013). In response to these stresses, *C. albicans* has developed several adaptive mechanisms, among which is the activation of stress response pathways that ensure the survival of the fungus in this stressful environment(A. J. P. Brown et al. 2014; Polke, Hube, and Jacobsen 2015).

One of the most well studied stress response system in *C. albicans* is the Hog1 pathway. Identified in a screen for genes that rescue osmosentivity in *Saccharomyces Cerevisiae*, the *ca*Hog1 (High Osmlolarity Glycerol) kinase is responsible for the response to osmotic, heavy

metal, and to a lesser degree, oxidative stress in *C. albicans* (Brice Enjalbert et al. 2006). Upon stimulation by external stress, Hog1 is activated via a phosphorylation cascade that results in its nuclear translocation, allowing it to activate the transcriptional expression of stress response genes, such as glycerol biosynthesis genes like *GPD1* and *GPP2* (San José et al. 1996; Rebeca Alonso-Monge et al. 2003). In *S. cerevisiae,* Hog1 is also important for the recovery of translation after down-regulation by osmotic shock (Warringer et al. 2010). A Similar observation was made in *S. pombe*, where the translation elongation factor 2, eEF2 in *S. pombe* and EF-2 in *S. cerevisiae,* are phosphorylated in a Hog1-dependent manner to repress translation in response to osmotic stress(Teige et al. 2001; Asp, Nilsson, and Sunnerhagen 2008).

In addition to a dynamic stress response, the ability of *C. albicans* to switch reversibly between a round yeast form and a filamentous hyphal form has contributed to its survival within its human host (Soll 2002). Generally, the growth of hyphae can be mechanistically separated into two temporally linked steps: initiation of hyphal growth and maintenance of elongation (Lu et al. 2011). The initiation step involves the transient down-regulation of the transcriptional repressor Nrg1while maintenance involves the stabilization of the hyphal machinery, both leading to the expression of hyphal genes and to sustain polarized growth. Removal of Nrg1 repression makes the promoter regions of Hyphal Specific Genes (HSGs) more accessible to transcription the transcription factors that promote hyphal growth. Furthermore, mutants that are unable to down-regulate Nrg1 do not activate the hyphal transcription machinery, resulting in a defect in hyphal formation (Lu et al. 2014). Therefore, the hyphal transcription machinery appears to be an important step in the initiation of hyphal growth. However, recent studies have identified important transcription-independent mechanisms involved in hyphal formation. For example, N-Acetyl-Glucosamine, a potent inducer of hyphal growth, can induce hyphal growth

in a mutant that fails to up-regulate transcription of hyphal genes; meanwhile, in a control WT strain, hyphal transcription occurs concurrently with hyphal formation (Naseem, Araya, and Konopka 2015).

The yeast-to-hyphae morphological transition is heavily influenced by environmental conditions found in the host niches. Several biological conditions, like temperature at 37°C and neutral-alkaline pH, promote filamentation; while quorum sensing molecules, like farnesol and acidic pH, inhibit filamentation (Sudbery 2011). It is well documented that when *C. albicans* are engulfed by macrophages, they respond by switching to the hyphal morphology(Jiménez-López and Lorenz 2013). However, the effect of several of these macrophage stresses, such as osmotic and oxidative stresses, on hyphal growth has been a relative mystery.

In this study, several cellular functions that are important for the initiation of hyphal growth were identified. It also revealed that stresses negatively inhibit the hyphal initiation process by inhibiting translation. Finally, this inhibition of hyphal initiation functions independent of the characterized hyphal transcriptional machinery. Together, this sheds new light on how *Candida albicans* responds to stress.

Results

Identification of processes involved in hyphal initiation: Studies in hyphal development generally observe long-term hyphal growth (≥3 hours in liquid medium or several days on solid medium), without distinguishing between early phase initiation and long term maintenance. To identify genes that are important for hyphal initiation, the Gene Replacement And Conditional Expression (GRACE) collection was utilized to screen for mutants that are defective in initiating hyphal growth. The collection consists of 2,356 heterozygotes mutants with one copy of each gene deleted and the remaining copy under a *TET* repressible promoter (Roemer et al. 2003). In

the presence of doxycycline, the *TET* promoter is turned off to prevent expression of the target gene and the mutant strain is observed for a phenotype. For this study, the mutant strains were grown in hyphal inducing conditions with/without 50μg/mL Doxycycline for 1 hour. The1 hour timing is essential to identify phenotypes that are specific to hyphal initiation before the cells switch to hyphal maintenance. The screen was performed in a 96-well plate format with a matching control without doxycycline to mimic a WT phenotype. In addition, each experimental (+Dox) and control (-Dox) plates contained an SC5314 WT control strain to ensure no off-target affects of Doxycycline. Mutants that were defective in the absence of Doxycycline were excluded from further analysis since we were only interested in DOX-dependent phenotypes. This screen identified 80 genes that were defective in hyphal initiation (Fig 1 and Table 1). These genes were mapped into different cellular processes using the Candida Genome Database GO Slim Mapper.

Among the different processes that were hyphal defective, we observed an enrichment of genes involved in cytoskeletal organization, specifically, genes that encode for subunits of the ARP2/3 complex to be defective in hyphal formation. Included in this list are *ARC15* (orf19.6151), *ARC18* (orf19.121), *ARC35* (orf19.2437), and *ARP3* (orf19.2289). The ARP2/3 complex is essential for remodeling the actin macromolecules along the cytoskeleton of the cell to initiate and has been shown to be important for sustaining polarized growth during hyphal initiation (Epp et al. 2010). Therefore our results serve to confirm the pre-existing data, which in turn validates the result from this screen.

A collection of genes involved in vesicle-mediated transport was also observed. Among these genes are orf19.1672, the *C. albicans* homolog of the *S. cerevisiae COP1* gene; orf19.4382, the *C. albicans* homolog of the *S. cerevisiae RET3;* and *CHC1* (orf19.3496) the clathrin heavy

chain protein. Other secretory-related genes were identified in this group: *SPC3* (orf19.4930) that cleaves the signal peptide on secreted proteins and *TIP1* (orf19.3951) a membrane protein involved in COPII vesicle biogenesis. Several genes within the ARP2/3 complex were also in this group as vesicular transport requires remodeling of the cytoskeleton to accommodate fusion and exiting the membrane.

Finally, the highest enrichment (~40%) of genes involved in protein synthesis, namely ribosomal biogenesis and translation, with 33 unique genes belonging to these groups out of 80 total genes defective in hyphal initiation were seen. This suggested to us that hyphal initiation is strongly influenced by the process of protein synthesis. Also observed were: 1) Several genes that were a part of Small Subunit (SSU) processosome, a 2.2 MDa ribonucleoprotein complex involved in the processing of the small subunit of the eukaryotic ribosome, such as *UTP 4* (orf19.1633), *UTP18* (orf19.7154), *SIK1* (orf19.7569), *NOP5* (orf19.1199). 2) Genes involved in nuclear transport, a process important for the maturation of ribosomes and transport of ribosomal RNA (rRNA) to the cytoplasm for translation into functional ribosomal subunits, such as *NOG2* (orf19.5732), *NIP7* (orf19.3478), *MEX67* (orf19.488), *GSP1* (orf19.5493). 3) Genes encoding ribosomal proteins to be important for hyphal initiation, such as *RPL3* (orf19.1601), *UBI3* (orf19.3087), *RPS3* (orf19.6312). In addition to ribosome biosynthesis genes, several translation factors were identified such as *RLI1* (orf19.3034), a Fe-S protein required for translation initiation and termination, translation initiation factors such as *FUN12* (orf19.5081) the translation initiation factor subunit eIF5b, *RPG1* (orf19.6345) the translation initiation factor subunit eIF3a, *SUI2* (orf19.6213) and *SUI3* (orf19.7161) the translation initiation factors eIF2a and eIF2b, respectively. Finally, genes encoding amino-acyl tRNA synthetases were represented in the results. Among them were *ALA1* (orf19.5746) for Alaninyl-tRNA, orf19.4931

for the putative Cysteinyl-tRNA, *CDC60* (orf19.2560) for Leucyl-tRNA, and *VAS1* (orf19.1295) for Valyl-tRNA. Overall, translation and its related genes appear to be important during the initiation step of hyphal growth.

Characterizing the role of translation during hyphal initiation: The enrichment of translation-related genes resulted in the examining the role of translation in hyphal initiation. To query this further, a *rio2* deletion mutant strain was utilized because, in *S. cerevisiae, RIO2* encodes a protein kinase involved in the processing of the 20S pre-RNA into the mature 18S rRNA during ribosome biogenesis (Geerlings et al. 2003). In *C. albicans,* loss of *RIO2* has been shown to reduce filamentation compared to a WT strain after a 1-hour hyphal induction (Blankenship et al. 2010). When inoculated into hyphal inducing conditions, the *rio2* mutant presented a delay in germ tube formation during the first hour (Fig 2). At 40 minutes after induction, there were noticeably less germ tubes in the mutant than in the WT strain. However, after 4 hours, the levels of filamentation in the *rio2* mutant had recovered to levels comparable to the WT strain.

An important aspect of hyphal growth is the activation of the hyphal transcriptional machinery, starting with down-regulating the transcriptional repressor Nrg1 via down-regulation of *NRG1* transcription or degradation of Nrg1 protein. This delay was independent of hyphal transcription as down-regulation of Nrg1 protein (Fig 3A) and *NRG1* transcription (Fig 3B), and up-regulation of hyphal genes such as *HWP1* and *UME6* were unaffected by loss of *rio2* (Fig 3C).

Ribosomal biogenesis is an early step of the translational response in the cell, but not the only step. To understand if other steps of translation have the same effect on hyphal growth, hyphal initiation was observed in the presence of Cycloheximide, a chemical inhibitor of the

elongation step of protein synthesis. Wild type cells were inoculated into media supplemented with 300ug/mL Cycloheximide and hyphal growth was observed over a 3-hour time period. In accordance with the *rio2* phenotype, addition of Cycloheximide delayed the initiation of hyphal growth (Fig 4), and the delay in hyphal initiation is independent of the hyphal transcription mechanism as Nrg1 down-regulation and hyphal genes transcription was unaffected by the addition of Cycloheximide (Fig 5 A, B, C). These results, in combination with the variety of genes observed from the screen, suggested that translation is important for efficient hyphal initiation, and inhibition of any step of translation impairs the cells' ability to initiate hyphal formation.

Discussion

The process of hyphal growth is important for *C. albicans'* survival and infection in the host as cells defective in this process are more susceptible to killing and show reduced virulence (Lo et al. 1997; Sudbery 2011). On the other hand, host conditions present a variety of stresses, including osmotic stress, that suppress hyphal growth (Gow et al. 2012). Ultimately, the survival of the host depends on the outcome of this struggle. The transition from yeast to hyphae involves significant change in *C. albicans'* cell state and involves expression of a new set of genes and generation of a new sets of proteins, some of which are specific to each cell state (Martin et al. 2013; Patricia L Carlisle and Kadosh 2013; Choi et al. 2003). Our observation that inhibition of translation results in a delay of hyphal growth suggests that a certain amount of translation is important for efficient hyphal initiation. In support of this, it has been shown that translation is increased within the first hour of hyphal initiation in the presence of N-Acetyl-Glucosamine and returns to baseline level after 2 hours (Torosantucci et al. 1984). Therefore, it is not surprising that genes involved in ribosomal biogenesis and translation were strongly enriched in our screen.

There are several possible reasons why translation is so important for the early steps of hyphal formation. Considering that the morphological switch involves hyphal-specific proteins that need to be synthesized during the hyphal growth, inhibition of translation might result in a delay or prevention of the biogenesis of these proteins, resulting in the delay. Also, translational regulation is linked to several cellular decisions such as growth rate, cell-cycle regulation (Pyronnet and Sonenberg 2001), and metabolic shift between nutrient sources (Kief and Warner 1981), among many other signals. These signals are important for hyphal formation, and could be affected individually or collectively upon inhibition of translation, resulting in a delay of hyphal growth. Further studies are required to truly understand this process.

We also identified that these stresses regulate hyphal initiation independent of the hyphal transcription machinery. Studies have showed that an important part of initiation is the down-regulation of the transcriptional repressor Nrg1 and the subsequent expression of hyphal genes, such as *HWP1, ECE1,* and *UME6* (Lu et al. 2011). Inability to down-regulate Nrg1 or ectopic over-expression of *NRG1* keeps the cells locked in yeast phase and prevents initiation of hyphal formation. So we find it interesting that osmotic and oxidative stresses and inhibition of translation have a negative effect on initiation without regulating this pathway. Other groups have identified regulation of hyphal growth independent of the hyphal transcription machinery. N-Acetyl-Glucosamine, a potent inducer of hyphal formation, has been shown to promote hyphal growth independent of the transcription mechanism (Naseem, Araya, and Konopka 2015). A *hxk1 nag1 dac1* triple mutant was able to grow mature hyphae upon addition of GlcNAc without expressing hyphal genes. Our observations add growing support for transcription-independent regulation of hyphal initiation.

Materials and Methods

Media and Growth Conditions. *C. albicans* strains were grown in Yeast Extract Peptone (2% Bacto Peptone, 1% yeast extract, 0.015% L-Tryptophan,) with 2% Dextrose as a carbon source (YPD) at 30°C to saturation (OD600 =10-12, ~ 17hrs). To induce hyphae, the saturated cultures were inoculated 1:50 into YPD media pre-warmed to 37°C. To determine the effects of stress, the pre-warmed medium was supplemented with either 0.3M NaCl, or 0.3mg/mL Cycloheximide (Sigma). Aliquots of growing cultures were collected at each time-point, washed with room temperature Phosphate Buffered Saline (PBS) solution, and re-suspended in PBS + 4% Formaldehyde to fix the cells before viewing on a microscope.

Strains: Kinase mutants (*rio2* and *hog1)* were obtained from the kinase mutants collection from the Fungal Genome Stock Center created by Blankenship JR, et al(Blankenship et al. 2010). To create the Nrg1-Myc tagged *rio2* strain, primers 1 and 2 from Yang Lu, et al(Lu et al. 2011) containing the C-terminal NRG1 coding region was inserted into the BamHI-MluI sites of plasmid pPR671 from Fang Cao, et al (Cao et al. 2006). The resulting plasmid was digested with Sac1 to target integration into its own locus to express Nrg1-13Myc.

Cell extraction and Immunoblotting: Cells containing a copy of *NRG1-13xMYC* were grown in yeast phase and at different times in hyphal inducing conditions, harvested by centrifugation, and washed twice in chilled PBS. They were re-suspended in lysis buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 0.1% NP-40) with glass beads and vigorously vortexed with a Fast-Prep system (FP120; Thermo Electron, Waltham, MA) for four 20-sec intervals with cooling on ice for 5 minutes between. The crude lysate was centrifuged to separate the lysate from debris, normalized, resolved by SDS-PAGE on an 8% gel, and transferred to a Nitrocellulose membrane. Nrg1 levels were determined by probing the membrane with a HRP-conjugated

mouse monoclonal antibody (Roche) against the c-Myc epitope. A control blot for PSTAIRE was done using a rabbit polyclonal primary antibody (Roche) and a goat anti-rabbit HRP-conjugated secondary antibody (BioRad).

Quantitative RT-PCR: RNA was extracted from yeast and hyphal cells using the Qiagen RNease Kit, and 2ug was reverse transcribed into cDNA using the BioRad iScript Reverse Transcription Kit. Quantitative PCR was performed on the BioRad iCycler using a BioRad SYBR Green Reaction Mix and the corresponding primers. Cycle parameter was 95°C for 1 minute, 39 cycles of 95°C for 10 seconds, 56°C for 45 seconds, and 68°C for 20 seconds.

Primers used for qRT-PCR are as follows:

ACT1 (F): 5' – TGGTGATGGTGTTACTCACG

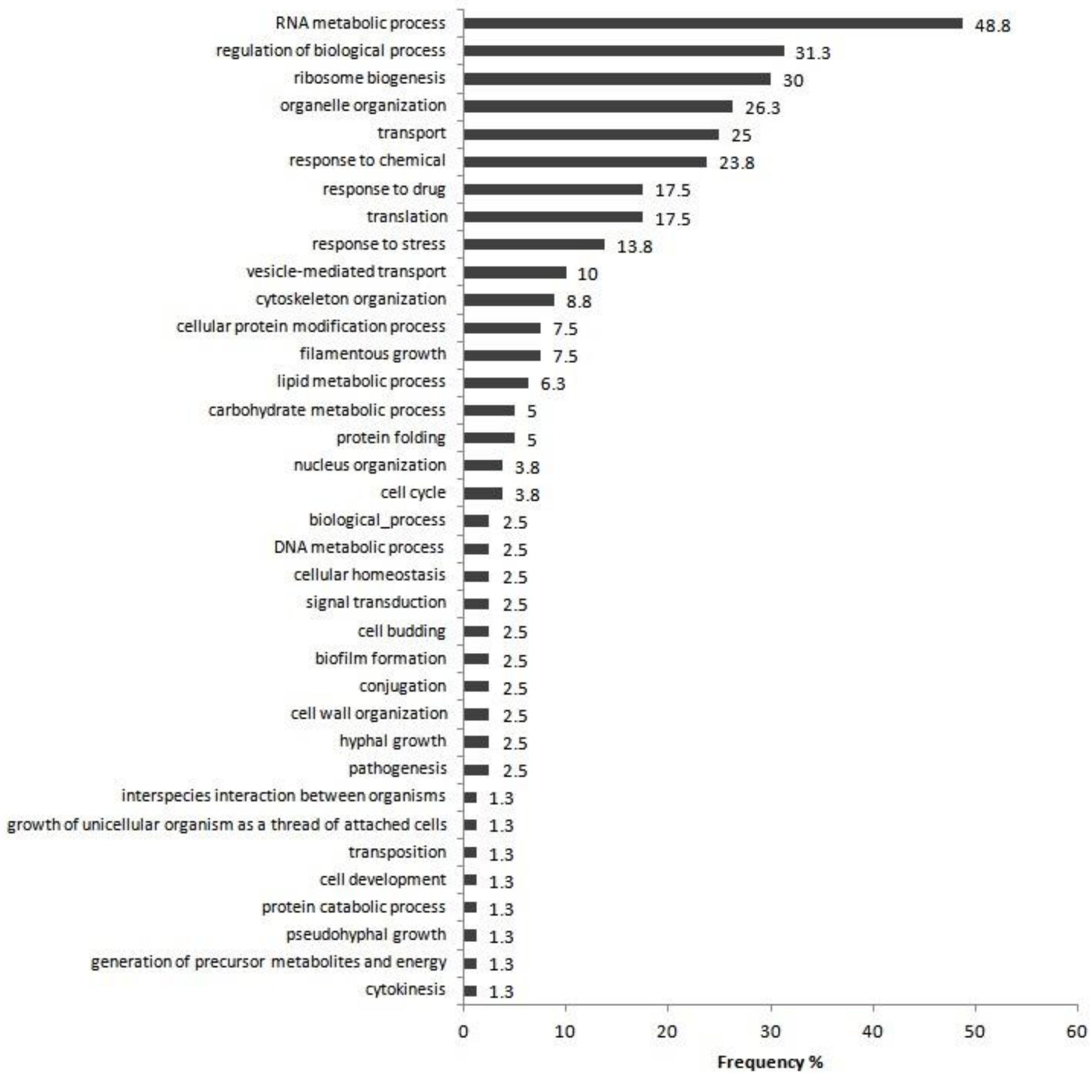
ACT1 (R): 5' – GACAATTTCTCTTTCAGCAC

NRG1 (F): 5' – GAATTCAAACCATCAACCAA

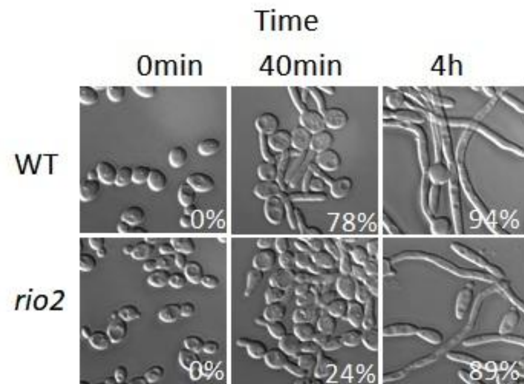
NRG1 (R): 5' – TGATTGTTGTGACAATGGAG

HWP1 (F): 5' – CCAGTTACTTCTGGATCATC

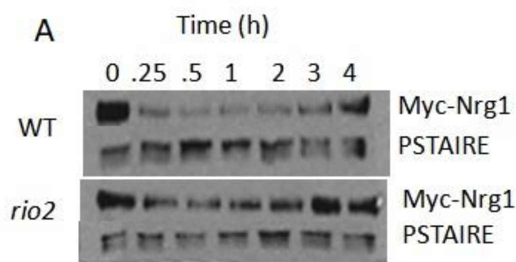
HWP1 (R): 5' - TCGGTACAAACACTGTTAGA



<u>Fig 1</u>. Cellular functions important for hyphal initiation. Functional analysis of mutants identified as important for hyphal initiation by Candida Genome Database GO Mapper program.



<u>Fig 2.</u> Deletion of *RIO2* gene delays hyphal initiation. (A) Morphology of WT and *rio2* mutants 0, 40 min, and 4h after hyphal induction. Overnight culture of WT was inoculated into fresh media pre-warmed to 37°C and collected and visualized after the indicated time. % of filamentous cells indicated in bottom right corner



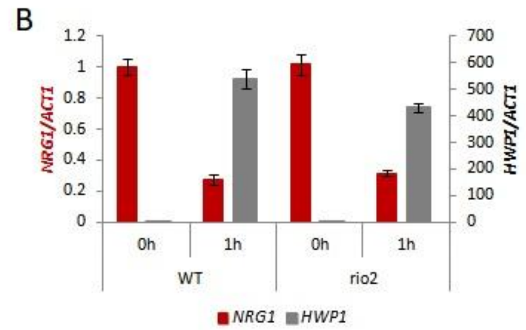


Fig 3. Hyphal delay in *rio2* mutant doesn't affect hyphal transcription. Nrg1 level is monitored by Western blot of WT and *rio2* mutant containing a copy of 13xMyc-Tagged Nrg1 after 0, 15min, 30min, 1h, 2h, 3h and 4h hyphal induction. PSTAIRE was used as a loading control (C) Expression of *HWP1* and *UME6* monitored by qRT-PCR in WT and *rio2* mutant cells from before (0h) and after (1h) hyphal induction. Levels of mRNA were normalized relative to *ACT1*

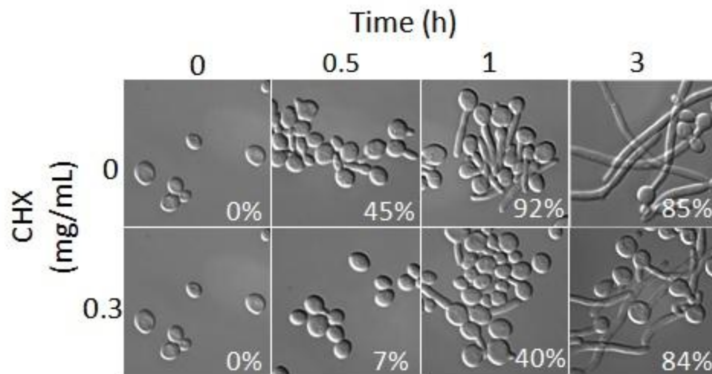


Fig 4. Cycloheximide stress delays hyphal initiation. (A) Morphology of WT cells contain grown in the absence and presence of 0.3mg/mL Cycloheximide (CHX) for up to 3h and visualized at the indicated time points. % of filamentous cells indicated in bottom right corner

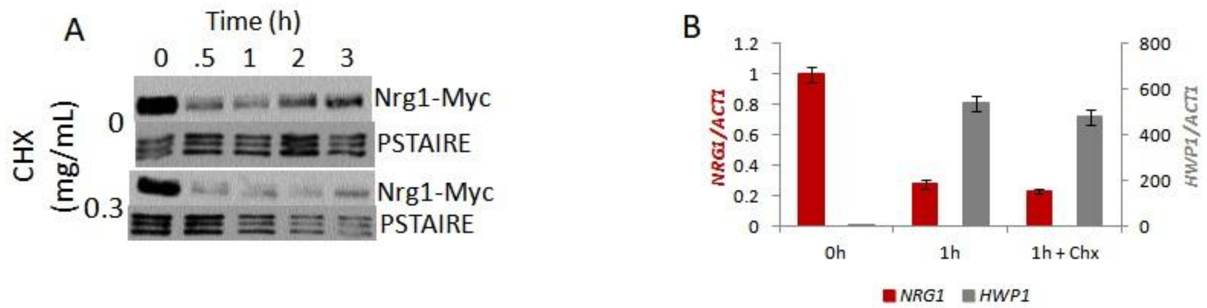


Fig 5. Hyphal delay in Cycloheximide doesn't affect hyphal transcription. A) Nrg1 level is monitored by Western blot of WT cells containing a copy of 13xMyc-Tagged Nrg1 after inoculation into YPD+/-CHX for 0h, 0.5h, 1h, 2h, and 3h. B) Expression of *HWP1* and *UME6* monitored by qRT-PCR in WT cells before (0h) and after (1h) inoculation into YPD+/-CHX. Levels of mRNA were normalized relative to *ACT1*.

CHAPTER 2

Acidic pH and other stresses inhibit hyphal initiation via Hog1-Sfl1

Introduction

Candida albicans is a commensal fungus that is part of the normal human microbiota. It can readily cause infections when fungal growth is unrestricted within hosts that have a compromised immune system, microbial imbalance, or damaged epithelial lining (Gow et al. 2012). *Candida* infections can be superficial on the skin and mucosal surfaces or systemic when the fungus disseminates through the bloodstream and colonizes vital organs. While superficial infections are relatively harmless, systemic infections can be life-threatening with mortality rates up to 40%(Pfaller and Diekema 2007). The prevalence of *C. albicans* infections has resulted in the increased interest in virulence factors and potential targets for drug therapies.

Within the human host, *C. albicans* cells are exposed to a variety of conditions, among which is the varying pH conditions within the different niches occupied by the fungus. *C. albicans* can colonize the stomach (pH~2)(Zwolinska-Wcisło et al., n.d.), the vagina (pH4-pH5.5)(Valore et al. 2002), the mouth (pH 6-7)(Arab et al. 2016), and the intestines of the GI tract (pH 8). Environmental pH influences many of *C. albicans* key biological functions such as white-opaque switching(Sun et al. 2015), cell-wall structures(Sherrington et al. 2017), filamentation(Buffo, Herman, and Soll 1984; Kullas, Martin, and Davis 2007), and nutrient acquisition(Wang et al. 2011; Baek, Li, and Davis 2008), making pH an important environmental factor in *C. albicans* biology. The diverse range of host pH that is tolerable by *C. albicans* suggests the presence of a dynamic pH response system, and the most well studied pH response is the Rim101 pathway. Mutants lacking a functional Rim101 show a growth defect when the cells are grown in alkaline

pH(M. Li et al. 2004; Kullas, Li, and Davis 2004), and transcription of certain pH-dependent genes is mis-regulated(Bensen et al. 2004). When the cells are in neutral- alkaline pH, Rim101 is activated via proteolytic cleavage at its C-terminal from an 85kD protein to a 74kD truncated form(M. Li et al. 2004). In *C. albicans,* Rim101 is also truncated to a 65 kD protein in acidic pH, which is proposed to be an inhibitory processing but the function of this event is still unconfirmed and unclear(M. Li et al. 2004). Activation of Rim101 promotes the expression of genes that facilitate survival within the host such as the cell wall β-Glycosidase *PHR1*(D. Davis, Wilson, and Mitchell 2000) that is essential for morphogenesis and systemic infection(Saporito-Irwin et al. 1995; Ghannoum et al. 1995); an agglutinin-like adhesion protein Als3(Nobile et al. 2008) that binds to endothelial N-Cadherins to facilitate endocytosis(Phan et al. 2007); several iron acquisition genes(Bensen et al. 2004) to counteract the iron starvation conditions within the host. Overall, the role of the Rim101 pathway is critical to survival and responding to changes in the external pH. While the Rim101 pathway is the most widely studied pH response system in *C. albicans,* other Rim101-independent pathways also exist that contribute to ensure the diverse cellular response to pH such as the Ca^{2+} dependent Crz1 pathway(Kullas, Martin, and Davis 2007), the cAMP/PKA pathway(Hollomon et al., n.d.), and the Mds3 and TOR pathways(Zacchi, Gomez-Raja, and Davis 2010), and the Vacuolar H+ ATPase pump(Dechant and Peter 2010) which serves to maintain pH homeostasis by pumping protons from the cytosol into the vacuolar compartments to keep the cytoplasm neutral .

In addition to a dynamic stress response mechanism, *C. albicans'* ability to switch between a unicellular yeast from and a filamentous form is another trait that is essential to its survival within its human host (Jiménez-López and Lorenz 2013; Lu, Su, and Liu 2014). Strains locked in either yeast or hyphal form are less virulent compared to WT cells (Lu, Su, and Liu 2014;

Murad, Leng, et al. 2001; Lo et al. 1997). Hyphal development has been shown to facilitate escape from the macrophages, function as an important virulence trait(Lo et al. 1997; Marcil et al. 2002), and is strongly influenced by common host conditions such as temperature(Mitchell and Soll 1979), the presence of serum(TASCHDJIAN, BURCHALL, and KOZINN 1960), pH(Buffo, Herman, and Soll 1984), hypoxia and 5% CO_2(Mock, Pollack, and Hashimoto 1990; Klengel et al. 2005; Lu et al. 2013), N-Acetyl-D-Glucosamine(Torosantucci et al. 1984; Su, Lu, and Liu 2016). Mechanistically, the regulation of hyphal development has two phases: initiation and maintenance (Lu et al. 2011; Lu, Su, and Liu 2012). The initiation step occurs within the 1st hour of hyphal growth *in-vitro* and involves the transient down-regulation of the transcriptional repressor Nrg1. This occurs through transcriptional down-regulation, protein degradation, and chromatin dissociation of Nrg1 from the promoters of hyphal genes(Lu et al. 2011). External conditions have been shown to target Nrg1 down-regulation to regulate hyphal growth. For example, elevated temperature (37°C) promotes the transcriptional down-regulation of *NRG1* via the cAMP/PKA pathway(Lu et al. 2011), and introduction of the quorum sensing molecule Farnesol promotes Nrg1 protein stability via Cup9 repression of the kinase Sok1(Lu et al. 2014). The regulation of its chromatin dissociation during hyphal initiation is still unknown. This phase of hyphal initiation serves as a window of opportunity to ensure that Nrg1 repression is sufficiently removed so as to maintain stable expression of hyphal genes and prevent Nrg1re-repression as Nrg1 levels begin to recover. During that window, the cell activates mechanisms that keep Nrg1 from the promoters of hyphal genes makes these promoters readily accessible to transcription factors that maintain hyphal growth. The mechanism for hyphal maintenance is dependent on the environmental condition sensed by the fungus. Growth in hypoxic conditions combined with 5% CO_2 maintains hyphae by stabilizing the protein levels of Ume6, a

transcription factor important for hyphal maintenance (Lu et al. 2013). Abundance of Ume6 is able to overcome the presence of Nrg1 repression to promote hyphal growth and transcription of hyphal genes independent of Nrg1 levels (Banerjee et al. 2008; P. L. Carlisle et al. 2009). Alternatively, growth in nutrient poor conditions or in the presence of serum in normoxic conditions promotes the expression and binding of the transcription factor Brg1 to the promoters of hyphal genes. Brg1 then recruits Hda1 to deacetylate the Yng2 subunit of the NuA4 histone acetyltransferase and remodels the chromatin region around the hyphal gene promoters, preventing Nrg1 re-association with the promoters. Brg1 expression also requires inactivation of the kinase Hog1 since the TOR pathway inhibitor Rapamycin promotes the expression of the phosphatases *PTP2* and *PTP3* to inhibit Hog1 basal activity, promote Brg1 transcription, and maintain hyphal growth. While classically associated with stress response, the Hog1 pathway has also been classified as a hyphal repressor in *Candida albicans,* as mutants lacking the *HOG1* gene have a higher tendency to form hyphae in certain conditions than a WT strain (R Alonso-Monge et al. 1999; Rebeca Alonso-Monge et al. 2003; Arana et al. 2005). This implication of Hog1 in hyphal growth might suggest a link between stresses and hyphal growth as well. While Nrg1 is the most studied hyphal repressor, several other factors that inhibit hyphal initiation have been identified that play a role in hyphal formation. The general repressor Tup1 binds to several factors such as Nrg1(Braun, Kadosh, and Johnson 2001), Rfg1(D. Kadosh and Johnson 2001), and Mig1(Murad, d'Enfert, et al. 2001)to inhibits hyphal initiation(David Kadosh and Johnson 2005); Sfl1 is a hyphal repressor that targets several hyphal transcription factors to prevent hyphal formation(Y. Li et al. 2007; Znaidi et al. 2013).

In this study, we screened a kinase and transcription factor collection for genes that repress hyphal initiation in acidic pH, identifying 1 transcription factor and 9 kinase mutants that

filament in acidic pH. We show the Hog1 pathway and the Sfl1 transcriptional repressor as a new genetic interaction in *C. albicans* that repress hyphal initiation. We also find that this new pathway functions independently of Nrg1 and must be inactivated for effective hyphal growth. Together, we have expanded the pathways involved in hyphal growth and identified

RESULTS

Acidic pH inhibits hyphal initiation independent of Rim101 processing.

It is well known that hyphal formation is inhibited by acidic pH(D. A. Davis 2009), however not much is known about mechanism for inhibition. Studies into the pH response pathways have postulated that the processing of Rim101 is a key pH response mechanism that is important for hyphal growth (D. A. Davis 2009). Therefore, we looked into its role in acidic pH inhibition of hyphal initiation by observing hyphal growth in a WT and *rim101* mutant strains at pH4 and pH7 (Fig. 1). As expected, the *rim101* mutant showed a defect at pH7 compared to the WT strain, highlighting its importance for filamentation in neutral pH. Moreover, pH4 was inhibitory to filamentation in both strains with the *rim101* mutant showing further sensitivity at acidic pH compared to the WT, suggesting the importance of additional pathways in acidic pH. Rim101 is proposed to be inhibitory in acidic pH and a shift to neutral-alkaline pH activates its transcription factor function via Rim13-mediated cleavage of an inhibitory C-terminal domain(M. Li et al. 2004). To determine if the lack of filamentation at pH4 was dependent on Rim101 inactivation, the *rim101* mutant and a *rim13* mutant were complemented with a copy of *RIM101ΔC,* an active Rim101 allele, and was grown to induce filamentation at pH4 and pH7 (Fig. 1). Rim13 is required for pH-dependent processing of Rim101(M. Li et al. 2004) therefore successful filamentation at pH7 confirms that *RIM101ΔC* restores Rim101 activity. The introduction of a

functional Rim101ΔC rescued filamentation in both mutant strains at pH7 but not at pH4. While Rim101 processing is essential for hyphal formation at neutral pH, its processing is not the mechanism by which acidic pH inhibits hyphal initiation and there exists a Rim101-independent pathway important for hyphal initiation in acidic pH.

The Hog1 pathway is involved in acidic pH inhibition of hyphal initiation:

To identify suppressors of hyphal initiation in acidic pH, we screened through a mutant collection containing 80 homozygous protein kinase and protein kinase related gene deletion strains to find mutants that could filament in medium at pH4. From the screen, we identified 9 mutants that were unable to repress filamentation in pH4 (Fig 2, Table 1). A Gene-Ontology analysis of these mutants showed that 7 out of 9 genes were associated with responding to stress in *C. albicans*, so we focused our studies on the *hog1* and *pbs2* mutants, which are involved in *C. albicans* central stress response pathway. When the *hog1* mutant was used for hyphal growth at pH7 and pH4, initiation of hyphae in pH7 was comparable between the mutant and WT strains. However, at pH4, the *hog1* and *pbs2* mutants lost the repression at pH4 compared to the WT (Fig. 3).The *pbs2* mutant phenotype indicated that it is the phosphorylation of Hog1, not just the presence of the Hog1 protein that is essential to the acidic pH inhibition of hyphal initiation (Fig. 3). In addition to the morphological changes as the cells switch from yeast to hyphae, pH also inhibits hyphal gene transcription during hyphal initiation(D. Davis, Wilson, and Mitchell 2000). To check if the pH repression of hyphal gene transcription is also mediated by Hog1 pathway, the WT, *hog1* and *pbs2* strains were transformed with a *HWP1p-GFP* reporter to determine transcription from the *HWP1* promoter, a hyphal specific gene that is highly up-regulated in the yeast to hyphae transition. In the WT strain, *HWP1p-GFP* was expressed in pH7, but this was

blocked in pH4 (Fig. 3). However, in the *hog1* and *pbs2* mutants, expression of *HWP1p*-GFP in pH4 and pH7 were indistinguishable, confirming the loss of hyphal repression at acidic pH (Fig. 3).

The observed de-repression of hyphal growth in the *hog1* and *pbs2* mutant strains suggested that the presence of Hog1 phosphorylation is inhibitory to hyphal initiation. It is well studied that Hog1 is rapidly and transiently phosphorylated in a Pbs2-dependent manner when the cells are challenged with osmotic or oxidative stress(Smith et al. 2004), so we performed a hyphal initiation in medium supplemented with 0.5M NaCl or 5mM H_2O_2 and observed the effects of stress on hyphal initiation (Fig 4). In the presence of stress, germ tube formation in the WT strain was delayed compared to medium without NaCl or H_2O_2. Within the 1st hour, about 90% of WT cells in medium alone had formed observable germ tubes and expressed *HWP1*p-*GFP* meanwhile only 12% of cells in NaCl medium and less than 5% of cells in H_2O_2 medium had observable germ tubes. Hyphal gene expression was also reduced in both stress conditions, especially in H_2O_2. The hyphal defects in the presence of stress were slowly recovered over time as the presence of germ tubes/hyphae and increase in *HWP1p-GFP* was observed at later time points. In the *hog1* strain, the effect of stress was attenuated as more cells showed germ tube formation (33% in NaCl and 26% in H_2O_2), higher amount of GFP expression within the first hour, and a faster recovery compared to the WT strain. These results indicate that activation of Hog1 via stress does have a negative effect on hyphal initiation.

Acidic pH prevents loss of Hog1 phosphorylation

Since loss of Hog1 phosphorylation could rescue filamentation in acidic pH, we looked to identify the effects of pH on Hog1 phosphorylation. Knowing that *hog1* mutants show a growth

sensitivity to stress conditions that require Hog1 phosphorylation, so we first checked the growth sensitivity of the *hog1* mutants to acidic pH stress (Fig. 5). The *hog1* mutant showed a mild growth phenotype at pH4 with smaller colonies compared to the WT strain and growth at pH 7. When acidic pH was combined to 0.5M NaCl and 5mM H_2O_2, we observed increased growth defect with the *hog1* strain when compared to the stresses at pH7, illustrating a combinatorial stress effect on the *hog1* mutant but not the WT.

This growth phenotype implied to us that acidic pH might be a stress sensed by the Hog1 pathway, and could promote Hog1 phosphorylation. We hypothesized that acidic pH activates Hog1 to respond to the acidic pH stress. Hog1 phosphorylation is regulated via two distinct mechanisms: A) upstream activation via the MAPK phosphorylation cascade or B) downstream dephosphorylation through the activities of phosphatases. We excluded the first option as inoculation of log phase cells into YPD at pH4 and pH7 did not induce phosphorylation. In contrast, inoculation into 1M NaCl strongly induced phosphorylation. Hog1 phosphorylation was absent in the *pbs2* mutant even in the presence of NaCl (Fig 6). We then checked to see if acidic pH could prevent the de-phosphorylation of Hog1. Through a time course monitoring Hog1 phosphorylation, we observed that cells that shifted to acidic pH were slower to deplete Hog1 compared to neutral pH7 (Fig. 7), suggesting that pH regulates the stability of Hog1 phosphorylation. Since de-phosphorylation is regulated by phosphatases, we checked the transcription levels of the phosphatases and observed a pH-dependent transcription of *PTP3,* with its transcript elevated in neutral pH and repressed in acidic pH (Fig 8). To confirm the role of the phosphatases in hyphal initiation, a *ptp2ptp3* double mutant lacking both Tyrosine phosphatases was used to induce hyphal growth in YPD at pH7 or pH4 or supplemented with 0.5M NaCl (Fig. 9). In all conditions, the *ptp2ptp3* double mutant showed a weaker ability to

grow hyphae compared to the WT. This was particularly noted in 0.5M NaCl where hyphal growth was completely blocked in the *ptp2ptp3* double mutant rather than the delayed observed in the WT. We concluded that the severity of these phenotypes were a consequence of a sustained Hog1 phosphorylation. To confirm this, a *ptp2ptp3hog1* triple mutant was used to determine the role of Hog1 in this phenotype and we observed that the deletion of Hog1 in the phosphatase double mutant completely alleviated the phenotype (Fig 9), affirming the role of Hog1 phosphorylation in pH and stress inhibition of hyphal initiation. Taken together, we conclude that acidic pH and stress inhibit hyphal initiation via Hog1 phosphorylation.

Hog1 functions independent of Nrg1 down-regulation

After identifying Hog1 as a mechanism for pH regulation of hyphal initiation, we sought to identify potential transcriptional effectors that function downstream of Hog1. One key regulator of hyphal initiation is the transcriptional repressor Nrg1, whose down -regulation is essential to effective hyphal initiation. So, we checked if acidic pH and Hog1inhibit initiation by preventing Nrg1 down-regulation. To test this, the drug farnesol, which prevents Nrg1 down-regulation, was used to assess the *hog1* mutant's ability to bypass Nrg1 repression (Fig 10). In the presence of Farnesol, the *hog1* and *pbs2* mutants failed to initiate hyphal growth, suggesting that Nrg1 could either function down-stream of Hog1to block hyphal initiation or it functions independently of the Hog1 pathway. In parallel, a *nrg1* strain was grown to induce hyphal initiation in acidic pH and in the presence of NaCl to confirm the dependence of Nrg1 in this regulation. The *nrg1* mutant shows a wrinkled surface on solid medium and the liquid overnight culture showed that the strain consisted of a mass of yeast cells aggregated together. When induced to grow hyphae, the *nrg1* mutant was able to form hyphae in pH7, pH4, and in the presence of NaCl, indicating

that loss of Nrg1 repression is required for filamentation in these conditions (Fig 11). Next, we looked at the down-regulation of a Myc-tagged copy of Nrg1protein in WT and *hog1*strains grown at pH7, pH4, and NaCl (Fig 12). Every condition showed a comparable down-regulation of Nrg1 protein 0.5h after inoculation in the WT, showing that they did not affect hyphal initiation through Nrg1, and this was unaffected by the loss of Hog1. Interestingly, the recovery of Nrg1 after down-regulation in pH4 occurs much faster and this appears to be Hog1-dependent as recovery returns to normal when Hog1 is absent. In agreement with the Nrg1-myc western, we also observed that neither pH nor Hog1 influence *NRG1* transcriptional down-regulation (Fig 13A) and the rate of degradation of Nrg1 protein (Fig 13B). Therefore we conclude that pH and Hog1 regulate hyphal initiation distinct from the down-regulation of Nrg1. A potential mechanism of regulation is also the Nrg1 dissociation from the promoter of hyphal genes, which has been shown to occur during hyphal initiation(Lu et al. 2011). To determine if pH and stress regulate Nrg1 dissociation, a chromatin immunoprecipitation assay was carried out on cells containing a tagged copy of Nrg1 in pH7, pH4, and NaCl, but neither of these stress conditions prevented the removal of Nrg1 from the *HWP1* promoter (Fig 13C). Therefore, we concluded that acidic pH and stresses regulate hyphal initiation independent of Nrg1.

Sfl1 represses hyphal initiation down-stream of Hog1.

Upon excluding Nrg1, we sought to find other down-stream factors through which Hog1 and pH regulate hyphal initiation. We performed a genetic screen through a collection of 165 transcription regulator mutants for mutants that filament in acidic pH and observed that *sfl1* (orf19.454) mutant was able to bypass acidic pH inhibition of hyphal initiation (Fig. 14A). In confirmation that it functions independently of Nrg1, its ability to initiate hyphae was repressed in the presence of farnesol, just like the *hog1* mutant was (Fig. 14A). To determine the epistatic

relationship between Hog1 and Sfl1, we checked the ability of *sfl1* mutant to reverse the inhibitory effect of activated Hog1 using 0.5M NaCl and 5mM H_2O_2, and observed that the *sfl1* mutant was de-repressed in hyphal formation, similar to the *hog1* mutant (Fig 14B). This confirmed to us that Sfl1 functions genetically downstream of Hog1 in repressing hyphal initiation.

Hog1 and Sfl1 partially mediate V-ATPase effect of hyphal initiation:

An essential process involved in cellular pH response is the Vacuolar H+ ATPase pump. with V-ATPase mutants show a growth phenotype when grown in alkaline pH, are sensitive to oxidative stress, and have a defect in hyphal development(Rane et al. 2013; Zhang et al. 2017). Studies in *Saccharomyces cerevisiae* have revealed that external pH regulates V-ATPase, with acidic pH4 promoting dissociation of the pump's V0 and V1 subunits and resulting in reduced proton pump activity(Padilla-López and Pearce 2006; Diakov and Kane 2010). To see if Hog1-Sfl1 pathway mediates VATPase effects in hyphal initiation, we monitored the mutants' ability to rescue the effects of the V-ATPase inhibitor Concanamycin A. In a WT strain, hyphal morphogenesis and hyphal gene transcription were repressed by addition of Concanamycin A. This inhibition was abolished in the *hog1* and *sfl1* mutants as, within the first hour, both mutants showed small germ tube formation in the presence of Concanamycin A that was absent in the WT and both mutants showed increased expression of HWP1p-GFP (Fig 14C). That trend continued at 2.5h with almost all the *hog1* and *sfl1* mutants showing elongated filamentous growth and strong HWP1-GFP expression, while the WT only showed a portion of filamentous cells (30%-40%) and very weak HWP1-GFP expression (Fig. 2C). From these data, we concluded that acidic pH and some

of its related functions inhibit hyphal initiation partly via the Hog1 and this inhibition requires the hyphal repressor Sfl1.

Discussion:

The process of hyphal growth is important for *C. albicans'* survival and infection in the host as cells defective in this process are more susceptible to killing and show reduced virulence (21, 31). On the other hand, host conditions present a variety of stresses that affect hyphal growth(Sudbery 2011). While it is well known that acidic pH suppresses hyphal growth, no mechanisms have been described for the regulation of hyphal initiation. In this study, we have discovered that several genes that mediate the inhibition of hyphal initiation in acidic pH. Hyphal morphology and transcription of hyphal genes are blocked in acidic pH and can be completely rescued by inactivating the central Hog1 pathway modules. In our screen of mutants that could initiate filamentous growth in acidic pH, the enrichment of genes involved in stress response suggests the possibility that acidic pH can be detected as a stress for *C. albicans,* resulting in activation of the Hog1stress response pathway. The Hog1 pathway has been characterized as a negative repressor of hyphal growth(R Alonso-Monge et al. 1999); therefore acidic pH activation of Hog1 could promote its repressive functions and prevent the initiation of hyphal growth. Surprisingly, other stresses that have been shown to hyper-phosphorylate Hog1 such as NaCl and H_20_2 delayed, rather than inhibited, hyphal initiation. This could indicate that Hog1activity can vary based on the external signal sensed by the cell and function in different ways to regulate different aspects of *C. albicans* biology. Rapid hyper-phosphorylation is necessary for the expression of stress response genes when the cells are challenged with NaCl or H_20_2(Smith et al. 2004), but our data shows that these stresses enact a moderate effect on hyphal initiation,

especially when compared to acidic pH. On the contrary, acidic pH appears to stabilize the basal levels of Hog1 phosphorylation by regulating the expression of the phosphatases, and deletion of Hog1 can rescue the phenotypes observed in a *ptp2ptp3* strain. A study by Chang Su et al showed that rapamycin also targets basal Hog1 phosphorylation during hyphal maintenance by regulating the expression of Ptp2 and Ptp3 phosphatases(Su, Lu, and Liu 2013). Taken together, it would seem that the levels of basal Hog1 phosphorylation rather than the inducible hyper-phosphorylation caused by other stresses regulate hyphal growth. Most studies on Hog1 phosphorylation focus on the role of hyper-phosphorylation, usually using NaCl or other stress inducers to activate Hog1.We present further evidence that the basal phosphorylation of Hog1 is another means of regulation that is important for the hyphal development program, and more studies into the dynamics of Hog1 phosphorylation in different conditions should shed more light into this. In addition to the Hog1 pathway, we identified 7 other kinases that are able to filament in the presence of acidic pH. Exploring the role of those other kinases identified from the screen would grant a greater understanding on how acidic pH regulates these different pathways to ultimately inhibit hyphal initiation. Pathways such as the Ca^{2+}/Calmodulin pathway (*CMK1)* (Wang et al. 2011; Kullas, Martin, and Davis 2007) have been connected with pH response in *C. albicans.* Since several of these kinases are involved in the response to stress, it is of interest to us if these kinases all regulate the Hog1 pathway. For example, Hog1 activation is dependent on AMPK/Snf1 activity in response to metabolic stress(Adhikari and Cullen 2014), and Kis1 functions as β subunit of the Snf1 complex(Corvey et al. 2005). Some of the genes identified in our screen (*DUN1* and *MEC1)* are involved in cell cycle regulation, a process that is intimately linked to hyphal formation yet function independently(Berman 2006). Hog1 pathway also plays a role in cell cycle regulation as stress activation of Hog1 results in cell cycle arrest, but Hog1

also facilitates the resumption of growth after cell cycle arrest(Correia, Alonso-Monge, and Pla 2010; Correia, Alonso-Monge, and Pla 2017).

From this study, we also determined that Sfl1 functions as a transcriptional regulator downstream of Hog1 to regulate hyphal initiation. Sfl1 has originally been identified as a hyphal repressor in *C. albicans* that binds to several hyphal gene promoters(Y. Li et al. 2007; Znaidi et al. 2013) but this is the first time it has been implicated in a regulatory pathway in this fungus. Studies in *Saccharomyces cerevisiae* have showed ScSfl1promoter binding to be regulated by the cAMP/PKA pathway phosphorylation, resulting in its dissociation from promoters(Pan and Heitman 2002). However, several results from our study suggest that this regulation, if present in *C. albicans,* is negligible during hyphal initiation at acidic pH. First, stimulation of PKA by addition of db-cAMP failed to initiate or enhance hyphal growth in the WT or *hog1* strains, indicating that activation of PKA does not inactivate Sfl1 repression. Second, in YPD medium, deletion of *sfl1* failed to initiate hyphal growth at the hyphal repressive temperature of 30°C. The temperature requirement of 37°C functions through the cAMP/PKA pathway to down-regulate the transcription of *NRG1,* removing a major inhibitor of hyphal growth(Lu et al. 2011). These two conditions suggest to us that Sfl1 and PKA are distinct in their regulation of hyphal initiation. While the upstream regulation of Sfl1 is not well studied, there is evidence in that suggests that Sfl1 could also be down-stream of the Hog1 pathway. First, while *S. cerevisiae* has only Sfl1, *C. albicans* has 2 homologs, Sfl1 and Sfl2, which can functionally complement an *S. cerevisiae sfl1* mutant to repress filamentation. However, Sfl1 and Sfl2 function differently in *C. albicans* with Sfl1 a hyphal repressor and Sfl2 a hyphal activator. This functional divergence in *C. albicans* could be a consequence of upstream regulation. It has been documented in *C. albicans* that Sfl2 functions downstream of the PKA pathway to promote hyphal growth when

the cells are exposed to elevated CO_2 conditions(Tao et al. 2017). We posit that Sfl1, in *C. albicans,* functions as the inhibitory arm of this system and it is a downstream effector of the hyphal inhibitory Hog1 pathway. In support of this, it was recently reported that the Ser556 of Sfl1, in *Saccharomyces cerevisiae,* is a downstream target of Hog1 in response to stress(Romanov et al. 2017). A sequence analysis of *C. albicans* Sfl1 and Sfl2 proteins revealed that the phosphor-residue is conserved only Sfl1 (Thr602), but not in Sfl2, supporting the hypothesis that in *C. albicans* Sfl1 is a target of Hog1. Further studies will be needed to clarify how this phosphorylation event regulates hyphal initiation and transcription. Therefore, it will be of great interest to identify the mechanistic link between Hog1, Sfl1, and hyphal initiation in *C. albicans.*

A key question that is of great interest to us is the relationship between Hog1-Sfl1 and Nrg1 in this pathway. Our data suggests that the two inhibitory pathways are independent of one another, and both inhibitions must be removed to ensure filamentation. Previous work from our lab had identified several conditions that target Nrg1 to regulate hyphal initiation(Lu et al. 2011; Lu et al. 2014), and this current work indicates that Sfl1 is responsive to stresses to inhibit hyphal initiation. In this system, Nrg1 is active in low temperature and in the presence of quorum sending molecules such as farnesol while Sfl1 functions as a stress responsive repressor that only inhibits in the presence of stresses such as acidic pH. Therefore, in the normal hyphal inducing condition of dilution into fresh medium at 37°C, the removal from farnesol during dilution and the increase in temperature inactivates Nrg1, and the absence of stress ensures the absence of Sfl1 repression, therefore efficient hyphal growth occurs. In acidic pH, activation of Sfl1 is enough to inhibit hyphal initiation even though temperature and fresh medium ensure removal of Nrg1 repression. The converse occurs when hyphal induction is performed in fresh medium

containing farnesol, with Nrg1 repression active but Sfl1 inactive. Overall, our results indicate that the presence of either one of these repressors is enough to inhibit hyphal initiation, making both of them key repressors that must be removed to ensure normal hyphal formation. Additional experiments are currently being performed to identify the details of these two independent regulations. This discovery opens the possibility to identify new environmental conditions that regulate hyphal growth through Sfl1 and not Nrg1.

Methods and Materials

Plasmid and Strain construction:

The *C. albicans* strains used in this study are listed in Supplementary table. To generate the *HWP1p-GFP-SAT1* (plasmid #1254), primers HWP1p-GFP-NAT F/R was used to PCR amplify the genomic sequence 1Kb upstream of the *HWP1* transcription start site. The fragment was incorporated into the plasmid by Gibson Assembly(Gibson 2011). The resulting plasmid was linearized by digesting with AflII and integrated into the endogenous *HWP1* promoter in the different strains and successful transformants were selected on YPD + 200ug/mL nourseothricin. To generate the *RIM101p-RIM101ΔC,* primers Rim101p F/R were used to amplify the genomic sequence 1KB upstream of the *RIM101* start site and inserted between the NotI and XbaI sites in plasmid BES116. Then primers Rim101 F & Rim101ΔC R were used to clone the first 1.4KB fragment of the Rim101 coding sequence (excluding the C-terminal) into the MluI and KpnI sites of the plasmid. The resulting plasmid was transformed into the *rim101(ura-)* strains and selected on SC – Uridine plates.

Mutant collection screening: To identify mutants that could filament in acidic pH, the kinase mutant collection generated by Blankenship JR, et al (2010)(Blankenship et al. 2010) and the transcription regulator mutant collection generated by Homann OR, et al (2009)(Homann et al. 2009) used for the screen. Mutants were grown in 96-well plates overnight in YPD medium until saturation and inoculated 1:50 into 100uL fresh YPD at pH4 pre-warmed to 37°C for 90 minutes and viewed under a microscope to observe filamentation. Filamentous strains were confirmed first in 1mL in 24-well plates and then in 10mL in glass flasks shaken at 200RPM in at 37°C water bath. Mutants that were filamentous in overnight culture were excluded from further analysis.

Media and Growth conditions: *C. albicans* strains were grown in Yeast Extract Peptone (2% Bacto Peptone, 1% yeast extract, 0.015% L-Tryptophan,) with 2% Dextrose or Maltose (for Promoter shut down assay) as a carbon source at 30°C to saturation (OD600 =10-12, ~ 17hrs). To induce hyphae, the saturated cultures were inoculated 1:50 into YPD media pre-warmed to 37°C and supplemented with the necessary stressor (HCl to pH4, NaCl, H2O2, Farnesol, Concanamycin A). Aliquots of growing cultures were collected at the indicated time-point, washed with once with water before viewing DIC and FITC fluorescence on a microscope.

Growth Spot Test: Strains were grown at 30°C to saturation and a 5-fold serial dilution, starting with 10^3 cells, were spotted on to YPD agar set to pH7 and pH4 and supplemented with either 0.5M NaCl or 5mM H_2O_2. The plates were grown at 30°C for 36 hours and were imaged.

Lysate Extraction and Immunoblotting: For Phospho-Hog1 detection, cells were grown to saturation and inoculated 1:50 into fresh medium at pH7 or pH4. At each time point, aliquots were collected in a 50mL conical tube with ice and centrifuged for 3 minutes at 4°C, then the

supernatant was aspirated and the cell pellet were flash frozen in liquid nitrogen and stored until cell lysis. The pellets were lysed by re-suspending in kinase buffer (50mM Tris-HCl pH7.5, 150mM NaCl, 10% Glycerol, 1% Triton X-100, 0.1% SDS, 5mM EDTA, 50mM EGTA, 50mM Sodium Fluoride, 0.1mM Sodium Orthovanadate, 10mM Sodium Pyrophosphate, 1mM PMSF) with glass beads and vigorously smashed with a Fast-Prep system (FP120; Thermo Electron, Waltham, MA) for four 20-sec intervals with cooling on ice for 5 minutes between. The crude lysate was centrifuged to separate the lysate from debris, normalized, resolved by SDS-PAGE on an 8% gel, and transferred to a Nitrocellulose membrane. Phospho-Hog1 levels were determined by blocking with PBST with 5% BSA for 1hr and then probed with Anti Phospho-p38 antibody in PBST+BSA overnight at 4°C. The membranes were washed and then probed with an anti-Rabbit IgG secondary antibody in PBST + 3% Milk for 1hr. For total Hog1 levels, the membranes were blocked for 1hr in PBST + 3% milk for 1hr and probed with an anti-Myc primary antibody pre-conjugated with HRP for 1hr. A control blot for PSTAIRE was done using a rabbit polyclonal primary antibody (Roche) and a goat anti-rabbit HRP-conjugated secondary antibody (BioRad).The membranes were washed in PBST 3x for 10 minutes, soaked in a chemiluminescence mix, exposed to a film and developed.

Promoter shutdown assay: CAI4 cells containing a copy of *MAL2p-NRG1-13xMYC* were grown overnight in YEP + 2% Maltose to over-express Nrg1-Myc and then inoculated 1:50 in fresh YEP+ 2% Dextrose for 1hr to shut down the *MAL2* promoter and observe the degradation rate of Nrg1-Myc. Aliquots were collected at each time point and centrifuged at 3500 RPM, the supernatant was aspirated and the cell pellets were flash frozen in liquid nitrogen until cell lysis. The pellets were lysed by re-suspending in lysis buffer and vigorously smashed with glass beads in a Fast-Prep system (FP120; Thermo Electron, Waltham, MA) for four 20-sec intervals with

cooling on ice for 5 minutes between. The crude lysate was centrifuged to separate the lysate from debris, normalized, resolved by SDS-PAGE on an 8% gel, and transferred to a Nitrocellulose membrane. Nrg1 levels were determined by probing the membrane with a HRP-conjugated mouse monoclonal antibody (Roche) against the c-Myc epitope. A control blot for PSTAIRE was done using a rabbit polyclonal primary antibody (Roche) and a goat anti-rabbit HRP-conjugated secondary antibody (BioRad).

Quantitative RT-PCR: RNA was extracted from yeast and hyphal cells using the Qiagen RNeasy Kit, and 2ug was reverse transcribed into cDNA using the BioRad iScript Reverse Transcription Kit. Quantitative PCR was performed on the BioRad iCycler using a BioRad SYBR Green Reaction Mix and the corresponding primers. Cycle parameter was 95°C for 1 minute, 39 cycles of 95°C for 10 seconds, 56°C for 45 seconds, and 68°C for 20 seconds.

Primers used for qRT-PCR are as follows:

ACT1 qPCR(F): TGGTGATGGTGTTACTCACG

ACT1 qPCR (R): GACAATTTCTCTTTCAGCAC

NRG1 qPCR (F): GAATTCAAACCATCAACCAA

NRG1 qPCR (R): TGATTGTTGTGACAATGGAG

PTP3 qPCR (F): GATATTAGACCATCTGCTC

PTP3 qPCR (R): CAGCAAATCTATCTCTCTG

HWP1p-GFP-NAT (F):

GCCAGTGAATTGTAATACGACTCACTATAGGGCGAATTGCCTTACACGCACATAAAT
TGC

HWP1p-GFP-NAT R:

ACACCAGTGAATAATTCTTCACCTTTAGACATTTTAATAATTGACGAAACTAAAAGC

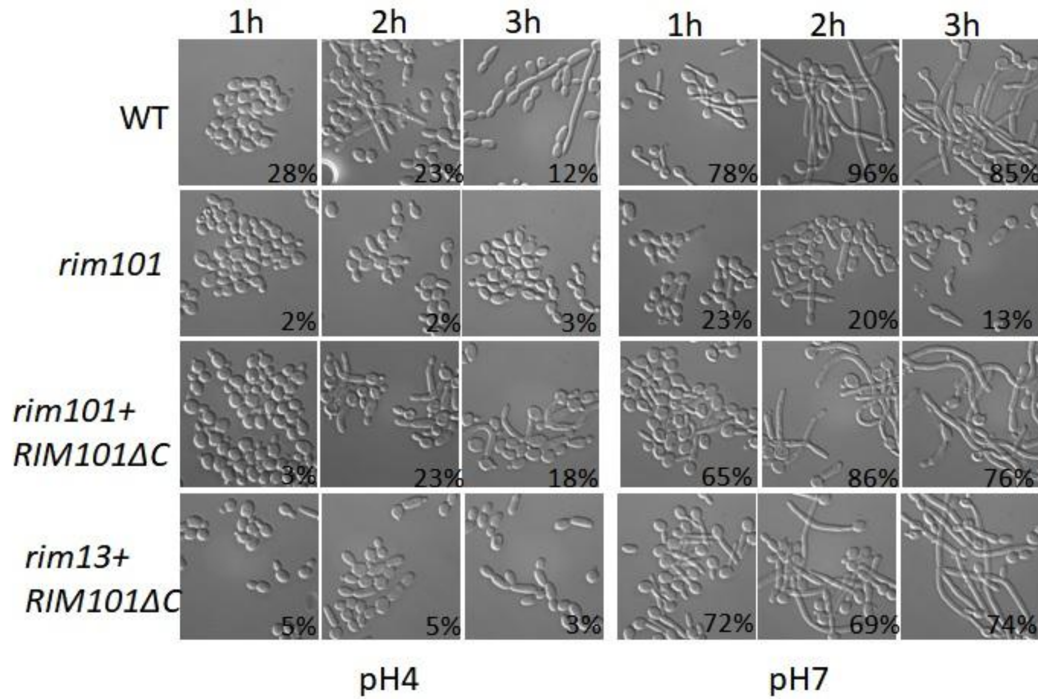
GAG

Rim101p F: TAAAGTTAGCGGCCGCTTACGCCAAAAGAACTCATCC

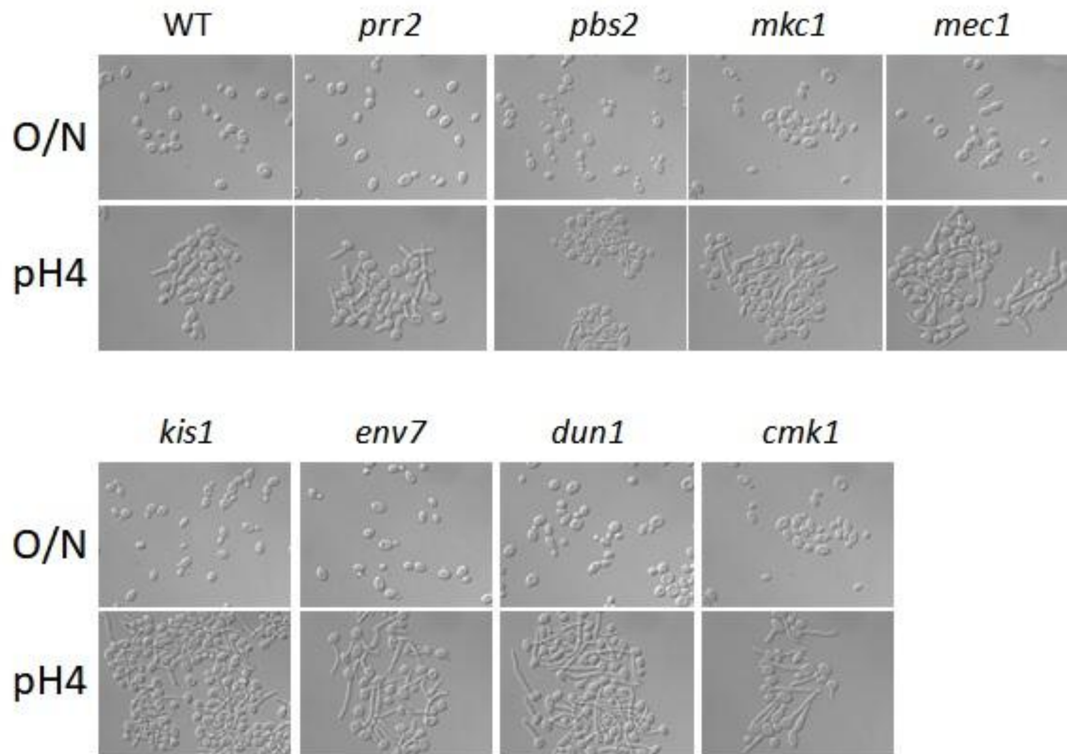
Rim101p R: GCTCTAGATGTCTAAAAATCTCGTTTGTCTAGG

Rim101 F: CGACGCGTCATGGTTCCAAGAAATCACCTT

Rim101ΔC R: TAGGTACCCCTAGGTTAGTCGATGGAATTCGATAAGG



<u>Fig 1:</u> Acidic pH inhibits hyphal initiation independent of Rim101 processing. A) WT SC5314, *rim101, rim101+RIM101ΔC*, *rim13+RIM101ΔC* cells were grown to induce hyphae in YPD medium pre-warmed to 37°C set at pH7 and pH4. Pictures of hyphae were taking at 1h, 2h, and 3h time points.



<u>Fig 2</u>. Morphology of kinase mutants that filament in acidic pH4. Morphology of overnight (ON) and hyphae after cells were inoculated for 90min into YPD medium at pH4 pre-warmed to 37°C.

orf19.895	***HOG1***	**MAP kinase of osmotic-, heavy metal-, and core stress response; role in regulation of glycerol, D-arabitol in response to stress; phosphorylated in response to H2O2 (Ssk1-dependent) or NaCl;**
orf19.7388	***PBS2***	**MAPK kinase (MAPKK); role in osmotic and oxidative stress responses, oxidative stress adaptation; required for stress regulation of Hog1p localization and activity;**
orf19.4084	*KIS1*	Snf1p complex scaffold protein; similar to S. cerevisiae Gal83p and Sip2p with regions of similarity to Sip1p (ASC and KIS domain);
orf19.4002	*DUN1*	Protein similar to S. cerevisiae Dun1p, which is a serine-threonine protein kinase involved in DNA damage cell-cycle checkpoint
orf19.1341	*PRR2*	Putative serine/threonine protein kinase;
orf19.7523	*MKC1*	MAP kinase; role in biofilm formation, contact-induced invasive filamentation, systemic virulence in mouse, cell wall structure/maintenance, caspofungin response;
orf19.1283	*MEC1*	Cell cycle checkpoint protein with a role in genome integrity;
orf19.5911	*CMK1*	Putative calcium/calmodulin-dependent protein kinase II;
orf19.7164	*ENV7*	Membrane-associated protein kinase localized in trans-Golgi network;

Table 1: Description of mutants that filament in acidic pH according to the *Candida Genome Database*.

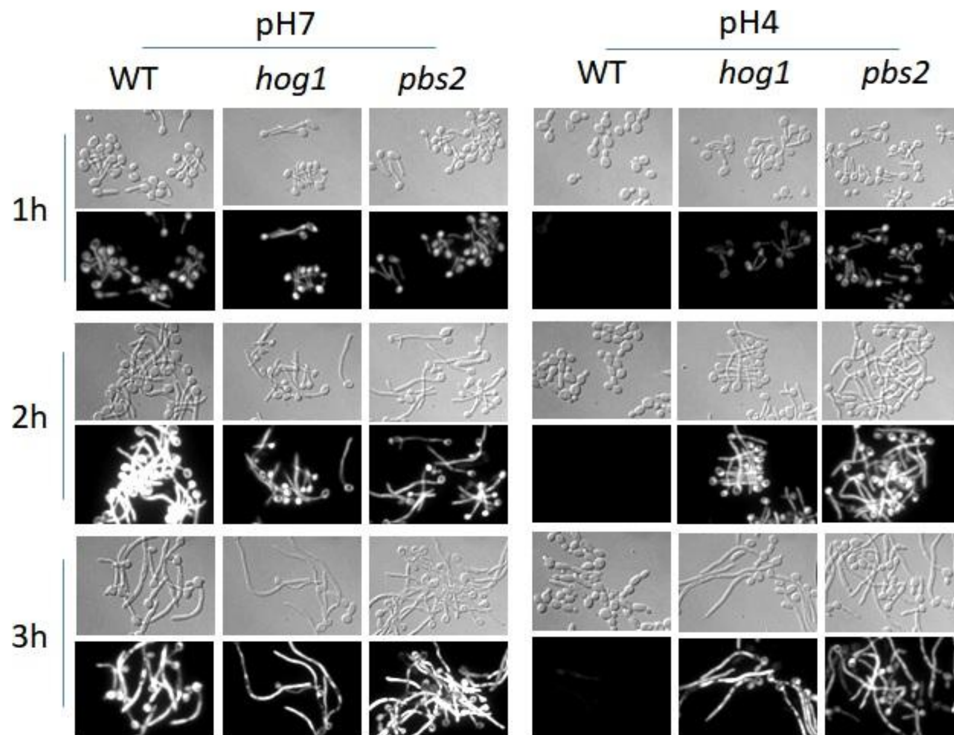


Fig 3: Loss of Hog1 phosphorylation promotes hyphal initiation in acidic pH. A) WT, *hog1,* and *pbs2* strains expressing a *HWP1p* driven GFP were hyphal induced in YPD set at pH4 and pH7. Pictures of hyphae and GFP fluorescence were taken at 1h, 2h, and 3h time points.

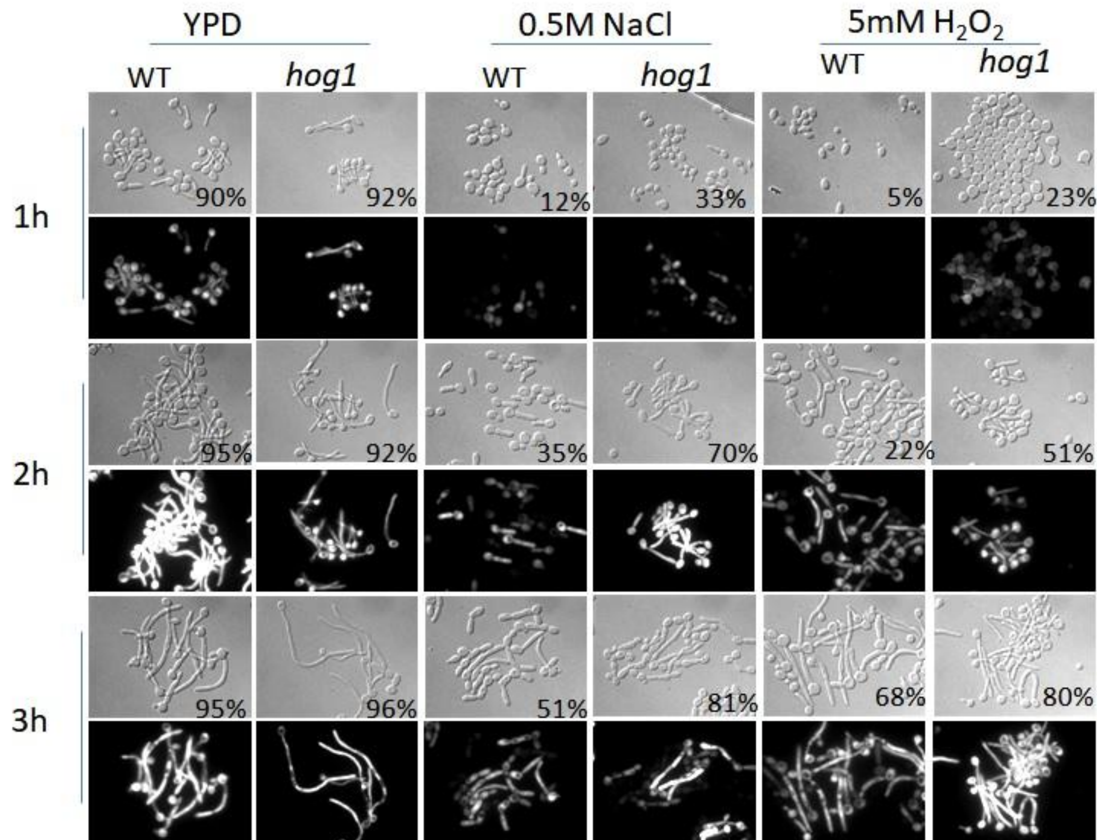
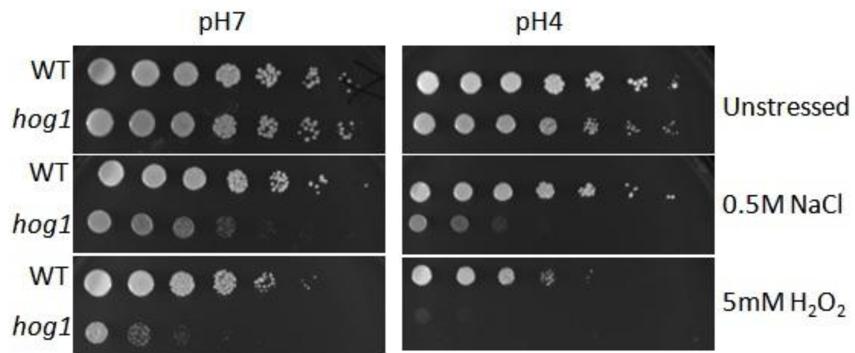


Fig 4: Hog1-activating stresses repress hyphal initiation. WT and *hog1* strains expressing a *HWP1p* driven GFP were hyphal induced in YPD supplemented with 0.5M NaCl and 5mM H_2O_2. Pictures of hyphae and GFP fluorescence were taken at 1h, 2h, and 3h time points.



<u>Fig 5:</u> *hog1* mutants show pH sensitivity. (A) Growth tests of WT and *hog1* cells on YPD plates for 36h at pH7 and pH4 with no stress, 0.5M NaCl or 5mM H_2O_2.

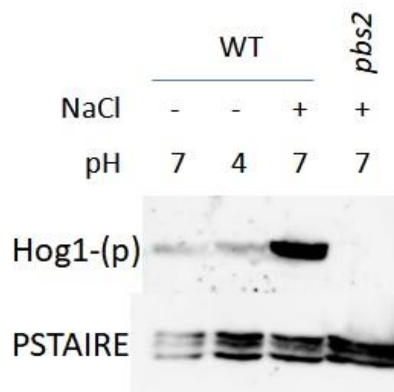
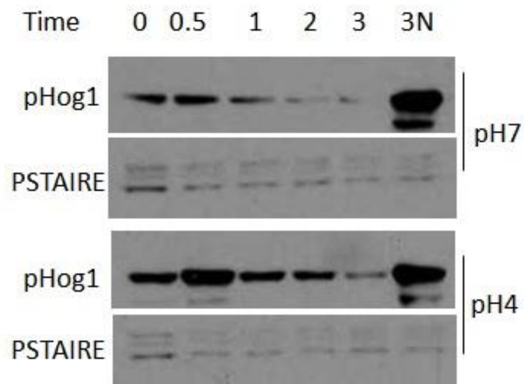


Fig 6: Acidic pH does not induce Hog1 phosphorylation. A Phospho-Hog1 immunoblot of logarithmic cells grown for 3h and inoculated into fresh YPD medium at pH4, pH7, and pH7 supplemented with 0.5M NaCl for 5 minutes. A *pbs2* mutant strain was induced in 0.5M NaCl as well as a negative control. A parallel blot was probed with anti-PSTAIRE as a loading control.



<u>Fig 7:</u> Acidic pH stabilizes Hog1 phosphorylation. A Phospho-Hog1 immunoblot of overnight cells (0h) inoculated into fresh YPD medium at pH4 and pH7 for 3h was carried out on aliquots collected every hour. At 3h, an aliquot of cells were shifted to medium with 1M NaCl (3+N) to induce Hog1 phosphorylation. A parallel blot was probed with anti-PSTAIRE as a loading control.

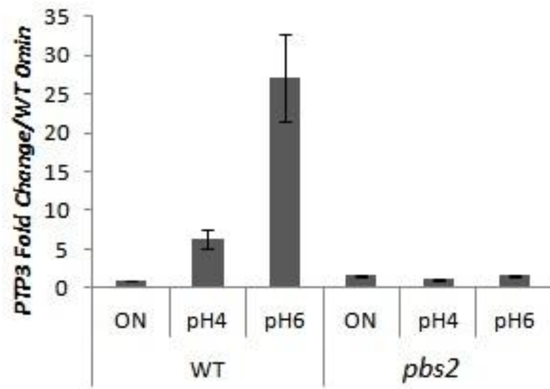
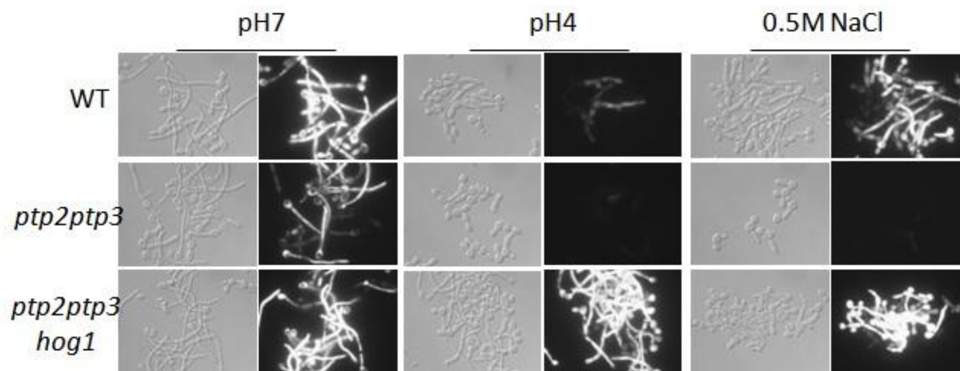


Fig 8: Acidic pH inhibits *PTP3* transcription. qRT-PCR of WT and *pbs2* cells to measure the levels of *PTP3* transcript after cells were grown in pH and pH7 for 15 minutes. qPCR values were normalized to *ACT1* for each samples, and overnight (ON) samples were set to a value of 1



<u>Fig 9:</u> Hog1 phosphorylation is repressive to hyphal growth. WT, *ptp2ptp3,* and *ptp2ptp3hog1* strains were used to induce hyphal growth in YPD medium at pH7, pH4, or supplemented with 0.5M NaCl. Pictures of morphology and GFP fluorescence were taken after 2h.

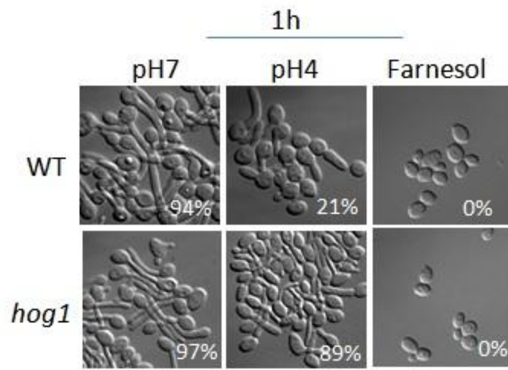


Fig 10: Farnesol inhibits *hog1* mutants filamentation. (A) Morphology of WT and *hog1* cells after 1h of hyphal induction in medium at pH7, pH4, or supplemented with 100μM Farnesol

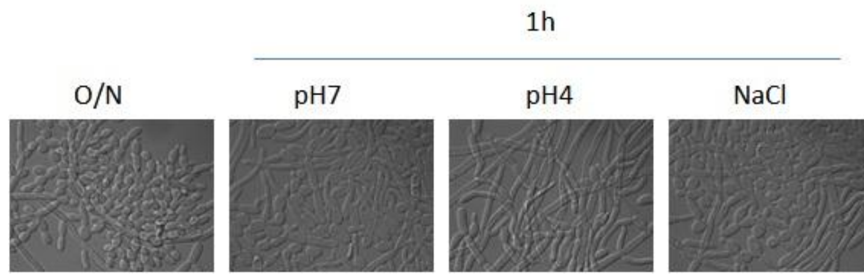
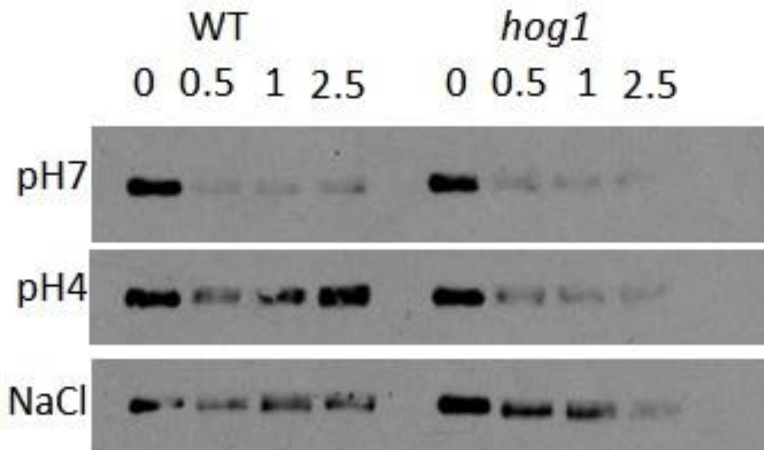


Fig 11: Morphology of *nrg1* mutant strain after 90min hyphal induction in YPD medium at pH7, pH4, and pH7 with 0.5M NaCl.



<u>Fig 12:</u> pH and stress do not prevent Nrg1 down-regulation. Western blot analysis of Nrg1-Myc levels during a 2.5h hyphal growth time course. Aliquots were collected at the indicated time points and analyzed via SDS-Page and immunoblotting for Myc. A parallel blot was probed with anti-PSTAIRE as a loading control.

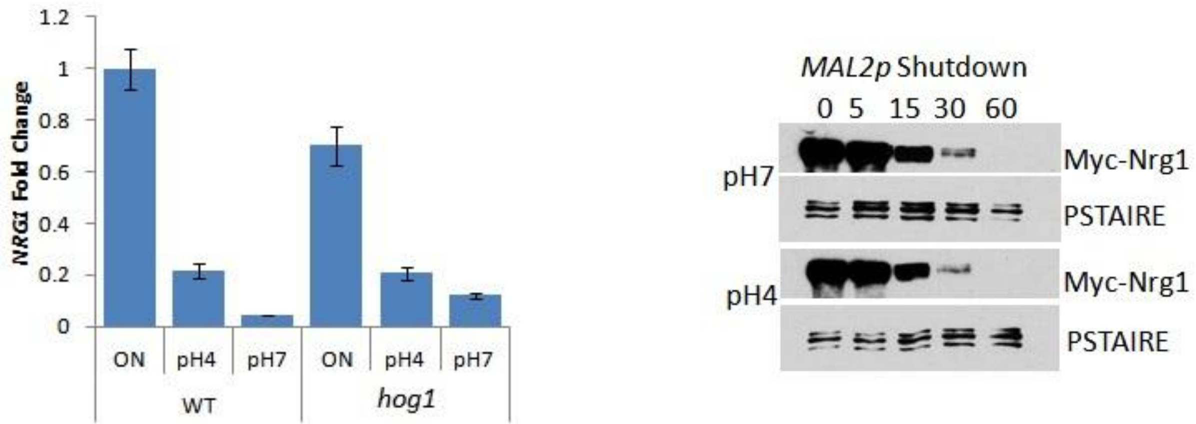


Fig 13: pH and Hog1 do not affect *NRG1* transcription and Nrg1 degradation (A) qRT-PCR of WT and *hog1* cells to measure the levels of *NRG1* transcript after cells were grown in pH4 and pH7 for 1h. qPCR values were normalized to *ACT1* for each samples, and overnight (ON) samples were set to a value of 1. (B) CAI4 strains containing a copy of *MAL2p-NRG1-MYC* were used for a promoter shutdown assay to observe degradation of Nrg1-Myc protein at pH4 and pH7. A parallel blot was probed with anti-PSTAIRE antibody as a loading control.

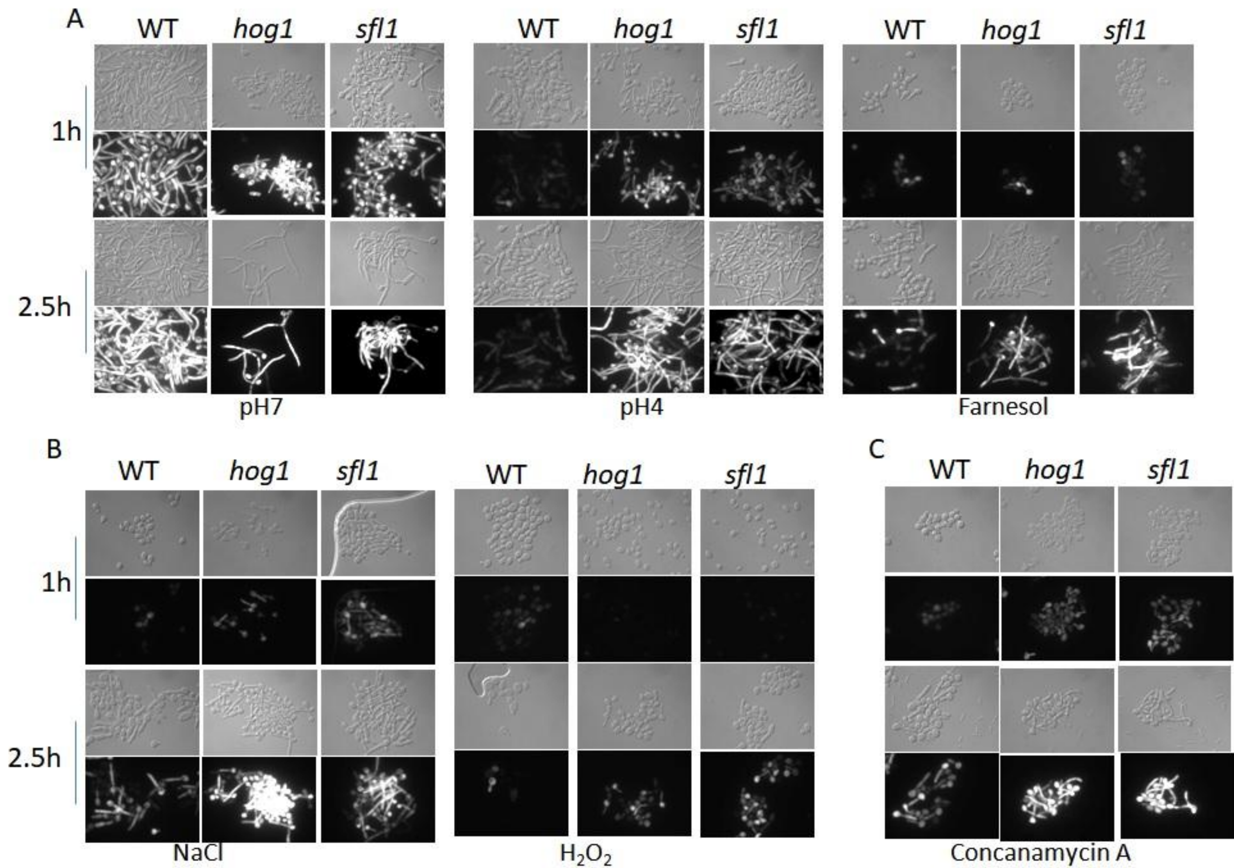
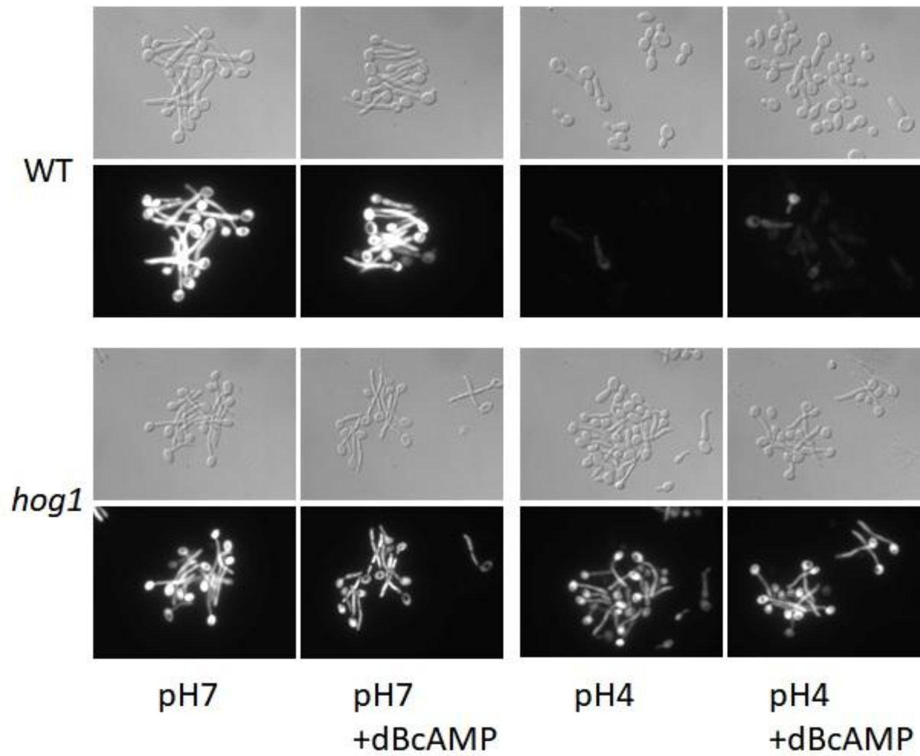


Fig 14: Sfl1functions genetically down-stream of Hog1. WT, *hog1,* and *pbs2* strains expressing a *HWP1p* driven GFP were hyphal induced in YPD set at (A) pH7, pH4, or pH7 supplemented with 100µM Farnesol, (B) 0.5M NaCl or 5mM H_2O_2 (C)5mM Concanamycin A. Pictures of hyphae and GFP fluorescence were taken at 1h and 2.5h time points.



<u>Fig 15:</u> Hog1 and pH functions independent of cAMP/Cyr1 pathway. WT and *hog1* mutants were grown in pH7 and pH4 with/without 10mM dB-cAMP for 90minutes.

CHAPTER 3

Characterizing the *C. albicans* cell wall response to acidic pH

Introduction

Candida albicans is one of the myriad of microorganisms residing within the human body. In healthy human hosts, it is a commensal organism that co-exists without any threat or damage to the host(F. C. Odds 1988). However, when the conditions are right, it can cause an infection ranging in severity from harmless superficial colonization of a particular niche to life threatening system infection as a result of invasion of critical organs. The understanding of *C. albicans* infection is critical as systemic infection have an increased mortality of ~30% in hospital cases(Gudlaugsson et al. 2003). A major defense in controlling the pathogenesis of *C. albicans* is the activity of the host immune system(Cheng et al. 2012). This is evident as hosts that have a compromised immune system, such as hospital patients undergoing immunosuppresive therapies or HIV patients, have an increased frequency of developing and dying from *C. albicans* infection.

The first line of defense against the fungal infection is the phagocytic cells that make up the immune system such as the macrophages and neutrophils that can uptake and kill the fungus(Höfs, Mogavero, and Hube 2016). Several studies have helped clarify some of the processes involved in host killing of the pathogen(Erwig and Gow 2016). First, phagocytosis of the fungus is dependent on the host cell recognition of the foreign pathogen. This is facilitated by the interaction between the multitudes of Pattern Recognition Receptors (PRRs) on the immune cells that recognize the many Pathogen Associate Molecular Patterns (PAMPs) on the fungal cell surface. These interactions have been very well reviewed in several publications, however the

most significant of these interactions is the recognition of the β1-6 Glucans on the fungal cell wall by the host surface receptor, Dectin-1(Taylor et al. 2007; Kennedy et al. 2007; Goodridge, Simmons, and Underhill 2007). This interaction is essential for recognition as macrophage Dectin-1$^{-/-}$ mutants or macrophages that are pre-treated with soluble β-Glucans show a severely attenuated ability to recognize and phagocytose *C. albicans.* Upon phagocytosis, the fungus is encapsulated in phagosomal compartment that is simply a membrane covered portion of the extracellular environment. Over time, this phagosome undergoes a maturation process that causes the compartment to become increasingly stressful to the internalized fungus(Bain et al. 2014). This phagosome maturation is essential to kill the fungus as genetic or chemical block of this process results in a reduced ability to kill the fungus. Phagosome maturation involves an increase in the phagosome's oxidative and nitrosative stress potential, uptake of hydrolytic enzymes that degrade several components of the fungal cargo, and an increase in the acidity of the phagosome, which contributes to the activation of the lytic enzymes(Mansour et al. 2013). This process is also dependent on Dectin-1, which signals to down-stream pathways required for full immune response. Activation of Dectin-1 signals to the kinase Syk1 to initiate a cascade of response such as expression of NFκB and the immune transcription factor family NFAT(Gantner et al. 2003; Goodridge, Simmons, and Underhill 2007), induction of the inflammasome(Cheng et al. 2011), and cytokine production(Ferwerda et al. 2008; Ghosh et al. 2010). Upon full immune response, the fungus within the phagosome is killed as a result of the stressful conditions, and the immune system is primed and ready for another encounter with the invading pathogen.

Upon being engulfed by macrophages, *C. albicans* can mount an active response to resist the phagosomal challenge and promote its survival. This is evidenced by the fact that a majority of live WT *C. albicans* that are engulfed by macrophages survive the host cell, compared to

macrophages that phagocytose heat inactivated fungal cells(Bain et al. 2014). Several fungal features have been identified that contribute to the survival of *C. albicans* inside a macrophage cell. First is its polymorphic ability to switch between yeast and hyphal morphology(Gow et al. 2012). Upon uptake, *C. albicans* rapidly induce hyphal growth within macrophages, and it was widely understood that the elongated morphology of hyphal cells results in mechanical lysis of the macrophage cells. However, research into the immunological impact of hyphal growth has revealed that there is more to hyphae than mechanical force. Reports have indicated that Dectin-1 better recognizes yeast cells than hyphal cells(Gantner, Simmons, and Underhill 2005), and it is well documented that yeast and hyphal cells induce different immune response, evidenced by different cytokine production(Han et al. 2013). Research into *C. albicans* interaction with macrophages proposed that hyphal formation is required for a non-lytic method of macrophage killing via the process of pyroptosis, a programmed cell death pathway in macrophages(Uwamahoro et al. 2014; Wellington et al. 2014; Krysan, Sutterwala, and Wellington 2014). However, a genetic screen of *C. albicans* mutants identified a combination of hyphal and pyroptosis defective mutants, hyphal competent and pyroptosis defective mutant, and hyphal defective and pyroptosis competent mutant, revealing that hyphal growth and pyroptosis may not be obligately connected(O'Meara et al. 2015). How these two processes are inter-regulated is still under investigation. Second, *C. albicans* cells can sense and neutralize the increasingly stressful environment within the macrophage phagosome. Osmotic and oxidative stresses are sensed by the Hog1 and Cap1 pathways, respectively, and promote the expression of the necessary stress response genes(A. Brown et al. 2012). In osmotic stress, the up-regulation of glycerol biosynthesis genes increases the amount of cellular glycerol, increasing the internal osmotic pressure and restoring osmotic homeostasis(San José et al. 1996; de Nadal and Posas

2015). Oxidative detoxification genes such as cellular superoxide dismutases (SODs) and Catalase1 facilitate the inactivation of reactive oxygen species within the cells(Dantas et al. 2015). Also, phagocytosis has been shown to cause a metabolic shift in *C. albicans,* in response to the nutrient-poor conditions found in the macrophage or neutrophil (Lorenz, Bender, and Fink 2004; Rubin-Bejerano et al. 2003). Lack of preferred carbon sources result in the up-regulation of genes that activate alternative metabolic sources including genes that function in gluconeogenesis and glyoxylate cycle, as well as the down-regulation of glycolysis genes. Nitrogen starvation in the macrophages induces arginine biosynthesis genes to increase intracellular nitrogen. Lastly, *C. albicans* is able to prevent/delay the maturation of the phagosome(Fernández-Arenas et al. 2009). In phagosomes containing live *C. albicans* cargo, acidification and recruitment of lysosomal proteins is inhibited compared to phagosomes containing dead cell. How this process is influenced by the fungus is not fully understood, but Dectin-1 recognition of β-Glucans and down-stream signaling is required for phagosome maturation(Mansour et al. 2013), and *C. albicans* mutants that have higher levels of exposed β-Glucans cause faster phagosome maturation(Bain et al. 2014). Therefore, it is hypothesized that *C. albicans* must have an ability to regulate the levels of exposure of its β-Glucans during phagocytosis.

In this study, we present evidence for a dynamic cell wall response that controls β-Glucan exposure in conditions found in the maturing phagosome, and we propose a genetic screen to identify pathways and genes involved in this regulation.

Results

Evidence of cell wall remodeling in acidic pH: Acidification of the environment is a critical stress condition for *C. albicans* as it correlates with phagosomal maturation and fungal killing in the macrophage. *C. albicans* has been shown to prevent this process during phagocytosis, making it an essential condition to understand the host-pathogen interaction. To understand the fungal response to acidification, we determined the level of exposed β-Glucans on the surface of live and heat-killed *C. albicans* after exposure to medium at increasing acidity, from pH7 to pH4, by observing the binding of fluorescent-labeled soluble Dectin-1 (Fig. 1). A 30 minute inoculation in the different pH media revealed that acidification of the environment resulted in an increased exposure of β-Glucans, and thus increased Dectin-1 binding, on the surface of heat-killed *C. albicans.* Remarkably, the increased exposure was absent in the live cells and they maintained the same amount of exposed β-Glucans regardless of medium pH. This supports the hypothesis that live *C. albicans* cells are able to protect themselves from threats perceived from the extracellular environment but dead cells cannot. Studies of β-Glucan exposure in *C. albicans* have showed that the β-Glucans are masked by a mask of mannan polysaccharides, and mutants that are defective in mannan branching have increased β-Glucans exposed. Analysis of the *C. albicans* mannan structure revealed a branch of the total mannan is located on a phosphate residue that is vulnerable to protonation, resulting in an acid-labile mannan branch, suggesting that extended stressing of the cell by acidic pH overwhelms this protection. Together, we propose that acidification of the fungal environment contributes to the exposure of antigenic β-Glucans by removal of the acid-labile mannans, and live cells are able to sense this reaction and enact a mechanism to prevent/counter this exposure.

Identification of β-Glucan protective mechanism: Our discovery that live cells can prevent acidic pH exposure of β-Glucans caused us to seek out the mechanism involved in this

regulation. *C. albicans* sensing of the environment is heavily dependent on its various MAPK pathways, and several lines of evidence supported the notion that they might be involved in this process. First, these pathways are important for survival in the macrophages. Second, several conditions within the macrophage activate the several MAPK pathways. For example, the Hog1 pathway is activated by the stress conditions in the phagosome. Third, studies have showed these pathways to be required for manna regulations. Together, we hypothesized that these pathways are involved in the protection of β-Glucans during acidic pH stress. To test this hypothesis, WT and mutants in the Hog1 pathway (*hog1* and *pbs2)* and the Cek1 pathway (*hst7*) were inoculated in medium at pH4 and pH7 for 30 minutes, and the levels of β-Glucan exposure was determined in live and dead cells. As expected, each of these mutants showed an increase in the basal level of β-Glucans because they are important for normal β-Glucan levels, but neither of them were defective in the protective mechanisms involved in acidic pH exposure of β-Glucans. Therefore the mechanism for protecting β-Glucans is still uncertain.

Future Work:

To identify genes involved in protection of β-Glucans, we propose a genetic screen of our kinase and transcription factor mutant collection for strains that are unable to protect themselves from acidic pH. The cells will be grown in medium at pH4 and pH7 for 30 minutes and the levels of exposed β-Glucans will be determined via Flow Cytometry. Mutants that are able to protect themselves should have no difference in the levels of exposure after 30 minutes in pH4 and pH7 (Fig 3 – Protected cells) but mutants that are required for protecting the β-Glucans will show increased exposure in pH4 but not in pH7 (Fig 3 – Unprotected cells). Using this profiling system, we aim to identify and map out the relevant pathway(s) involved in actively protecting the cell wall β-Glucans from environmental pH insults.

Upon identifying these mutants, the importance of this protective mechanism will be evaluated by macrophage phagocytosis of these mutants, and evaluating their ability to survive, form hyphae *in-vivo,* prevent phagosome maturation, and elicit an immune response from the host cell. We expect that these mutants will have a reduced ability to counter the macrophage's stress conditions and should be more vulnerable to killing than a WT counterpart. To assess the specific role of this protection, we will observe these effects compared to mutants like *hog1* and *hst7* that have a higher amounts of exposed β-Glucans but are still able to execute the active protection mechanisms. From this data, we hope to gain a better understanding of the very dynamic interaction that occurs between host and pathogen that is essential for the survival of both and the regulation of infection within the human body.

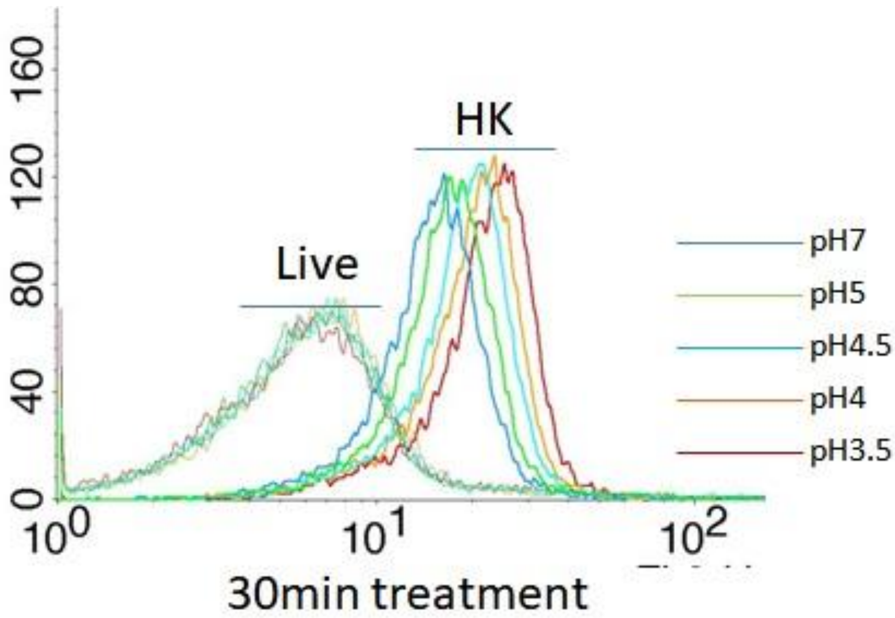


Fig 1: *C. albicans* protect from β-Glucan exposure in acidic pH. Live and Heat Killed (HK) WT cells were inoculated in medium at the indicated pH for 30 minutes, and the levels of exposed β-Glucans were determined by binding of soluble Dectin-1 and analyzed via Flow Cytometry

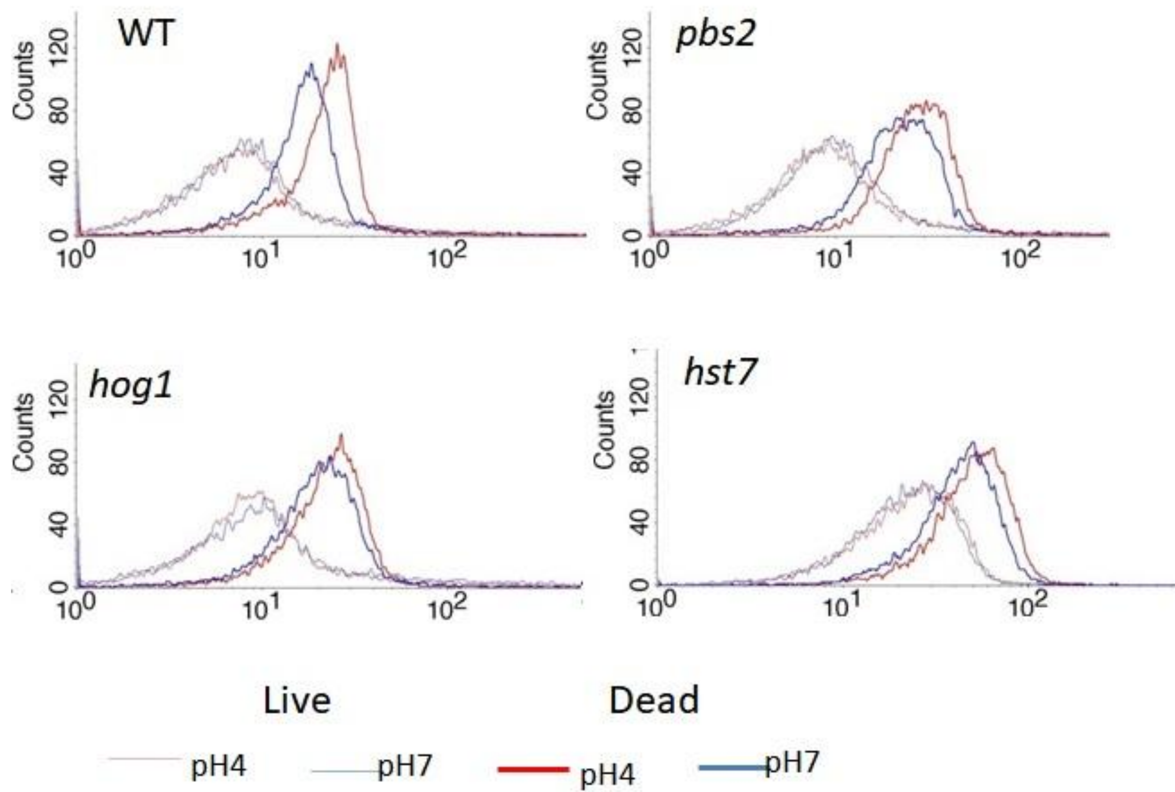


Fig 2: The Hog1 and Cek1 pathways are not involved in β-Glucan protection. Viable and Dead cells of the WT, *hog1, pbs2,* and *hst7* strains were inoculated in medium at the indicated pH for 30 minutes, and the levels of exposed β-Glucans were determined by binding of soluble Dectin-1 and analyzed via Flow Cytometry

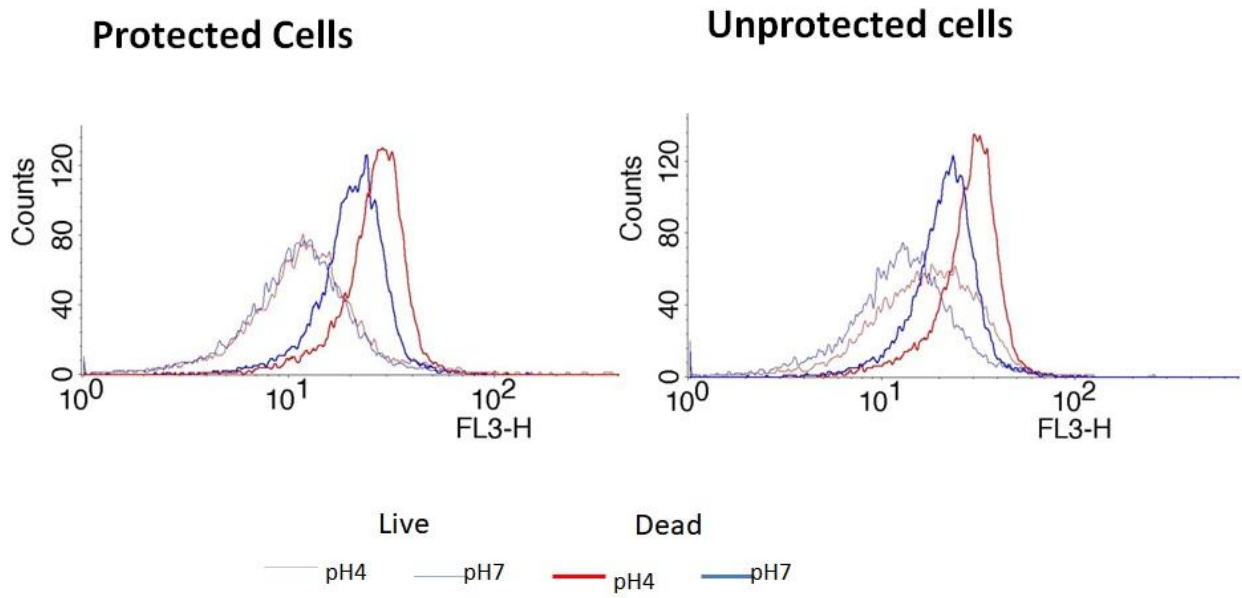


Fig 3: Representative profiles of strains that are dispensable or essential for β-Glucan protection.

CHAPTER 5

Summary and Conclusions

The goal of this dissertation is to investigate and identify new regulations involved in initiating hyphal formation in *C. albicans.* In this, we sought to determine the effects and mechanisms by which stress conditions regulate hyphal growth. This is the first study to directly query the influence of stressful conditions on hyphal formation. Previous studies that that concluded the effects of stress often concluded indirectly based on the hyphal phenotypes of genes that were involved in stress response. For example, Hog1 was identified as a hyphal repressor because *hog1* mutants formed invasive colonies compared to WT strains on solid media, and its function as a stress responsive gene suggested to others that stress activation of Hog1 might be inhibitory to hyphal growth, but there was no data to confirm that until this study.

Due to our focus on conditions that are inhibitory to hyphal formation, we have identified several new regulatory networks that are relevant to hyphal formation. Very often, studies in hyphal formation seek to identify positive regulators that lose the ability to filament in hyphal inducing conditions. Those studies have contributed to the valuable wealth of knowledge about the activating processes required for hyphal growth. However, the process of hyphal formation is a complex relationship between the activation of positive regulators and inactivation of inhibitors. Hyphal repressors such as Nrg1 and Tup1 often present a hyper-filamentous morphology even in the absence of hyphal stimulation, indicating that they are general repressors. However, not much is known about signal specific inhibitors. Therefore, our identification of relevant inhibitory networks and the individual signals that regulate them will improve our understanding

of hyphal growth and provide new potential targets for therapeutic development against candida infections.

Stress regulation of hyphal initiation:

Our studies have revealed that addition of stress to the environment has a negative impact on hyphal growth. Using cycloheximide and NaCl, we observe that these stress negatively impacts morphogenesis and hyphal gene transcription respectively. In Chapter 2, inhibition of translation delayed morphological developments without impacting the transcription of hyphal genes, suggesting that translation is required for the morphological aspect of hyphal initiation. However, addition of NaCl stress (0.5M) in Chapter 3 resulted in both morphological and transcriptional inhibition of hyphal growth. NaCl inhibitions showed a dependence on Hog1 as its deletion or inactivation resulted in increased frequency of hyphal formation in the presence of salt. Transcription of hyphal genes requires the removal of transcriptional repressors and the activation of transcription factors, and we have identified Sfl1 as a stress regulated transcriptional repressor that functions downstream of Hog1. However, it is still unclear how Hog1 and Sfl1 are regulated in the presence of stress. One possibility is the intensity and duration of Hog1 phosphorylation caused by different levels of stress. As the concentration of NaCl increases, the level and duration of Hog1 phosphorylation increases as well. Our unpublished data have showed that 0.3M NaCl induces a level of Hog1 phosphorylation that is rapidly lost by 30 minutes. In contrast, addition of 1M NaCl induces a Hog1 phosphorylation that is maintained even after 90mins. This increasing level and duration of Hog1 activation could directly correlate with repression of hyphal initiation and may influence regulation of Sfl1. Studies in *Saccharomyces cerevisiae* have showed that Sfl1 is a target of Hog1, so the possibility of this regulation exists in *Candida albicans* as well.

New players in pH regulation of hyphal growth:

Our studies have also identified new genes that are involved in acidic pH inhibition of hyphal initiation. Our kinase screen identified 7 other mutants that can filament in acidic pH, with each mutant functioning in distinct pathways, and our transcription factor screen identified Sfl1 as a transcriptional repressor in acidic pH. The focus of this study characterized the function and relationships of Hog1 and Sfl1 within acidic pH regulation, with our data indicating that Sfl1 functions down-stream of Hog1. While the genetic relationship between Hog1 and Sfl1 is clear, the mechanism of regulation in acidic pH is still unclear. Hog1 is shown to be hyper-phosphorylated in the presence of stress, but that regulation is absent in acidic pH. Moreover, levels of stress required to hyper-phosphorylate Hog1 only delay, rather than inhibit hyphal initiation. Together, these suggest that Hog1 phosphorylation may not be the main mechanism of hyphal repression in acidic pH. One possibility is that Hog1 functions in partnership with these other mutants to ensure Sfl1 repression. In this condition, loss of one or multiple of these players removes Sfl1 repression and promotes hyphal initiation. Alternatively, some of these kinases could function in an uncharacterized relationship, either directly or indirectly regulating each other to ensure Sfl1 repression. Therefore, the next set of experiments will explore the activities of these kinases in acidic pH, if and how they regulate each other, and how their activities influence Sfl1 repression of hyphal growth and transcription.

Sfl1 and Nrg1 as two parallel repressors of hyphal initiation:

Previous studies from our lab has identified that a key step in the initiation of hyphal growth is the removal of Nrg1 repression. Now, results from my work have showed that Sfl1 is another hyphal repressor that must also be removed to ensure hyphal initiation. How these two key repressors function in relation to one another is essential to understanding the dynamic regulation

of hyphal initiation. We have seen that pH and Hog1, which function upstream of Sfl1, do not prevent Nrg1 down-regulation, indicating an independent regulation. Interestingly, deletion of *NRG1* appears to promote filamentation in acidic pH, suggesting that removal of its repression is required for hyphal formation. Together, we conclude that these two pathways receive signals from different stimuli to regulate hyphal growth. Work from our lab have showed the mechanisms through which temperature and release from quorum sensing regulate Nrg1.Next, we will like to explore the stress signals regulate the activity of Sfl1 during hyphal initiation.

<u>The dynamic response to macrophage engulfing</u>

The interaction between host phagocytes and *C. albicans* is essential to understand the fungal pathogenesis. From our preliminary studies, we have discovered a dynamic protection that the fungal cells utilize to survive the host conditions, in this case acidic pH. A key question that we have yet to answer is how does *C. albicans* sense that it is within the phagosomal compartment. One possibility is the ability to sense damage to its external cell wall. In acidic pH, we see that there is an exposure of β-Glucans, which we suspect occurs as the acid labile mannans are removed. Therefore, from our proposed screen, we hypothesize that some of the genes important for protection of the cells should be involved in mannan regulation, which should further elucidate how the *C. albicans* cell wall is regulated and modified. In addition, the phagosome has a higher oxidative, nitrosative, and osmotic potential than the extracellular environment, suggesting that these stresses could also serve as additional signals in modifying the response to macrophage engulfing.

In addition, our experiments on hyphal growth have showed that stresses that are relevant *in-vivo* can influence hyphal formation, which is an important mechanism for surviving macrophages. It will be of interest to study the dynamics of hyphal formation within the

macrophages, especially as it is exposed to macrophage stresses, how that affects β-Glucan exposure, and macrophage survival. Other groups have explored the effects of hyphal formation in macrophage engulfing and killing, however these are done using mutants that are locked in yeast phase. Since Candida-Macrophage interactions are very dynamic, the opportunity to explore the effects of these stresses within the system as they are interacting will enable us to better understand this relationship.

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