Title
Msb2 is a signaling molecule required for temperature-dependent developmental responses in the fungal pathogen Histoplasma capsulatum

Author
Rodriguez, Lauren

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Msb2 is a signaling molecule required for temperature-dependent developmental responses in the fungal pathogen *Histoplasma capsulatum*

by

Lauren Rodriguez

DISSERTATION

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DOCTOR OF PHILOSOPHY

in

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of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

To my family.
Thank you for so much love and support!
I love you!

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Msb2 is a signaling molecule required for temperature-dependent developmental responses in the fungal pathogen *Histoplasma capsulatum*

Lauren Rodriguez

ABSTRACT

Dimorphic fungi are temperature-sensitive organisms that couple their cell shape with their environment. One of these fungi, *Histoplasma capsulatum*, exists as both a soil-dwelling hypha and a host-associated yeast. Here I examine the genetic elements required for hyphal growth. Insertional mutagenesis identified a mutant that is disrupted in the promoter region of the previously uncharacterized gene *MSB2*, whose ortholog in the model yeast *Saccharomyces cerevisiae* is a known signaling component in the high osmolarity glycerol (HOG) pathway and filamentous growth (FG) pathway. Through genetic analysis I confirmed *MSB2* is necessary and sufficient for filamentation in *H. capsulatum.* To identify elements of the transcriptional program dependent on *MSB2*, I took both a targeted and global approach. Previous work has established 4 transcription factors, *RYP1-4*, as regulators of yeast phase growth and probing for their expression here shows that *MSB2* is required for appropriate *RYP* expression at 37°C. Additionally, RNA-seq provided insight on a small number of genes affected by *MSB2* expression. This analysis identified *STU1*, an APSES transcription factor, as dependent on *MSB2* expression. It was previously shown that *STU1* overexpression is sufficient for filamentation in *H. capsulatum* at 37°C. To identify signaling elements between the transmembrane protein

MSB2 and the transcription factor *STU1*, I looked at the 4 MAP kinases in *H. capsulatum* and in the RNA-seq data found that one, called *HOG2*, is dependent on *MSB2* expression. I conclude with showing *HOG2* is required for filamentation and for appropriate expression of *STU*1 at room temperature. This genetic analysis outlines a pathway for regulation of filamentation in *H. capsulatum* through *MSB2*, *HOG2*, and *STU1*.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER TWO

CHAPTER THREE

CHAPTER FOUR

LIST OF FIGURES

CHAPTER ONE

CHAPTER TWO

CHAPTER THREE

CHAPTER FOUR

CHAPTER FIVE

CHAPTER 1

Introduction

One of the remarkable observations of the fungal kingdom is the diversity that it encompasses. It is easy for the mind to go straight to the plethora of beautiful mushrooms with the mention of the word fungus, however there is much more to the kingdom than basidiomycetes. The fungus *Histoplasma capsulatum*, a dimorphic pathogen in the Ascomycota phylum, establishes two different morphological states depending on its environment. The two states, hyphae and yeast, may not be as other-worldly as other fungal structures but how these states are established is incredibly intricate and remains largely unclear.

The mystery of how the morphology of *H. capsulatum* is regulated caught my interest because although it sounds like a simple process, it is actually quite complex. This fungus isn't simply just changing shape spontaneously; its morphology change has important characteristics attributed to each of the two states. In the soil, *H. capsulatum* is in a multicellular hyphal stage where it produces vegetative spores to aerosolize and disperse geographically or potentially to an unassuming mammalian host. If these spores do make it to a host, once inhaled, they are engulfed by alveolar macrophages in the lungs and mammalian body temperature (37°C) induces a transition to the unicellular budding yeast phase. It is in this phase that genes known to be required for host colonization and virulence are expressed and the yeast can intracellularly replicate, lyse the macrophages and go on to infect other cells and eventually other tissues.

This shows that the cell shape change is coupled to the temperature of its environment as well as the virulence state of the fungus. These factors make the study of the regulation of *H. capsulatum* morphology a lot more involved. However, that is what drew me to this question. Something seemingly so simple is actually excitingly intricate.

Histoplasmosis is the disease caused by the fungus *Histoplasma capsulatum*

Within the United States, *H. capsulatum* is endemic to the Ohio and Mississippi River valleys where an estimated 60-80% of people have been exposed to the fungus sometime during their lifetime (Manos, Ferebee, & Kerschbaum, 1956). With about 200,000 to 500,000 new cases per year (Centers for Disease Control and Prevention), understanding how to effectively treat the disease is imperative. Primarily contracted from aerosolized soil particles, the fungus is particularly successful at growing in bird and bat droppings as well as caves.

Unlike pathogens that take advantage of a compromised immune system, *H. capsulatum* is a primary pathogen that can colonize immunocompetent hosts (Kauffman, 2007; Pfaller & Diekema, 2010). However, patients with HIV/AIDS or undergoing immunosuppressive therapy are at a higher risk than those with an intact immune system. In Latin America, histoplasmosis is one major contributor to deaths of HIV/AIDS patients with about 30% succumbing to *Histoplasma* infection (Colombo, Tobon, Restrepo, Queiroz-Telles, & Nucci, 2011).

***H. capsulatum* is a thermal dimorphic, mammalian pathogen**

H. capsulatum is a fungus that has two morphologic states that are specialized for the environments that it resides in (Figure 1-1). In the soil, the mold state exhibits a long multicellular filamentous structure where only the terminal cells of the filament actively divide. This mold phase is able to produce vegetative spores in the soil. These spores, called conidia, come in two varieties: macroconidia and microconidia (Figure 1-2) and the biological differences between the two remains unclear. Also, fungal spores are specialized for dispersal more than they are specialized to withstand harsh conditions. Once aerosolized, they have the possibility of encountering a mammalian host via inhalation. This is when transition to yeast occurs, triggered

by the mammalian body temperature. While it remains unclear if other environmental cues can induce the transition from spore to yeast, temperature is a sufficient signal to trigger morphology change. Additionally, by simply switching the temperature between room temperature and 37°C, the morphology change can be observed between hyphae and yeast. Germination of spores can result in yeast if grown at 37°C and hyphae at room temperature. Since spores are considered a dormant stage of the fungus with the purpose of securely dispersing the organism, this highlights the thermoresponsive nature of *H. capsulatum*.

Figure 1-1. Morphological states of *H. capsulatum*

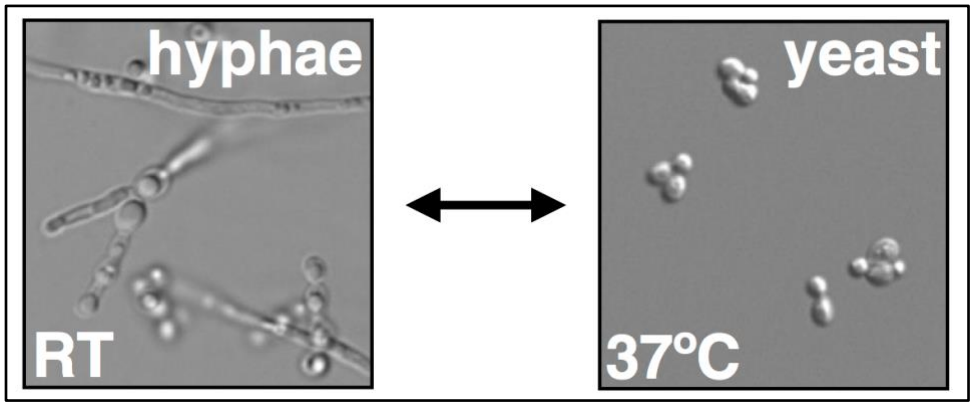
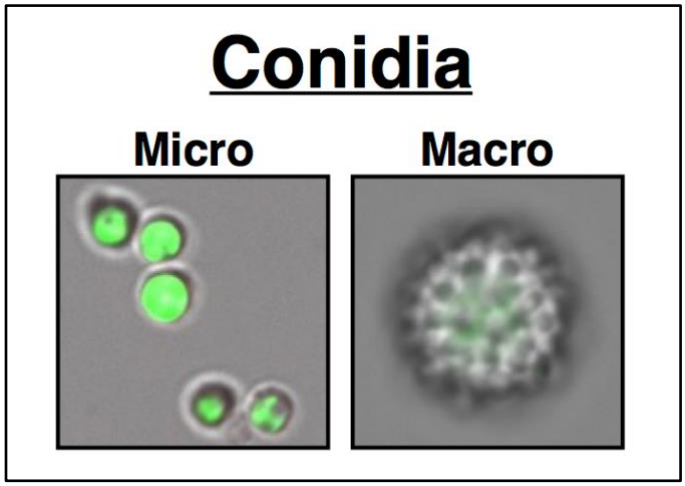


Figure 1-2. Macroconidia vs microconidia morphology



***H. capsulatum* has a temperature-dependent transcriptional profile**

Previous work in the lab has identified genes that are specifically expressed in either the yeast or hyphal phase. Microarray analysis of 37°C-grown yeast and room temperature-grown hyphae shows about 15% (about 1300 genes) of the transcriptome is temperature-dependent (Beyhan, Gutierrez, Voorhies, & Sil, 2013). This is a considerable percentage of the transcriptome keeping in mind that this is a eukaryotic organism with a decent sized number of genes.

Genes that are temperature-dependent according to transcriptomics approaches have been confirmed via qPCR to understand the dynamics of temperature responsiveness. There are genes like *MS95* that have been shown to be upregulated transcriptionally very quickly after transition to room temperature and other genes like *STU1* that take almost a week to increase transcript level at room temperature. On the other hand, *CBP1* is a secreted protein that is the most highly expressed gene at 37°C.

H. capsulatum yeast lyse macrophages

Once inhaled by a host, the fungus encounters the first line of innate immune defense. Yeast cells, and spores in the process of transitioning to yeast, are engulfed by alveolar macrophages in the lungs. In the macrophage, the fungus resides in a tight phagosome where it blocks fusion with the lysosome, avoiding acidification and making it a favorable environment for yeast growth and replication. Through a mechanism still not completely understood, the buildup of yeast in the macrophage will eventually trigger lysis, allowing the now extracellular yeast to go infect neighboring cells.

The routine assay to observe macrophage lysis is performed via cell culture using bone-marrow derived macrophages (BMDMs). These cells are differentiated from bone marrow collected from mice and can be plated in order to perform an infection in vitro. An infection of macrophages with *H. capsulatum* yeast at a multiplicity of infection of 1 results in intracellular fungal growth (Figure 1-3) and host cell lysis (Figure 1-4). Fungal growth is measured by lysing macrophages with water to release the intracellular fungal cells. They are then plated in order to count colony forming units over the course of infection. This is done at each timepoint up until the macrophages start lysing from infection in order to avoid counting extracellular yeast. Host cell lysis is measured using a lactate dehydrogenase (LDH) assay. LDH is an intracellular enzyme that can be detected using a colorimetric assay. Lysis is quantified by calculating the amount LDH released from the host cell into the culture medium compared to the amount of LDH in the cells initially plated.

Host cell lysis has been shown to be dependent on the yeast-specific gene *CBP1* (Isaac et al., 2015). Infection with a mutant strain lacking *cbp1* expression lyses cells at a rate similar to uninfected cells. This has been confirmed both *in vitro* and *in vivo* through a mouse model of

histoplasmosis. While it has been well established that this is a *CBP1*-dependent process, the mechanism by which *CBP1* mediates macrophage lysis has yet to be determined. Work done recently in the Sil lab elucidated a *CBP1*-dependent induction of the integrated stress response of mammalian macrophages upon infection (English, Van Prooyen, Ord, Ord, & Sil, 2017). While this has added to what is known about yeast-promoted lysis of mammalian host cells, the question of how it is successfully accomplished remains to be fully understood. Other questions of interest on this subject include the localization of Cbp1 protein during infection, what host components the protein may interact with, and what exactly the protein is doing to induce the integrated stress response in the host.

Figure 1-3. Macrophage infection: fungal growth via CFU analysis

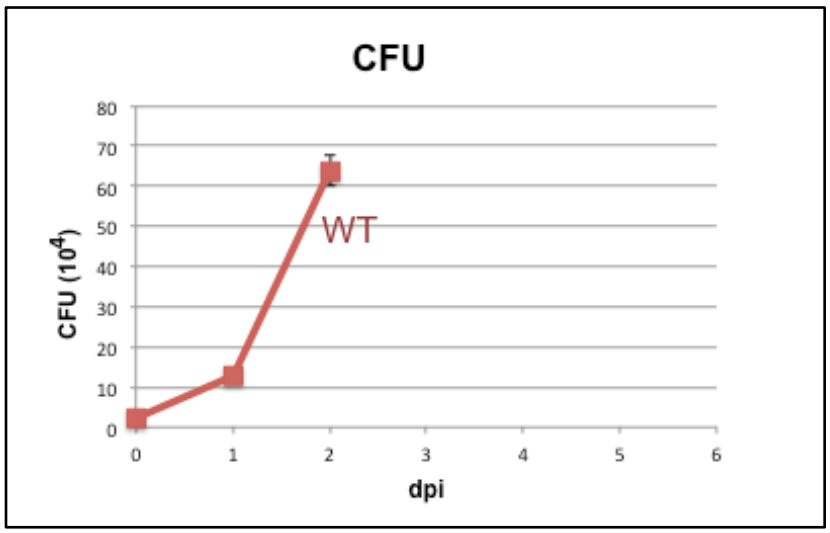
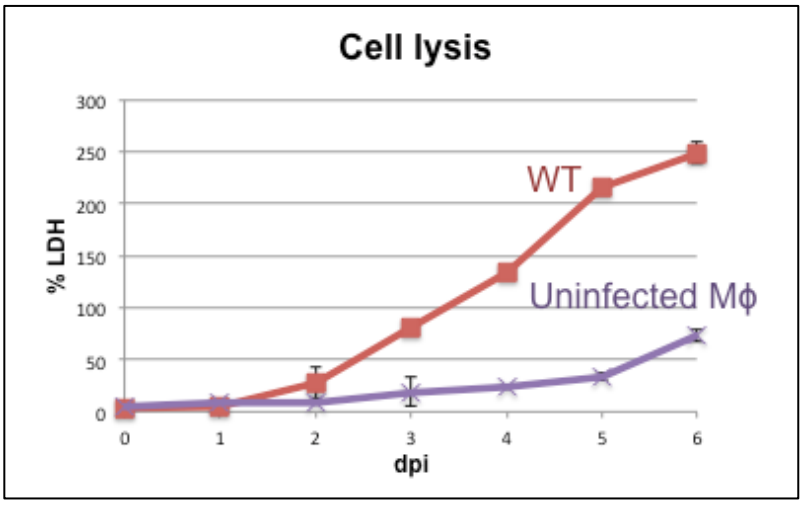


Figure 1-4. Macrophage infection: host cell lysis via LDH quantification



Genes regulating morphology in *H. capsulatum*

Along with the morphology difference between the two stages of *H. capsulatum*, there is a remarkable transcriptional response to temperature as well. In order to understand the gene regulation of *H. capsulatum*, a microarray was performed using RNA isolated from yeast, hyphae and spores (Inglis, Voorhies, Hocking Murray, & Sil, 2013). This analysis defined gene sets for each cell shape. Additional insight has been shown through RNA sequencing analysis that revealed ~2% of the transcriptome actually undergoes reprogramming of transcript lengths between the hyphal and yeast states (Gilmore, Voorhies, Gebhart, & Sil, 2015). This supports an additional mechanism of gene regulation for *H. capsulatum*. While it remains unclear the effect transcript length has on transcription, it has potential to contribute to the intricate regulation of expression of phase-specific genes.

An extensive sequencing analysis of hyphae and yeast of two different strains of *H. capsulatum* has provided more data on the dimorphic transcriptome (Edwards et al., 2013). The

motivation was to examine if there were differences in the expression profiles of the pathogenic state (yeast) of strains that differed in virulence. The study concluded that within one strain, 6-9% of the transcriptome is phase specific and comparing the yeast phases of the two strains showed that 7.6% of genes are lineage specific.

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CHAPTER TWO

Characterization of a yeast-locked mutant

Introduction

The basis of dimorphic fungi is their ability to transition from one morphological state to another. While this sounds like a simple process, the field has made little progress in understanding the regulation of this central characteristic of dimorphic pathogens. The Sil lab utilizes basic genetic approaches to identify genes and pathways required for regulation of morphology and here I describe my work done to contribute to the growing picture of how dimorphic pathogens sense and respond to their environment. This work includes characterizing a mutant to identify a gene that is required for filamentous growth. Through molecular cloning I was able to confirm the role of *MSB2* in filamentation. I continued the characterization by surveying any phenotype of the mutant in our normal assays such as the macrophage model of infection as well as spore production. In this chapter, I outline my work on the role of *MSB2* in morphology establishment.

Materials and Methods

Strains

Histoplasma capsulatum G217BΔura5 was used for the insertional mutagenesis screen which was performed using the *Agrobacterium tumefaciens* strain LBA1100 containing the plasmid pRH5b as described (Marion, Rappleye, Engle, & Goldman, 2006; Sullivan, Rooney, & Klein, 2002; Zemska & Rappleye, 2012). All strain manipulations were done in the Histoplasma capsulatum G217BΔura5 background. Sub-cloning was performed in *E. coli* DH5α. Strains used in the characterization of *MSB2* can be found in Table 2-1.

Media

Wildtype G217B strain was grown in liquid or solid Histoplasma Media (HMM), shaking or in a standing incubator, respectively (Worsham & Goldman, 1988). G217BΔura5 and insertional mutants were grown in HMM media supplemented with 2.5mg/ml uracil. Bone marrow derived macrophages were grown in Bone Marrow Media (BMM) as described previously (L. H. Hwang, Mayfield, Rine, & Sil, 2008).

Construction of strains

Knockdown strains were produced using the RNAi method as previously described (Rappleye, Engle, & Goldman, 2004). Multiple 500bp regions of each gene were chosen as RNAi targets. Transformants were screened for knockdown efficiency through qPCR of target gene compared to an empty control vector (pSB200). *MSB2* complementation strains were constructed through amplification of *MSB2* genomic region including 2490bp upstream of the start codon from wildype G217B gDNA. Cloning was done via the Gateway cloning method (Katzen, 2007) into a

destination vector (pDG33) carrying the URA marker. The resulting episomal expression vector was linearized and electroporated into the yeast-locked mutant SG1 and plated onto HMM agarose plates without uracil to select for URA5-containing transformants. Complementation was determined by the ability to filament at room temperature. Overexpression strains were constructed by amplifying the coding region of the gene and cloning into an expression vector with a strong promoter (pACT1, pGAPDH). The control vectors for these strains are pTM4 for pACT1 and pSB275 for pGAPDH. Cell morphology of transformants was screen through growth at appropriate temperature. Primers are shown in Table.

Imaging

Imaging was performed on a Zeiss AxioCam MRM microscope DIC at 100X magnification.

Fungal cell collection

H. capsulatum cultures were collected by filtration with a disposable filtration apparatus either in Biosafety Level 2 laboratory for growth at 37°C or in the Biosafety Level 3 laboratory for growth at room temperature. At each time point, 10mL of fungal culture was passed through a filter (Thermo Fisher), the cells were scraped off with a cell scraper, placed into a conical with 1mL trizol reagent (Qiazol) and flash frozen in liquid nitrogen. Samples were stored at -80°C until all time points were collected.

RNA extraction

Total RNA was extraction from fungal cells using a trizol-based RNA extraction protocol. Frozen, resuspended pellets of cells were incubated at room temperature for 5 minutes to thaw. The lysate was bead beat with 300-500ul zirconia beads for 2 minutes. Place on ice immediately after the 2 minutes of beating. Add 200ul of chloroform, vortex 15 seconds, incubate at RT for 5 minutes, and centrifuge at 12000g for 20 minutes at 4C. The aqueous phase contains the RNA. Transfer this phase to a new tube, add 600ul of 100% ethanol, quickly vortex and load onto an Epoch RNA column. Wash filter with 300ul of 3M NaOAC, pH 7.5 and centrifuge at max speed for 30 seconds. Wash filters with 800ul of wash buffer (10mM TrisCl, pH7.5, 80% EtOH). Centrifuge at max speed for 30 seconds. Spin columns at max speed to remove excess ethanol. Treat each column with 40ul Purelink DNase/buffer mix for 20 minutes at RT. Put 300ul 1:1 mixture of guanidinium lysis butter and 100% EtOH and spin at max speed for 30 seconds. Wash filters with 3M NaOAC, pH 7.5 and centrifuge at max speed for 30 seconds. Wash filters with 800ul wash buffer and spin at max speed. Spin at max speed for 1 minute to dry columns. Elute with 50ul RNase-Free H_20. RNA quality was determined with a High Sensitivity DNA bioanalyzer chip from Agilent.

Expression level by qRT-PCR

Relative gene expression was observed using qRT-PCR. RNA extraction was performed as stated above and cDNA was prepared by priming 2ug DNase treated total RNA with oligodT/pdN9 and 10uM dNTPs for 5 minutes at 65°C. Samples were brought down to room temperature and 1ul RNaseOUT Recombinant Ribonuclease Inhibitor from ThermoFisher and 0.5ul Thermo Scientific Maxima H Minus Reverse Transcriptase. Negative controls include

samples without the addition of reverse transcriptase. Samples +RT were diluted 1:50 and samples –RT were diluted 1:10. qPCR reactions were set up using 96-well plates with Roche FastStart Universal SYBR Green Master (Rox) and 1.5uM primers (found in Table 2-2) and plates were read using Mx3000P machine (Stratagene) and analyzed using MxPro software (Stratagene).

Protein extraction

Organic fractions from trizol RNA extractions were stored at -20°C until protein extraction was performed. To remove DNA from the organic fraction, 300 ul of 100% ethanol was added, inverted to mix and incubated at room temperature for 5 mins. Tubes were then spun at 2000g for 5 mins at 4°C. The phenol-ethanol supernatant contains the proteins so they were moved to another tube. 1.5 ml isopropanol was added to the tube of supernatant and the samples were incubated for 10 minutes at room temperature. The samples were transferred to 2 ml screw cap tubes and spun at 12,000g for 10 minutes at 4°C to pellet the protein. This was repeated as many times as necessary to pellet all protein. The pellet was then washed with 2ml wash solution (0.3M guanidine thiocyanate in 95% ethanol). After incubation for 20 minutes at room temperature, the tube was spun at 7,500g for 5 minutes at 4°C. The solution was discarded and the wash step was repeated for a total of 3 times. 2 ml of 100% ethanol was added to the protein which was then vortexed and then incubated for another 20 minutes at room temperature. The protein was pelleted by centrifugation at 7,500g for 5 minutes at 4°C. The ethanol was discarded and the pellets were left to air dry for 10 minutes at room temperature. Finally, the pellet was resuspended in 100 ul to 1ml urea lysis buffer. If necessary, the pellet was incubated at 50°C for 10-20 minutes in order to successfully resuspend in the urea buffer. Once resuspended, the

sample was boiled for 5 minutes and spun down at 12,000g for 2 minutes at room temperature. The supernantant contained the protein and it was transferred to a new tube for quantification by the Pierce 660nm assay (ThermoFisher).

Expression level by Western blot

Following quantification of protein, 20ug was aliquoted in a total of 20 uL of urea lysis buffer and 6uL NuPAGE LDS Sample Buffer (Novex). The samples were boiled for 5 minutes and placed immediately on ice. The prepared protein was loaded into a 10-well NuPAGE 4-12% BT SDS-PAGE gel (Novex) in MOPS running buffer and run at 150V. After the run, the protein was transferred to a nitrocellulose membrane at ~40V for 2 hours. The blot was blocked with blocking solution (1g milk powder in 100ml wash buffer [0.1% tween-20 in PBS]) for an hour and then incubated in the primary antibody in wash buffer overnight at 4°C. The primary antibody was washed 3 times in wash buffer for 10 minutes each. The secondary antibody was added to the blot for 1 hour at room temperature followed by another 3 washes with wash buffer for 10 minutes each. Protein bands were then detected using chemiluminescence following directions provided by the SuperSignal West Pico kit (ThermoFisher).

Southern Blot

Southern blots were performed following the protocol described using the DIG kit purchased from Sigma-Aldrich.

Macrophage infection

Bone marrow-derived macrophages (BMDMs) were plated on a 48-well plate at a concentration of $7.5x10^4$ cells in 0.25ml bone marrow media (BMM) per well. They were allowed to adhere to the plate overnight. Yeast, at OD_{600}=5-7, were prepared for infection by pelleting at 2500rpm, for 5 mins, resuspended in BMM, and sonicated for 3 seconds to break apart cell clumps into individual yeast. Yeast were counted and diluted to the appropriate multiplicity of infection (MOI) and added to the adhered macrophages. Phagocytosis of yeast occurred for two hours, after which the cells were washed once with phosphate buffered saline (PBS) and in a final volume of 375ul BMM. On days 0 and 1, wells to be quantified that day were fed with 125ul fresh BMM to match the final volume of the wells later in the infection timecourse. On day 2, cells in all remaining wells were fed with 125ul BMM. To quantify cell lysis, at each 24 hour timepoint 175ul supernatant was collected. An assay quantifying lactate dehydrogenase present in the media was performed to measure macrophage cell lysis.

Spore production, harvest, and viability testing

Strains of *Histoplasma capsulatum* yeast were grown at 37°C, 5% CO_2 in HMM media for 2 days. The OD_{600} of each strain was measured and the cultures were diluted to OD_{600}=1. 150ul was plated on Modified Cysteine-Glutamine + Agar medium as described (Kwon-Chung & Bennett, 1992). Each plate was parafilmed individually and placed at room temperature in the Biosafety Level 3 facility. 20 plates were prepared for each strain. After 3 weeks, spores were harvested by flooding the plates with 5ml PBS+ 1M sorbitol. The surface of the plates was gently scraped with a glass rod. The 5mL of PBS+ 1M sorbitol was collected and pooled from all 20 plates of each strain and filtered through a syringe with glass wool. Filtrate was spun down at

2000rpm, 10mins, 4°C. The pellet of spores was resuspended in 500ul PBS. Final concentration of spores was determined by dilution of 1:1.5 in lactophenol blue and quantification on a hemacytometer. To test for viability, serial dilutions were plated onto both HMM and Sabouraud plates at room temperature and 37°C to determine germination at both relevant temperatures.

Results

Agrobacterium-mediated insertional mutagenesis produced a mutant unable to form hyphae at room temperature

Identifying genes and pathways involved in morphology of *H. capsulatum* is a central theme in the Sil lab. In order get the full picture of how the pathogen successfully colonizes a host, it is imperative to understand how the pathogen itself functions. This remains unclear in all of the dimorphic fungal pathogens.

To probe for players in the regulation of morphology, the Sil lab, quite successfully, has utilized forward genetics to identify genes that are involved in the process. Previous studies have identified and characterized genes required for the yeast phase of *H. capsulatum* (L. Hwang et al., 2003; Inglis, Voorhies, Hocking Murray, & Sil, 2013; Nguyen & Sil, 2008). These studies have shed light on the regulation of yeast phase growth however the regulation of filamentation remains largely unclear.

By screening mutants unable to transition from the pathogenic yeast state to the infectious filament state, we sought out to elucidate genes required for this transition. *Agrobacterium*-mediated insertional mutagenesis was performed on *H. capsulatum* yeast and mutants that retained their yeast morphology after growth of 6 weeks at room temperature underwent characterization. We went on to characterize one of these yeast-locked mutants. When the mutant yeast culture was grown under hyphae-promoting conditions, it failed to transition (Figure 2-1) and instead actively divided as yeast (Figure 2-2) as seen through a performing a growth curve.

Figure 2-1. Mutant retains yeast morphology at room temperature

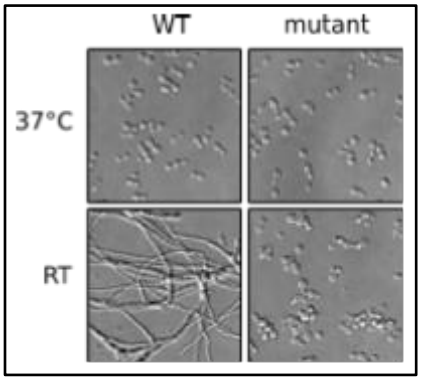
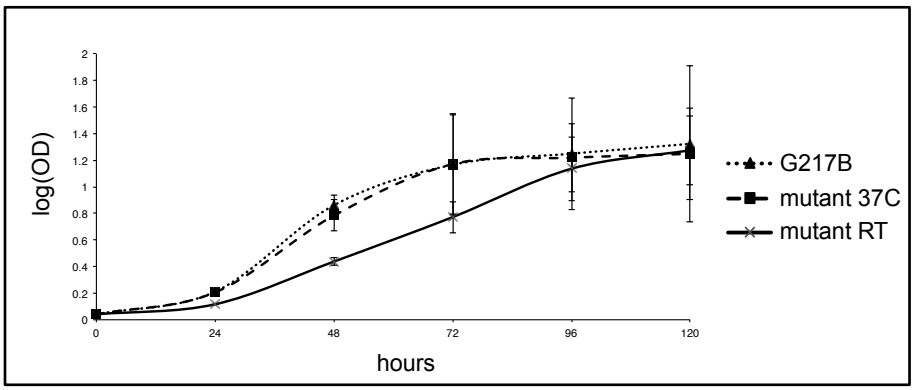


Figure 2-2. Growth curve of mutant at room temperature



Insertional mutants grow as yeast at room temperature after growth in both liquid and solid media

To examine the extent of the yeast-locked phenotype of this mutant, the strain was grown up at room temperature on Human Macrophage Media (HMM) agarose plates and tested for growth either straight to liquid HMM at room temperature or to liquid HMM at 37°C and then transitioned to room temperature for two days. The rationale for this was to determine if the mutant can robustly grow from a single colony to a successful yeast culture at room temperature.

Additionally, by initially growing the culture at 37°C and then transitioning to room temperature, we can examine the ability for the mutant to retain yeast morphology after establishing that growth at 37°C (Figure 2-3). The results showed that regardless of whether the room temperature culture was grown directly from an HMM plate (Figure 2-4) or transitioned from a 37°C growing liquid culture (Figure 2-5), the mutant retained its yeast morphology compared to wild type.

Figure 2-3. Schematic of yeast-locked mutant growth characterization

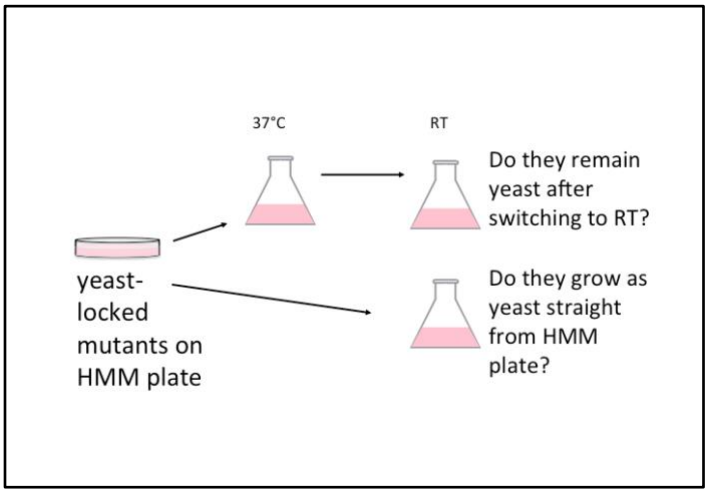


Figure 2-4. Growth of yeast-locked mutant at room temperature from solid media

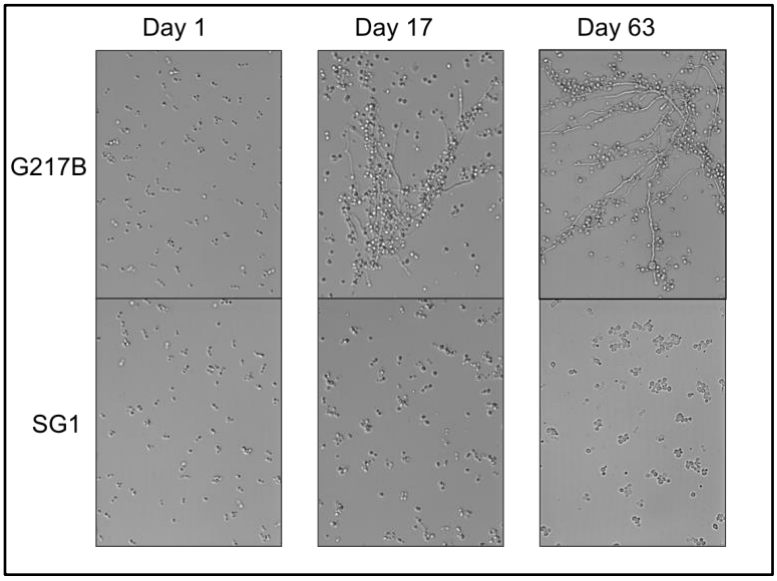
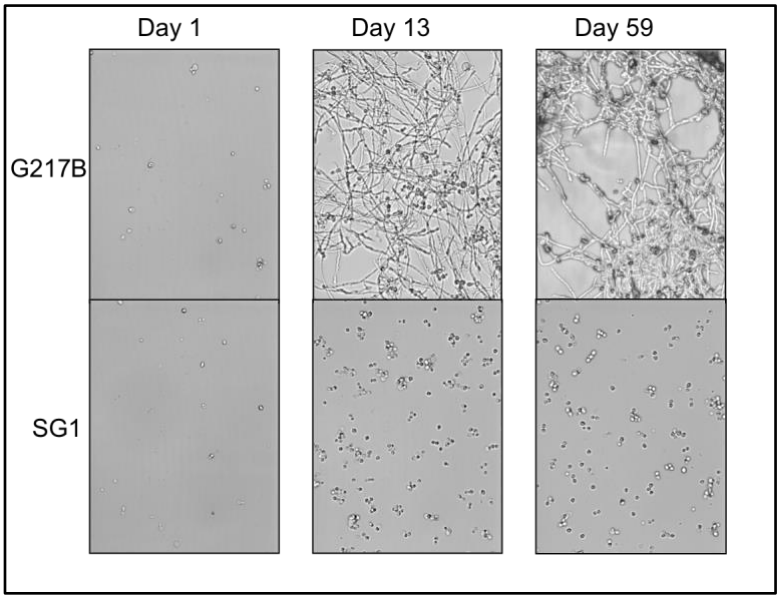


Figure 2-5. Growth of yeast-locked mutant at room temperature from liquid media



Mapping the mutation to the gene *MSB2*

To begin understanding why this mutant is unable to transition to hyphae at room temperature, it was imperative to know that there indeed was one gene disrupted in this strain. I did a Southern blot to probe for the tDNA insertional element used to produce the mutants in the screen. Using the method previously established (Southern, 1975), it was determined that one t-DNA element was present in the yeast locked mutant strain (Figure 2-6).

Further characterization of the mutant via whole-genome sequencing led to the identification of the transposon insertion site in the promoter region of the *MSB2* gene (Figure 2-7). The function of *MSB2* in *H. capsulatum* is unknown, however, it has orthologs in the distant fungal species *Candida albicans* (Roman, Cottier, Ernst, & Pla, 2009; Sorgo et al., 2010), and *Saccharomyces cerevisiae*, where its function has been more aggressively examined (Bender & Pringle, 1992; Cullen et al., 2004; O'Rourke & Herskowitz, 2002; Pitoniak, Birkaya, Dionne,

Vadaie, & Cullen, 2009; Tatebayashi et al., 2007; Vadaie et al., 2008). Using a wildtype copy of *MSB2,* we were able to complement the mutant phenotype and restore filamentation at room temperature (Figure 2-8). Additionally, we were able to confirm the contribution of *MSB2* to filamentation by constructing an RNAi knockdown strain. *H. capsulatum* strains deficient in *MSB2* transcription through RNAi exhibited a yeast-locked phenotype during growth at room temperature (Figure 2-9). The characterization of the yeast-locked mutant has led us to conclude that *MSB2* is required for filamention at room temperature in *H. capsulatum*.

Figure 2-6. Southern blot analysis on yeast-locked mutant genomic DNA

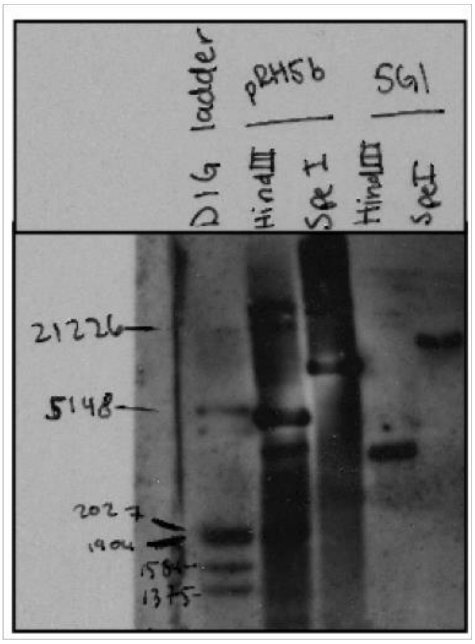


Figure 2-7. *MSB2* gene disruption

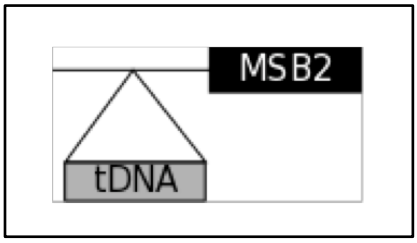


Figure 2-8. Complementation morphology

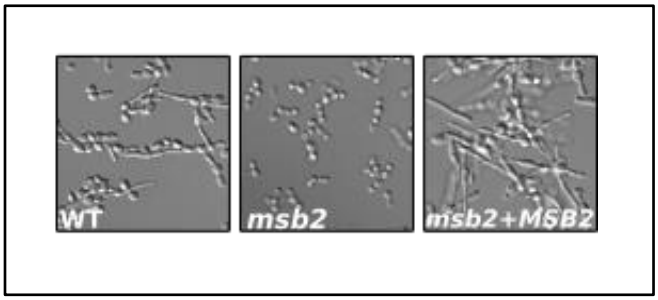
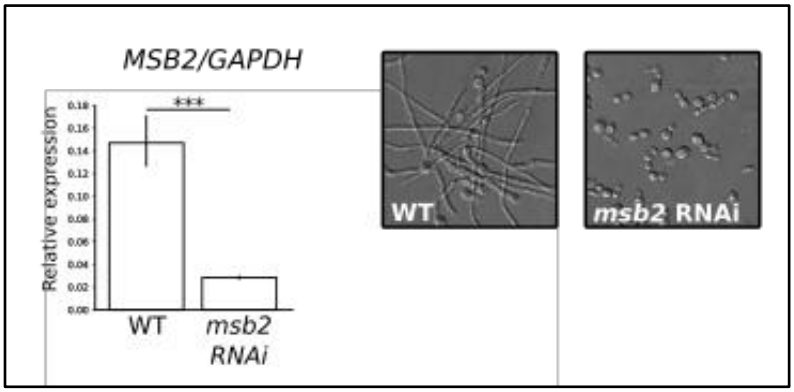


Figure 2-9. RNAi knockdown morphology



H. capsulatum has an *MSB2*-dependent response to osmotic stress

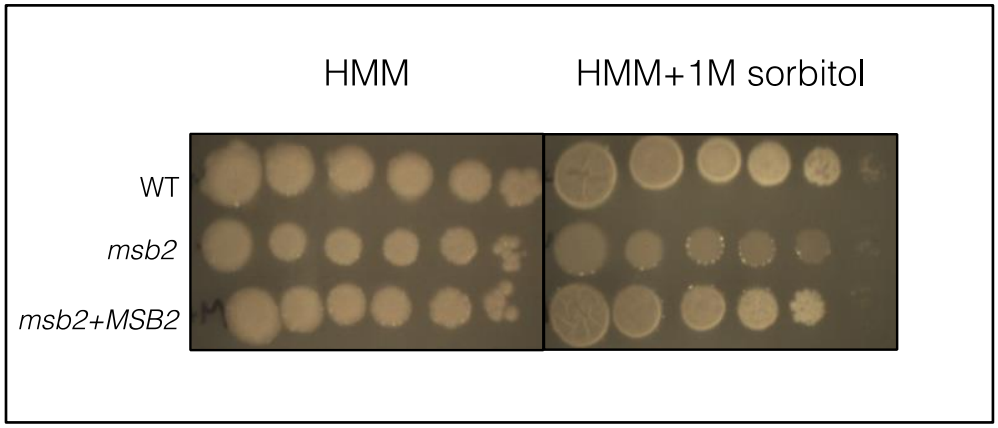
MSB2 has orthologs in other fungi that have been more extensively studied. In *S. cerevisae*, *MSB2* has been characterized as a transmembrane signaling protein with a mucin-like extracellular domain and relatively small cytoplasmic domain. The extracellular domain is serine/threonine-rich, making it a target for glycosylation. While *MSB2* was initially identified as a multicopy suppressor of a *cdc24* budding defect (Bender & Pringle, 1992), it was later found in a screen for genes involved in the response to osmotic stress (O'Rourke & Herskowitz, 2002).

Additionally, the *Candida albicans MSB2* ortholog has also been surveyed for a role in osmotic stress response. It was first identified in a study of the secretome but was confirmed as a transmembrane protein very similar to that of *S. cerevisiae* (Sorgo et al., 2010). After confirming its participation in the HOG pathway, analysis of mutants in the pathway revealed that *MSB2* is involved in the osmotic stress response as well as invasion of solid media and cell wall biogenesis (Roman et al., 2009).

Since the function of *MSB2* in *H. capsulatum* was still unknown, we decided to simply observe the growth of the mutant strain in the presence of 1M sorbitol. In *S. cerevisiae* the mutant phenotype of the Δ*msb2* strain grown on solid media with 1M sorbitol was a growth defect; *MSB2* is required for growth in the presence of osmotic stress. We performed a dilution series of *H. capsulatum msb2* yeast to identify any growth defect the mutant might have when stressed with sorbitol, a sugar alcohol. However, instead of a growth defect, a morphology defect was observed (Figure 2-10). The size of the colonies was similar between wildtype, *msb2,* and the *MSB2*-complemented strain. The difference can be seen in the wrinkly colony morphology and presence of hyphae on their perimeter in the wildtype and complement strains but not in the

mutant strain. This shows filamentation is an *MSB2*-dependent osmotic stress response in *H. capsulatum*.

Figure 2-10. Osmolarity response of *msb2*



Macrophage infection

H. capsulatum is a mammalian pathogen and one hallmark of its virulence is the ability to replicate and eventually lyse host alveolar macrophages. An established *in vitro* model of this interaction is through infection of bone marrow derived-macrophages (BMDMs) with the pathogenic yeast phase of the fungus. We used this model to examine the virulence of *msb2* grown at both 37°C and room temperature (Figure 2-11).

The phenotype in both conditions is yeast, however from sequencing data, *msb2* yeast grown at room temperature looks transcriptionally more similar to wildtype hyphae than wildtype yeast (Figure 2-12). BMDMs were prepared as previously reported (L. H. Hwang et al., 2008; Isaac et al., 2015) and infected with wildtype yeast grown at 37°C, *msb2* grown at 37°C or room temperature, and the *MSB2* complement strain at 37°C. While the mutant strain was

prepared at room temperature in one condition, once the infection was performed, the cells were kept at 37°C in order for the macrophages to remain in the biologically relevant condition. Over the course of 7 days, host cell lysis was observed though the quantification of lactate dehydrogenase, a mammalian intracellular enzyme, released into the medium. Wildtype *H. capsulatum* yeast steadily lysed the macrophages as previously reported (L. H. Hwang et al., 2008). The mutant strain, independent of growth temperature, lysed the macrophages at a rate similar to wildtype (Figure 2-13). This suggests that even though *msb2* grown at room temperature transcriptionally resembles hyphae, its pathogenic genes required for host cell lysis remain coupled to the yeast morphology.

Intracellular fungal growth was then measured in order to see if *msb2* has any effect on yeast growth regardless of its absence of a lysis phenotype. The result showed a growth defect in *msb2* yeast grown at 37°C (Figure 2-14). While the explanation for why that is the case is unclear, comparing the overall transcriptional profile of wildtype yeast to *msb2* yeast at 37°C shows that they are quite different regardless of being in the same culture conditions and exhibiting the same morphology (Figure 2-15).

Figure 2-11. Macrophage infection setup

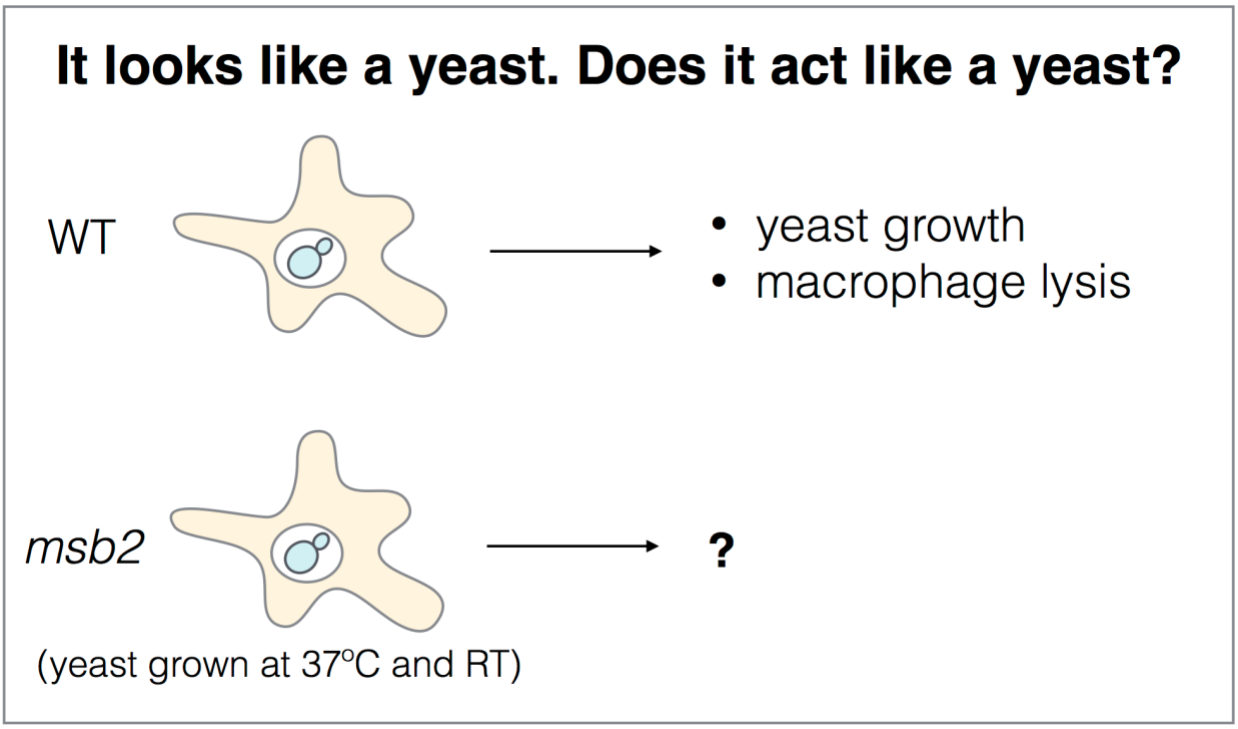


Figure 2-12. Transcript abundances comparing wildtype hyphae and *msb2* at room temperature

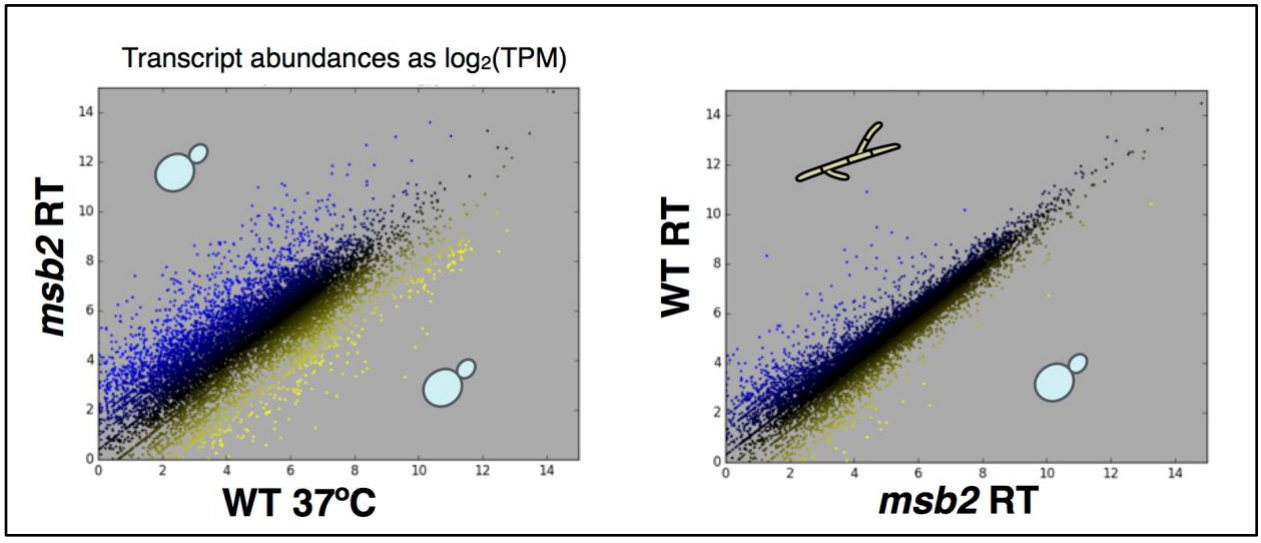


Figure 2-13. Infection: Macrophage lysis

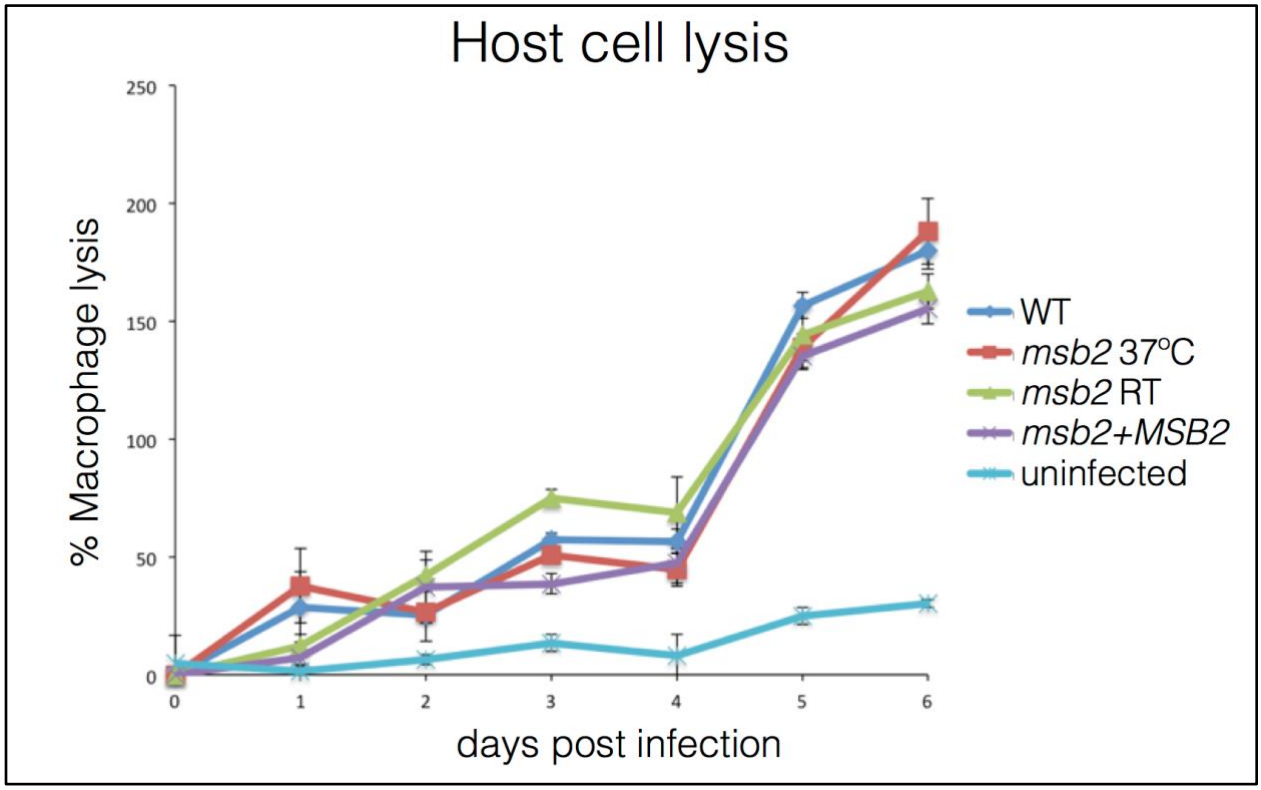


Figure 2-14. Infection: Intracellular fungal growth

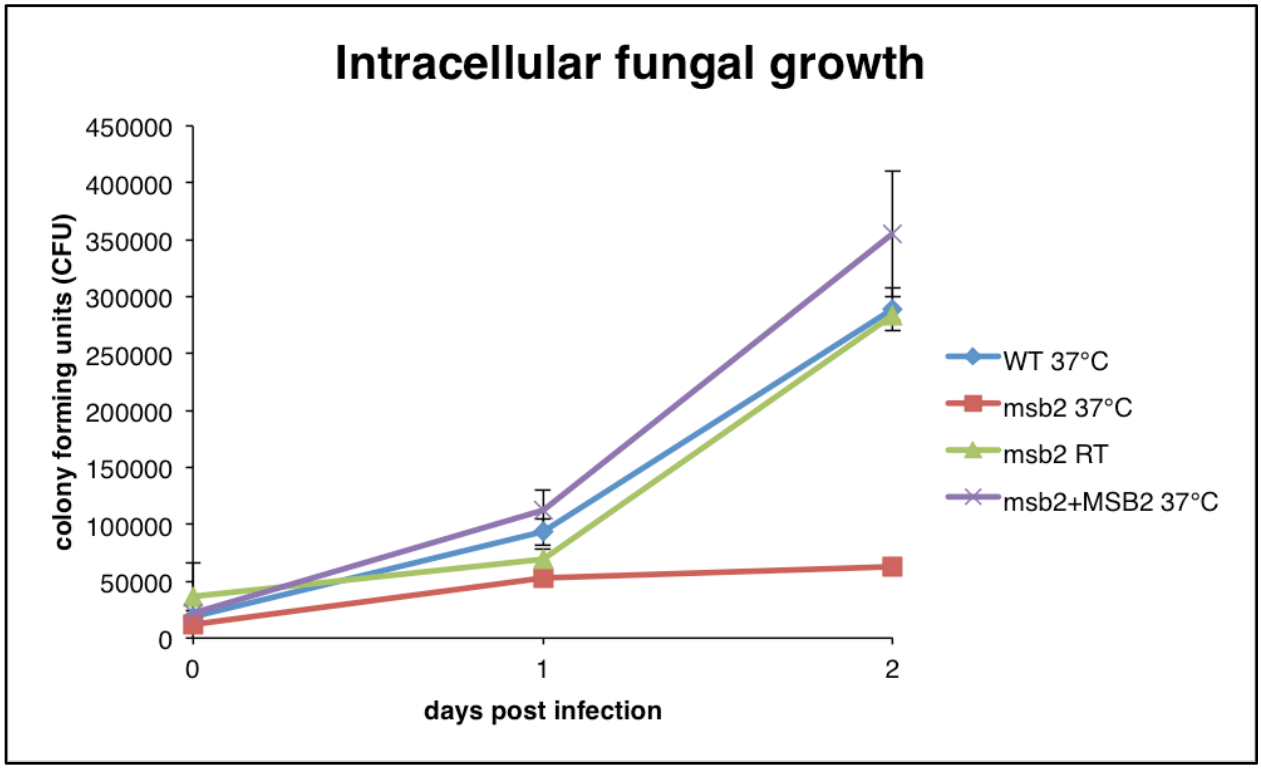
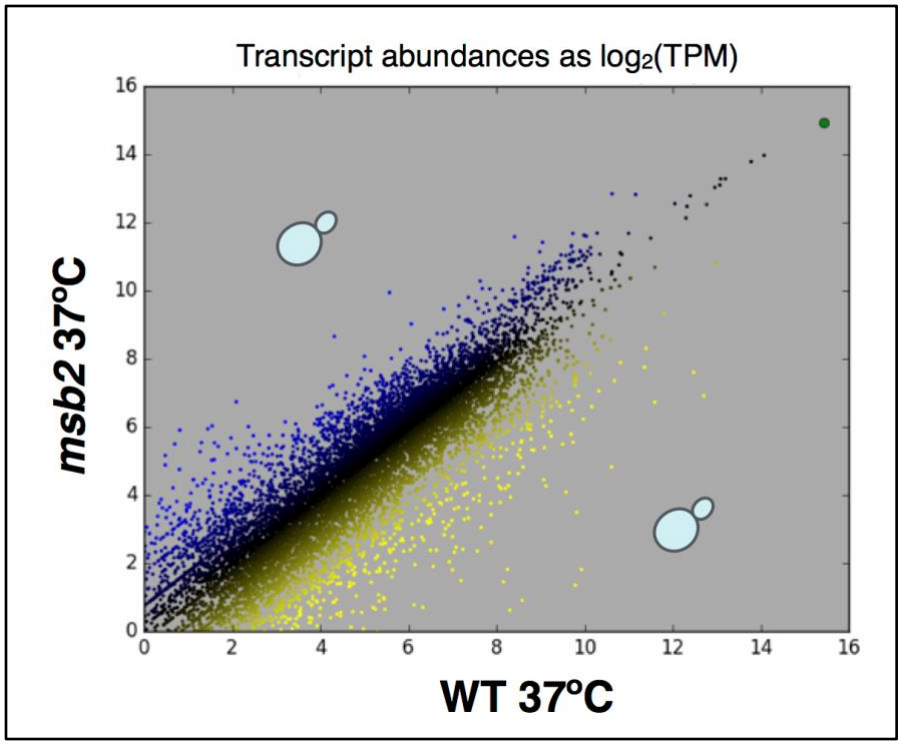


Figure 2-15. Transcript abundances comparing wildtype yeast and *msb2* at 37°C



Spore growth

Dimorphic fungi transition between two physical states and in the case of *H. capsulatum* as well as other fungal species, a third spore state is produced that is specialized in dispersion (Howard, 1962). These airborne spores, or conidia, are considered dormant structures that are incredibly protective from the harsh elements of the environment. In *H. capsulatum* the hyphae and yeast can transition between morphological states simply through undergoing a temperature change. However, with hyphae, specific environmental conditions at room temperature also induce conidiation (Dowding, 1948). The resulting conidia are temperature responsive and have their own transcriptional profile (Inglis et al., 2013). Since *msb2* is in the yeast state at room temperature but transcriptionally resembles hyphae, we asked whether *msb2* would be able to produce conidia.

In order to induce sporulation, yeast cultures are diluted to an optical density of 1 and 150ul are plated on sporulation solid media as described in the Materials and Methods. The plates were then placed at room temperature and after 2 months the conidia were harvested. The conidiation plates looked very similar at the time of harvest. They both showed the characteristic lawn of fungal growth (Figure 2-16). After harvest, the spores were counted and *msb2* produced the same amount of total spores as wildtype (Figure 2-17).

There are two types of spores: microconidia and macroconidia. Their biological differences remain unknown but their physical differences are obvious as discussed in the introduction. Even though the total number of conidia produced by the mutant is comparable to wildtype, we looked to see how the fraction of macroconidia compared in both strains. The resulting analysis showed that, just like the total spore number, the percentage of macroconidia is similar in the strains (Figure 2-18). This suggests that although yeasts lacking *msb2* are unable to

produce hyphae, they are able to bypass this stage and produce total conidia and macroconidia to wildtype levels.

After seeing that *msb2* can produce conidia in the proper conditions, we tested the germination of the mutant spores. They were plated on Sabouraud plates at room temperature to identify the viability and resulting cell morphology in hyphal-inducing conditions. When the plates were examined, there was much less hyphal growth on the *msb2* plate than wildtype at room temperature (Figure 2-19).

It is not uncommon for *H. capsulatum* mutants to produce spores but when germinated, are found to be unviable. To determine viability of the mutant spores, they were also grown on HMM plates at 37°C in 5% CO_2. Unfortunately, it is difficult to quantify an accurate percent yield do to varying plating efficiencies of spores. There is so little known about the biology of these structures that it is unclear whether macroconidia or microconidia germinate at the same rate. Therefore, it is standard practice to simply qualitatively determine if they produce yeast colonies or not following growth at 37°C. Growth of 10-fold dilutions of spores for both wiltype and *msb2 H. capsulatum* produced yeast cultures at 37°C (Figure 2-20). However, it is worth mentioning that *msb2* spores produced a much smaller number of yeast colonies suggesting they have lower viability.

Figure 2-16. *msb2* conidiation plates

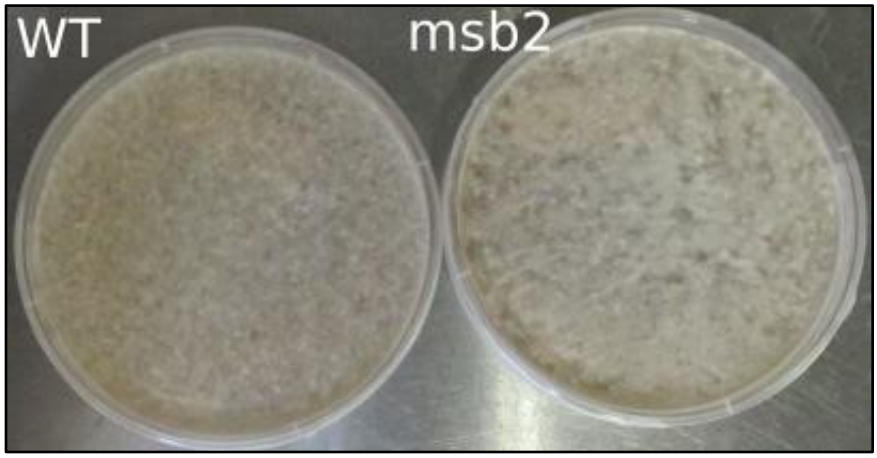


Figure 2-17. Total spore count of *msb2* after harvest

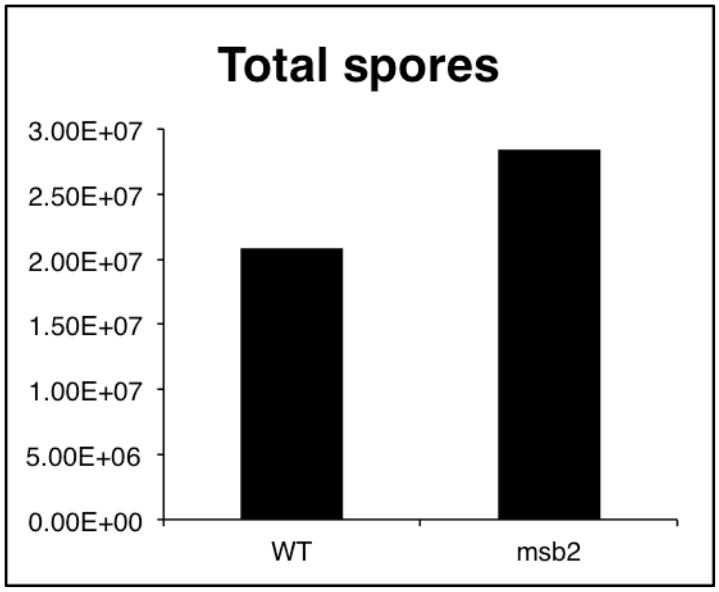


Figure 2-18. Percent macroconidia

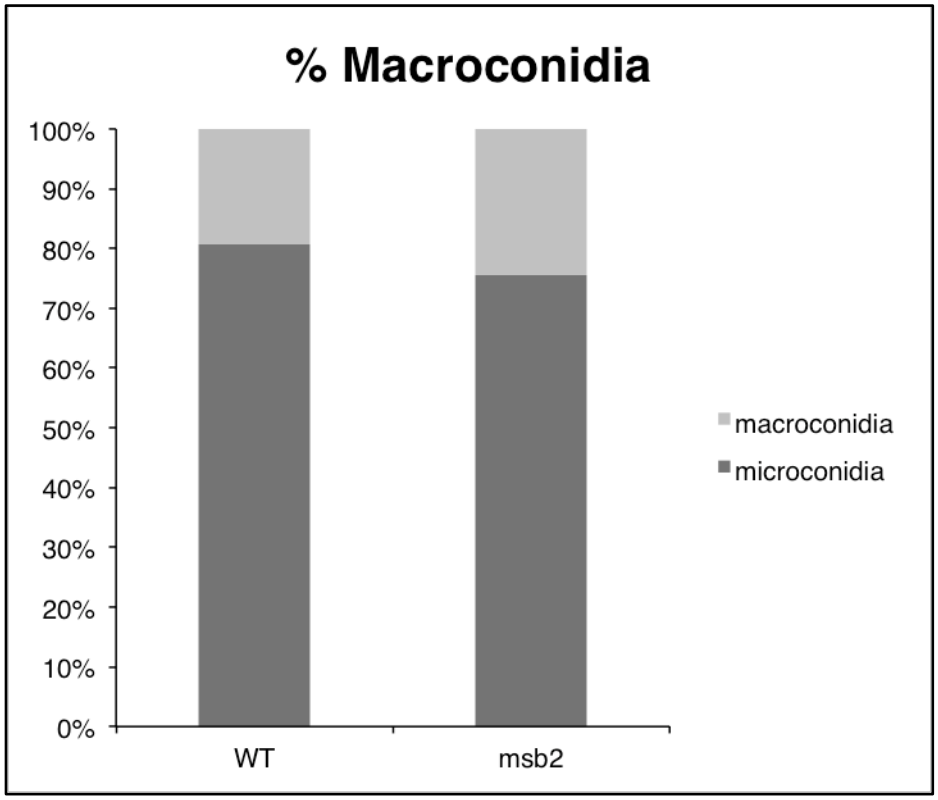


Figure 2-19. *msb2* conidia germination at room temperature

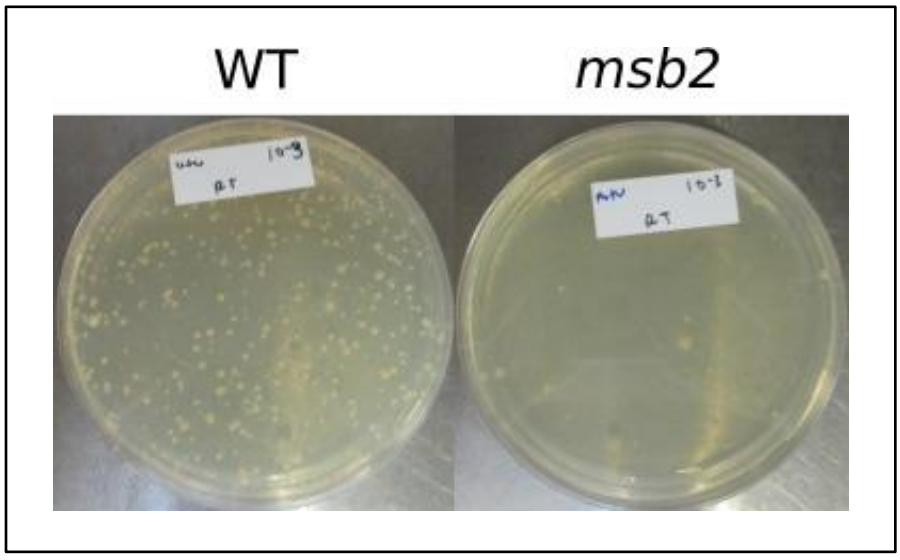
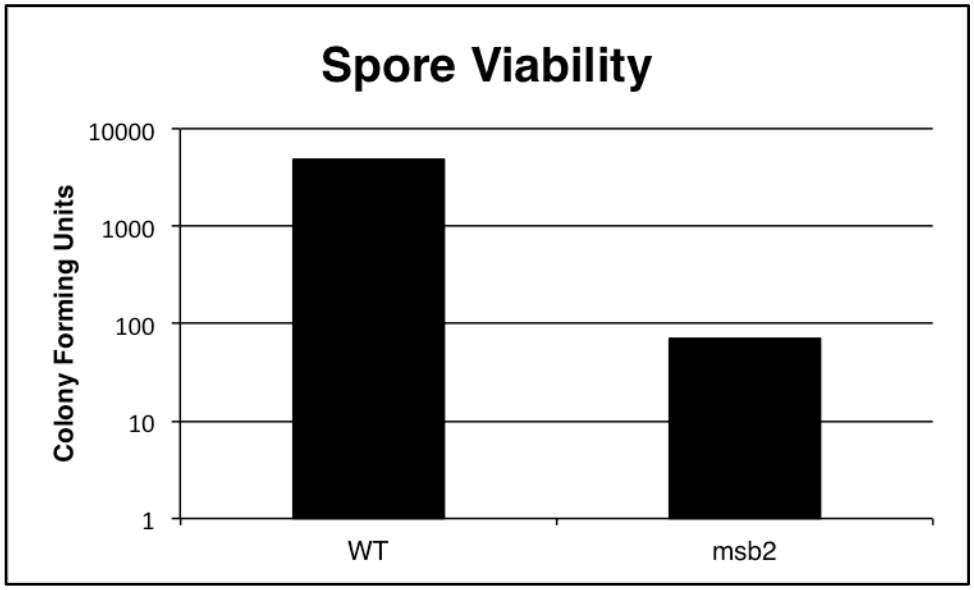


Figure 2-20. *msb2* conidia germination at 37°C to test viability



Cbp1 in supernatants

One of the hallmarks of *H. capsulatum* yeast is the ability to successfully colonize a host. Alveolar macrophages have been identified as one of the first cells that come in contact with *H. capsulatum* yeast following inhalation by a mammalian host. The gene *CBP1*, which encodes a secreted protein, is the most highly expressed in the yeast phase and has been found to be required for proper macrophage lysis as well as dissemination of disease and ultimately animal death (Isaac et al., 2015). Since it is thought of as a yeast-specific protein, we wanted to see if Cbp1 expression is coupled to the yeast morphology in the mutant which we can grow at room temperature and grow as yeast. Cbp1 is a secreted protein so supernatants were collected from wildtype, *msb2* mutant, and *MSB2*-complemented strains, concentrated, and run on a coommassie gel. At about 7kDa, Cbp1 protein can be seen absent from wildtype and complement strains but the *msb2* mutant still highly expressed Cbp1 (Figure 2-21). This supports the claim that Cbp1 expression is coupled to morphology and it is still expressed independent of

the culture temperature. Additionally, supernatants of cultures grown at 37°C were also examined for Cbp1 protein levels where the *msb2* mutant resembles the wildtype and complemented strains very closely (Figure 2-22).

Figure 2-21. Cbp1 expression in *msb2* supernatants at room temperature

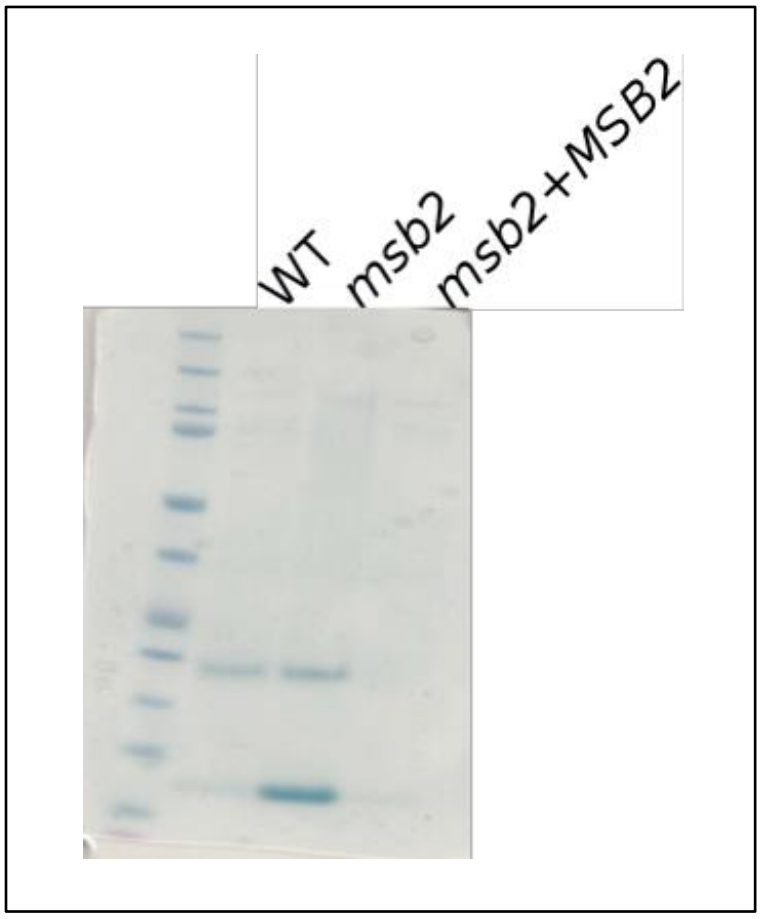
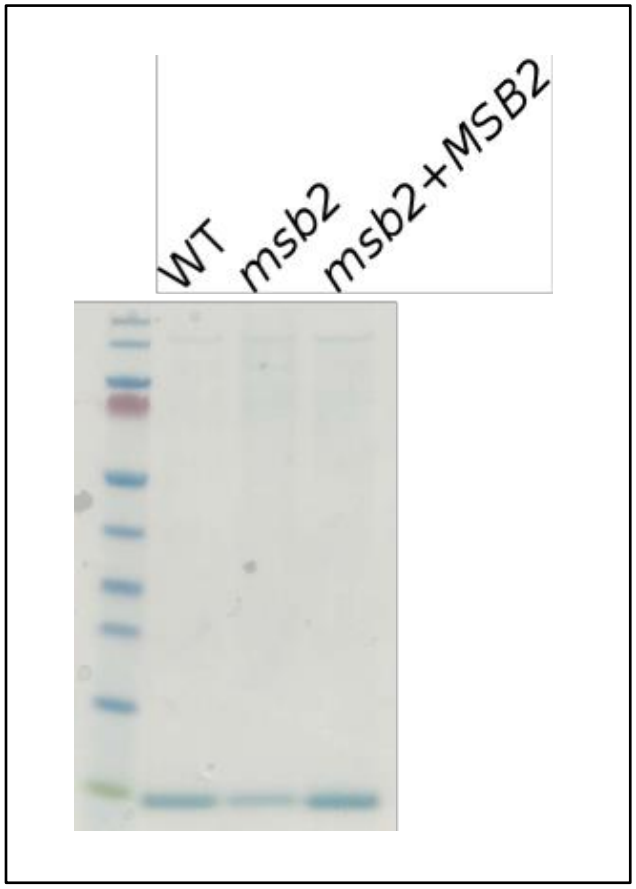


Figure 2-22. Cbp1 expression in *msb2* supernatants at 37°C



***RYP* gene expression at room temperature**

Genes previously identified as promoters of yeast phase growth were probed for expression in the yeast-locked mutant following steady state growth at room temperature. To determine whether these yeast specific genes were misregulated in a strain that remained yeast at room temperature, we explored transcript level by qPCR. We found that mRNA levels in *msb2* at room temperature more closely reflect what we see in wildtype yeast grown at 37°C (Figure 2-23). When a wildtype copy of *MSB2* was added back to *msb2*, wildtype expression level was restored (Figure 2-24).

Figure 2-23. *RYP1-4* mRNA levels at room temperature and 37°C

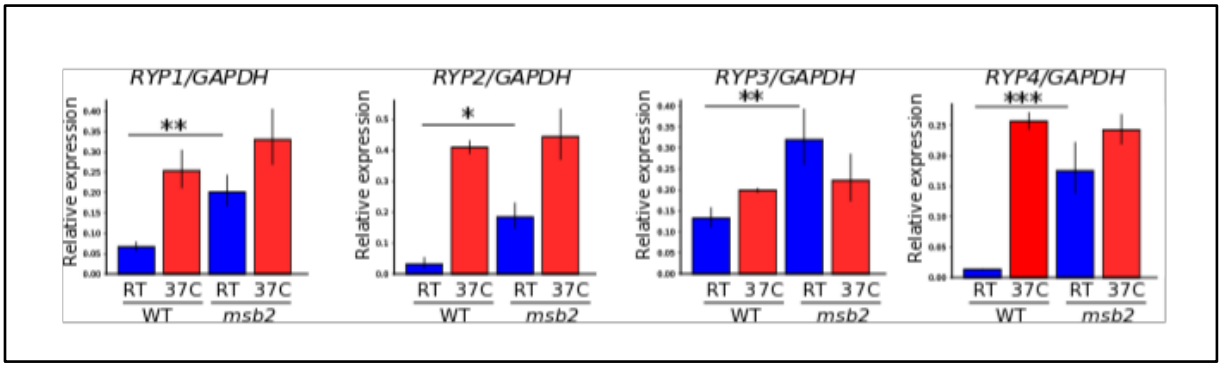


Figure 2-24. *RYP1-4* mRNA levels in MSB2 complementation strain

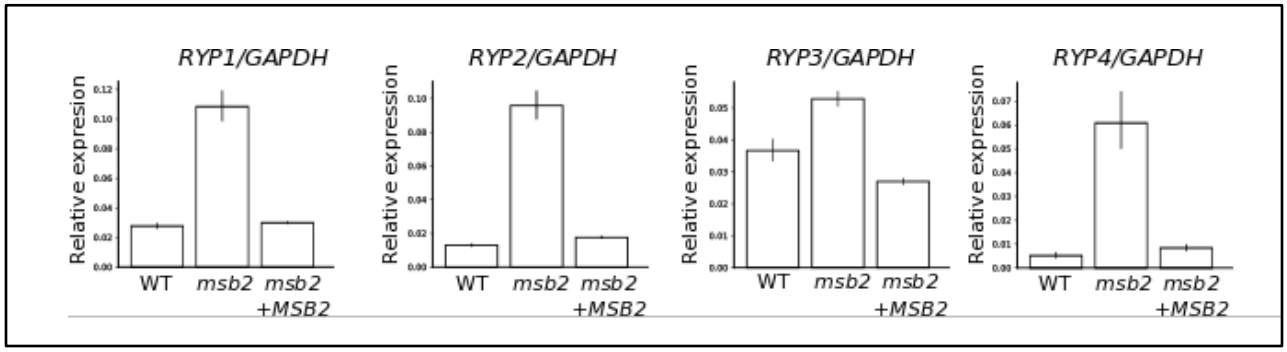


Table 2-1. Primers used for cloning *MSB2*	
Cloning for MSB2 complementation	
forward	GGGGCAACTTTGTACAAAAAAGTTGCCTCCGTACGAAGTGCGAGAGAGAGTCCCA
reverse	GGGGCAACTTTGTACAACAAAGTTGTTACACATACACATACGTCATATAGATCTGT
Construction of MSB2 RNAi	
forward	GGGGCAACTTTGTACAAAAAAGTTGCCTCCATCACTTAAGCTTCCGGACG
reverse	GGGGCAACTTTGTACAACAAAGTTGTTAGTGGAATTGCCAGGGGC
qPCR primers for MSB2 RNAi knockdown validation	
forward	TCCTACCTCCACTTCCACC
reverse	TGTGATGGGCAAAGACTGG
qPCR primer for HOG2 RNAi knockdown validation	
forward	GACCTGTGCCGGAGAGCGTAT
reverse	GTCATGCCTTGGTGCTGTGAT
qPCR primer for RYP1 expression levels	
forward	GGACTCTTACGGGTTCAAGG
reverse	CTGCATCGGGGTATGAAGG
qPCR primer for RYP2 expression levels	
forward	CGGCTCGAGAGATGAAGTCGTT
reverse	AAGTGTACGGGCTTCCTTCCG
qPCR primer for RYP3 expression levels	
forward	CCAAAGGCCAAGATGGAGAAGG
reverse	GGAAATGAGAGGAAGGGGGAAAGA
qPCR primer for RYP4 expression levels	
forward	GAACTTGATGAGTGGCAGAGG
reverse	ACTAGGGCCAGTATGGAAGG

Table 2-2. *H. capsulatum* strains used in the characterization of *MSB2*	
Strain	Genotype
WT in screen	G217B*ura5*$^-$
mutant from screen	G217B*ura5*$^-$ *msb2*::TDNA
WT vector control for complemenation	G217B*ura5*$^-$ + *URA5*
mutant vector control for complemenation	G217B*ura5*$^-$ *msb2*::TDNA + *URA5*
MSB2 comp strain	G217B*ura5*$^-$ *msb2*::TDNA + *MSB2 URA5*

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CHAPTER THREE

Transcriptional profiling to identify *MSB2*-dependent genes and pathways

Introduction

Transcriptional analysis is a massive tool that can be used to ask a variety of questions. To maximize the amount of data and the diversity of questions that could be asked, I set up a timecourse after a temperature shift in two different media, of two different genotypes. This allowed me to find genes that depended on the temperature, the time after transition, the media, as well as *MSB2*. While all of this data is readily available now, I decided to focus on the genes that depended on *MSB2* to hopefully find pathways involved in filamentation. Here I discuss the experimental setup of the RNAseq portion of my project and summarize the gene set that arose from the analysis.

Material and Methods

Culture growth protocol

G217B, *msb2, msb2+MSB2* yeast cells were inoculated off of a plate into 5ml HMM liquid medium and incubated at 37C, shaking, with 5% CO_2. Liquid cultures were passaged 1:25 after 48 hours into HMM media. They were passaged 1:25 again and expanded to 50ml into HMM liquid media with glucose or GlcNAc as carbon source. They were grown for another 48 hours at 37C, 5% CO_2 after which time point 0 was taken. The cultures were then transferred to a room temperature shaker. Time points were taken after 2, 6, and 8 days of growth at room temperature.

Cell collection

Yeast and hyphal cells were collected by filtration with a disposable filtration apparatus either in Biosafety Level 2 laboratory for growth at 37°C or in the Biosafety Level 3 laboratory for growth at room temperature. At each time point, 10mL of fungal culture was passed through a filter (Thermo Fisher), the cells were scraped off with a cell scraper, placed into a conical with 1mL trizol reagent (Qiazol) and flash frozen in liquid nitrogen. Samples were stored at -80°C until all time points were collected.

RNA extraction

Total RNA was extracted from fungal cells using a trizol-based RNA extraction protocol. Frozen, resuspended pellets of cells were incubated at room temperature for 5 minutes to thaw. The lysate was bead beat with 300-500ul zirconia beads for 2 minutes. They were placed on ice immediately after the 2 minutes of beating. 200ul of chloroform was added, vortexed for 15 seconds, incubated at RT for 5 minutes, and centrifuged at 12000g for 20 minutes at 4C. The

aqueous phase contains the RNA. This phase was transferred to a new tube, 600ul of 100% ethanol was added, quickly vortexed and loaded onto an Epoch RNA column. The columns were washed with 300ul of 3M NaOAC, pH 7.5, spun at max speed for 30 seconds then washed with 800ul of wash buffer (10mM TrisCl, pH7.5, 80% EtOH) and spun at max speed for 30 seconds. The columns were spun at max speed to remove excess ethanol. Next they were treated with 40ul Purelink DNase/buffer mix (Thermo Fisher) for 20 minutes at RT. Put 300ul 1:1 mixture of guanidinium lysis butter and 100% EtOH and spun at max speed for 30 seconds. Columns were again washed with 3M NaOAC, pH 7.5, spun, 800ul wash buffer, spun, and spun once more at max speed for 1 minute to dry columns. Finally, RNA was eluted with 50ul RNase-Free H_20. RNA quality was determined with a High Sensitivity DNA bioanalyzer chip from Agilent.

cDNA production

20ug of total RNA was purified for mRNA through polyA selection with Oligo-dT Dynabeads as described in the Thermo Scientific protocol. Ribosomal RNA depletion was confirmed with an RNA 6000 nano bioanalyzer chip from Agilent Technologies. Samples were acceptable for library preparation at 0-4% rRNA.

Library preparation

Libraries for RNAseq were prepared using the NEBNext Ultra Directional RNA Library Prep Kit (New England Biolabs). Individual libraries were uniquely barcoded with NEBNext Multiplex Oligos for Illumina sequencing platform. Average fragment size and presence of excess adapter was determined with High Sensitivity DNA bioanalyzer chip from Agilent Technologies. Libraries had an average fragment length of 300-500bp. The concentration of the

individual libraries was quantified through Qubit dsDNA High Sensitivity Assay (Thermo Fisher). 5ng of each library was pooled into a final library and ran on a High Sensitivity DNA bioanalyzer chip to determine the average fragment size of the final pooled sample. Final library was submitted to the UCSF Center for Advanced Technology for sequencing on the Illumina HiSeq4000 sequencer.

RNA sequencing (RNAseq)

Libraries were multiplexed and subjected to 50 bp single-end sequencing in three independent single-lane runs on an Illumina HiSeq4000 sequencer (Illumina, San Diego, CA), yielding 2 to 16 million reads per sample for a total of 116 samples. All sequencing was performed at the Center for Advanced Technology (CAT) at UCSF.

Transcriptome quantification and abundance filtering

Transcript abundances were quantified based on version ucsf_hc.01_1.G217B of the *Hc* G217B transcriptome (S5 Data of (Gilmore, Voorhies, Gebhart, & Sil, 2015), http://histo.ucsf.edu/downloads/ucsf_hc.01_1.G217B.transcripts.fasta)

Relative abundances (reported as TPM values (Li & Dewey, 2011)) and estimated counts (est_counts) of each transcript in each sample were estimated by alignment free comparison of k-mers between the preprocessed reads and assembled transcripts using KALLISTO version 0.43.0 (Bray, Pimentel, Melsted, & Pachter, 2016).

Further analysis was restricted to transcripts with TPM >= 10 in at least one sample.

```
kallisto quant -i ucsf_hc.01_1.G217B.idx -t 4 -b 100 \
--single --rf-stranded -l 250 -s 50 \
-o SAMPLE.kallisto SAMPLE.fastq.gz
```

Identification of differentially expressed genes

Differentially expressed genes were identified by linear regression on four independent factors using LIMMA version 3.26.1 (Ritchie et al., 2015; Smyth, 2004).

Specifically, KALLISTO est_counts were rounded to the nearest integer and imported as counts per million (CPM) in a DGEList in R. Samples were TMM normalized with calcNormFactors and VOOM (Law, Chen, Shi, & Smyth, 2014) was used to estimate the mean/variance trend.

Samples were classified on four factors (sup_table_factors): batch (1 or 2), genotype (WT or msb2), media (glcNAc or glucose), and time (0, 2d, 6d, or 8d). For each transcript, the observed counts were fit to a model assuming a mean transcript abundance plus independent corrections for each factor using lmFit. Shrinkage of the individual gene variances over the full data was applied with eBayes, and significantly differential genes were identified by extracting genes with a significantly non-zero genotype coefficient (at 5% FDR) with an effect size of at least 2x (absolute log2 fold change >= 1) using topTable.

```
dge <- DGEList(counts)
dge <- calcNormFactors(dge)
```

```
time <- as.factor(time)

genotype <- as.factor(genotype)

media <- as.factor(media)

batch <- as.factor(batch)

d <- model.matrix(~time+genotype+media+batch)

v <- voom(dge, d)

fit <- lmFit(v, d)

fit <- eBayes(fit)

topTable(fit, coef="genotypeMSB2", n = 20000, lfc=1, p.value = .05)
```

Results

msb2 mutant fails to produce hyphae over 8 day timecourse at room temperature

We have previously shown that mutant strains unable to transition from hyphae to yeast at 37°C result in global disruption of the transcriptome (Beyhan, Gutierrez, Voorhies, & Sil, 2013). This provided precedent to survey the transcriptional profile of the yeast-locked mutant in comparison to the wildtype strain over the course of normal morphological transition. This would allow us to identify genes and pathways required for hyphal growth. Yeast cultures were grown in normal yeast-promoting conditions (37°C, 5% CO_2) and transferred to room temperature without CO_2 to promote filamentation (Figure 3-1). This timecourse was performed in two different carbon sources, glucose (Figure 3-2) and N-Acetylglucosamine, (Figure 3-3). GlcNAc has been shown to expedite the transition from yeast to hyphae at room temperature (Gilmore, Naseem, Konopka, & Sil, 2013) and these results reproduce that observation with wildtype and the complement strain producing filaments after 2 days at room temperature. However, GlcNAc was unable to override the morphology defect in the yeast-locked mutant at room temperature which remained yeast over the timecourse. RNA was isolated from wildtype, *msb2*, and the *MSB2* complement strain and we performed RNAseq analysis in both media at 37°C and 2, 6, and 8 days post transition to room temperature.

Figure 3-1. RNAseq timecourse: experimental design

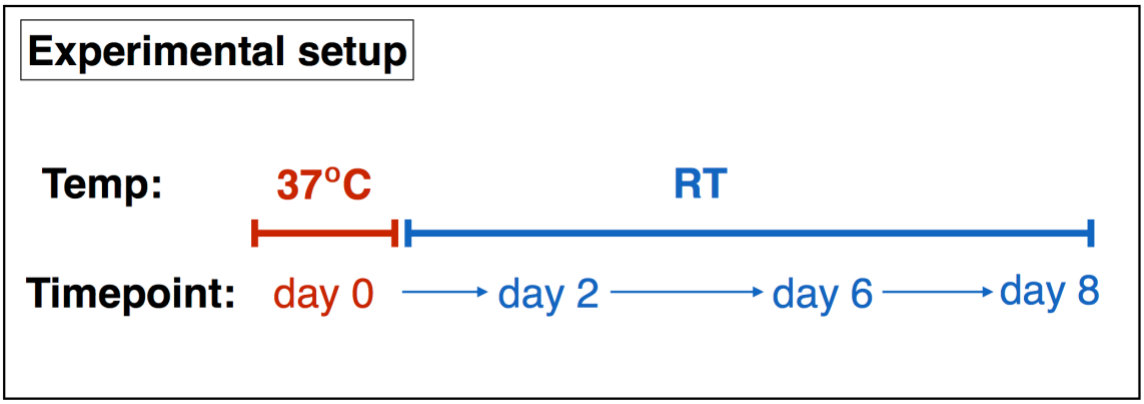


Figure 3-2. Cell morphology: glucose

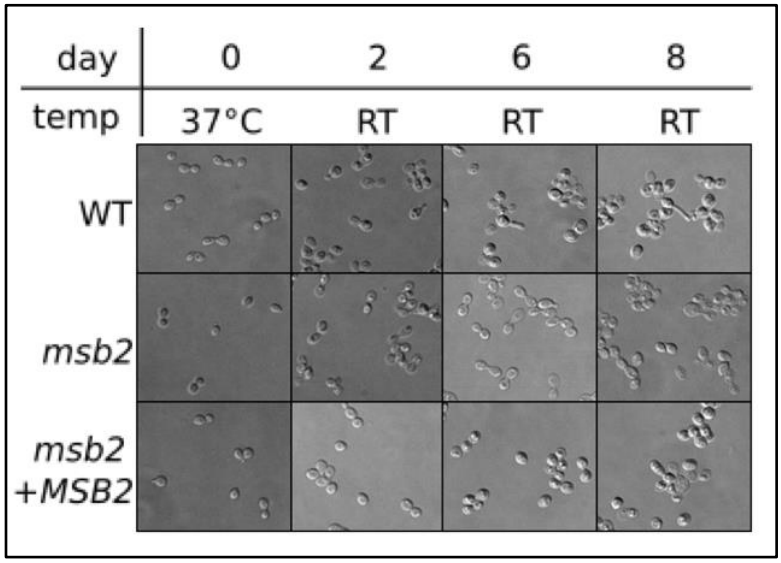
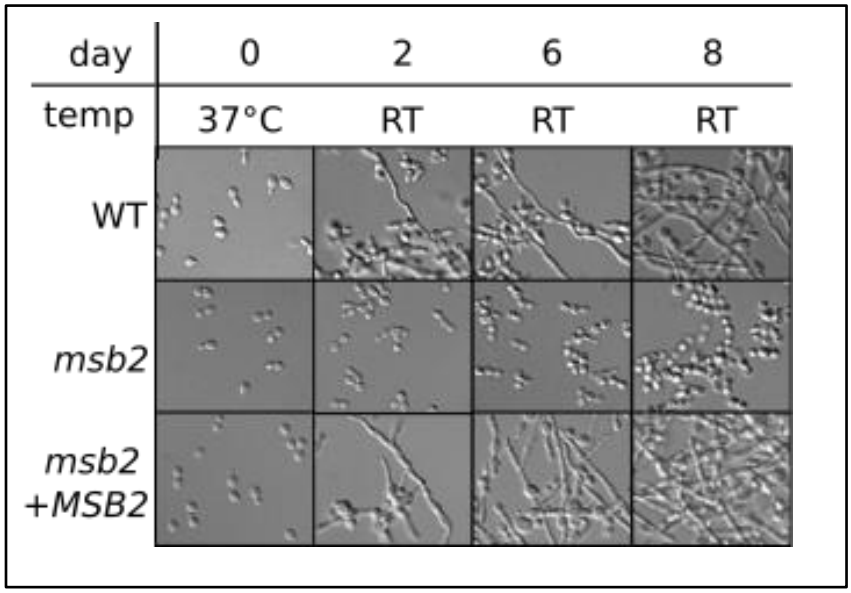


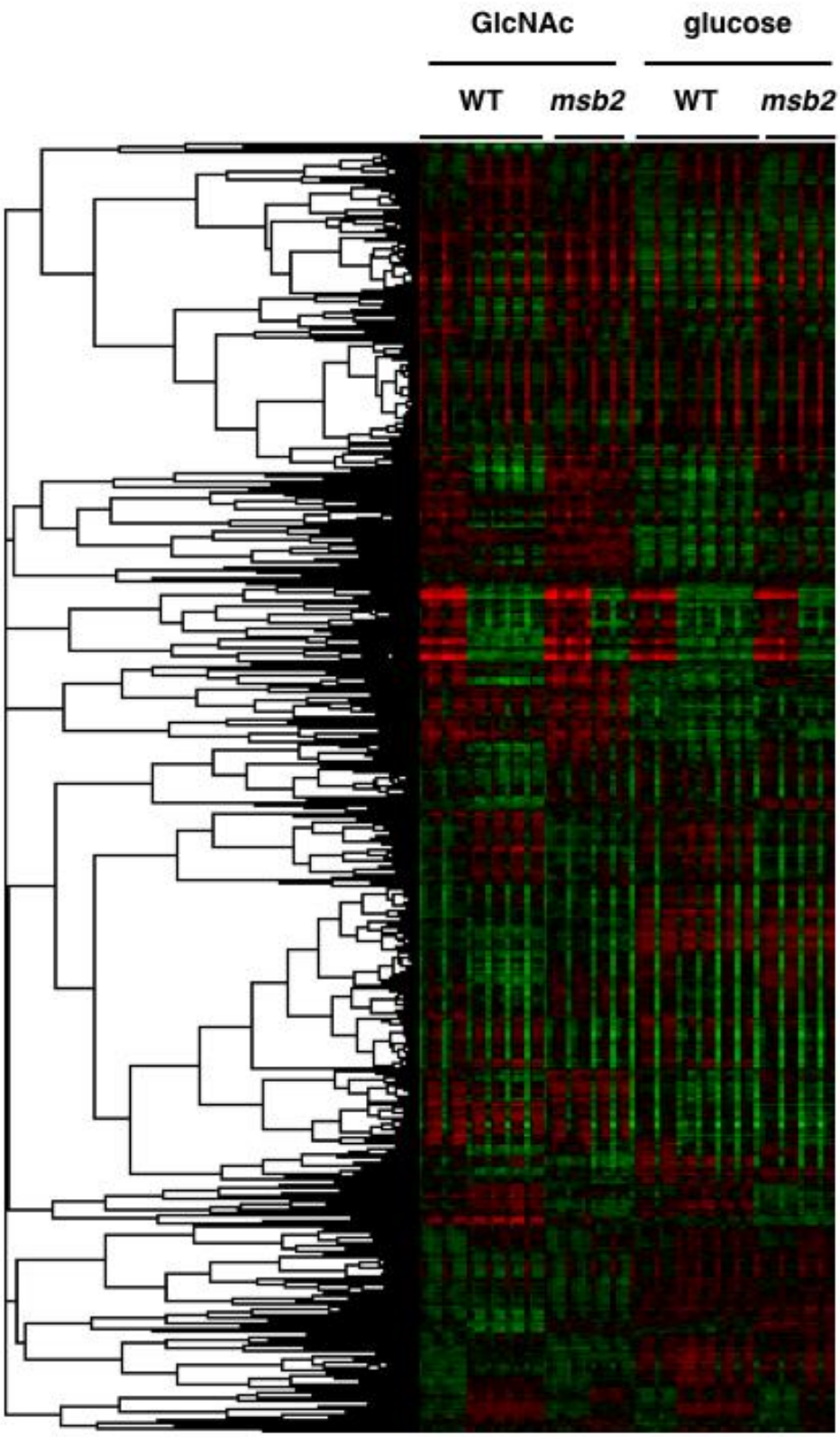
Figure 3-3. Cell morphology: glcNAc



Transcriptional profiling of entire *H. capsulatum* transcriptome identifies 11,618 genes

The global transcriptional profile was observed after the data was processed through an established pipeline as we describe in the methods. The transcripts were normalized to the wildtype level at 37°C to identify genes that deviated from the normal expression pattern after transition to room temperature. In the overall heatmap, red indicates genes that have increased expression at room temperature and green indicates decreased expression at room temperature (Figure 3-4).

Figure 3-4. Heatmap of the entire transcriptional profiling dataset



Identifying genes that are dependent on *MSB2*

Since we are interested in the genes dependent on *MSB2,* we compared transcript abundances between various conditions to identify incorrectly expressed genes in the yeast-locked mutant. When wildtype yeast and wildtype hyphae were compared, the transcript abundances were obviously very different between cell morphologies (Figure 3-5). This result is comparable to the phase specific gene number we have identified in previous datasets (Gilmore et al., 2015; Inglis, Voorhies, Hocking Murray, & Sil, 2013). We also show the comparison between wildtype yeast at 37°C and mutant yeast that had been grown for 8 days at room temperature (Figure 3-6). This comparison shows that while the morphology is the same, the transcriptional profile is very different. When wildtype hyphae and mutant yeast from day 8 at room temperature was examined, these morphologically distinct strains are surprisingly, transcriptionally similar (Figure 3-7). We conclude from these data that the majority of the temperature-dependent transcriptional program remains intact in the yeast-locked mutant and the small set of genes that are *MSB2*-dependent are candidates for regulators or effectors of filamentation.

Figure 3-5. Transcript abundance comparison: wildtype yeast vs wildtype hyphae

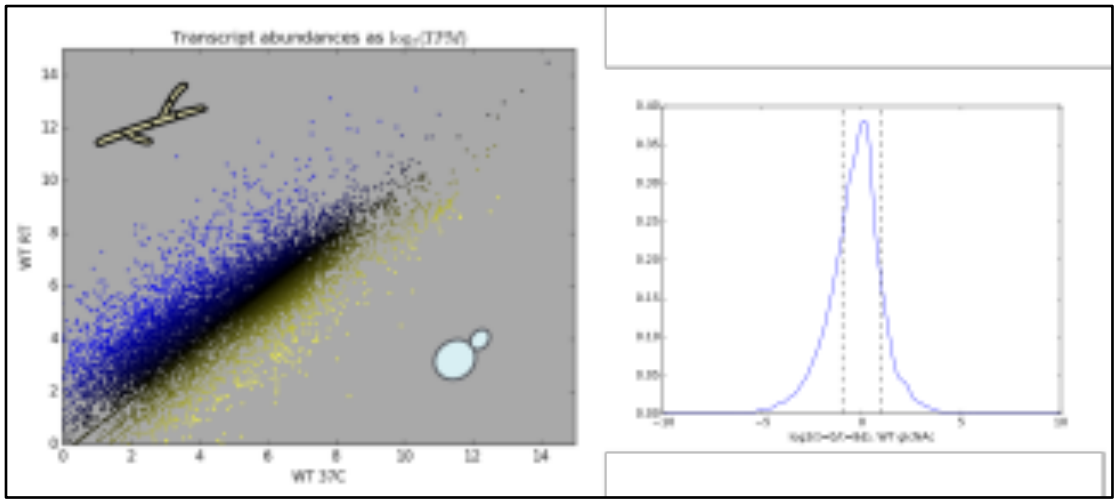


Figure 3-6. Transcript abundance comparison: wildtype yeast vs *msb2* room temp. yeas

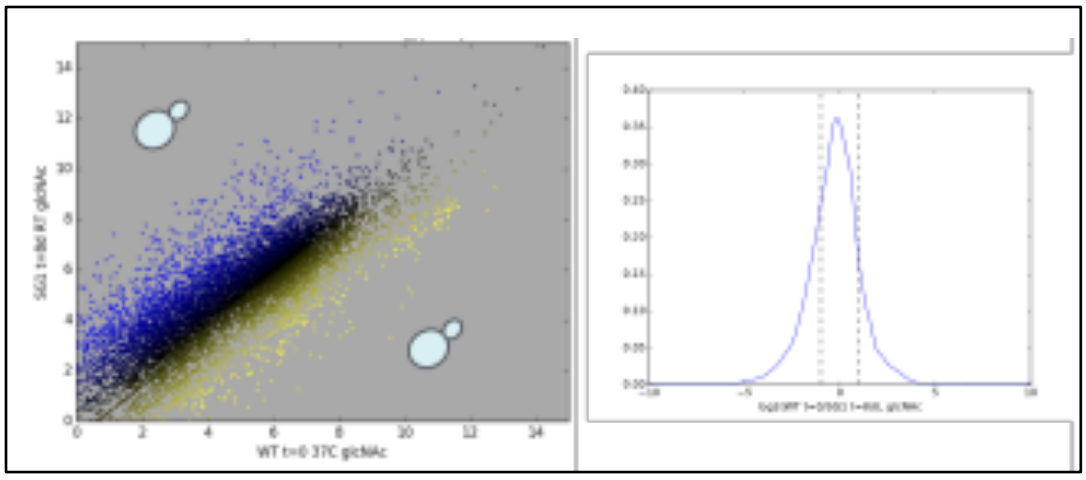
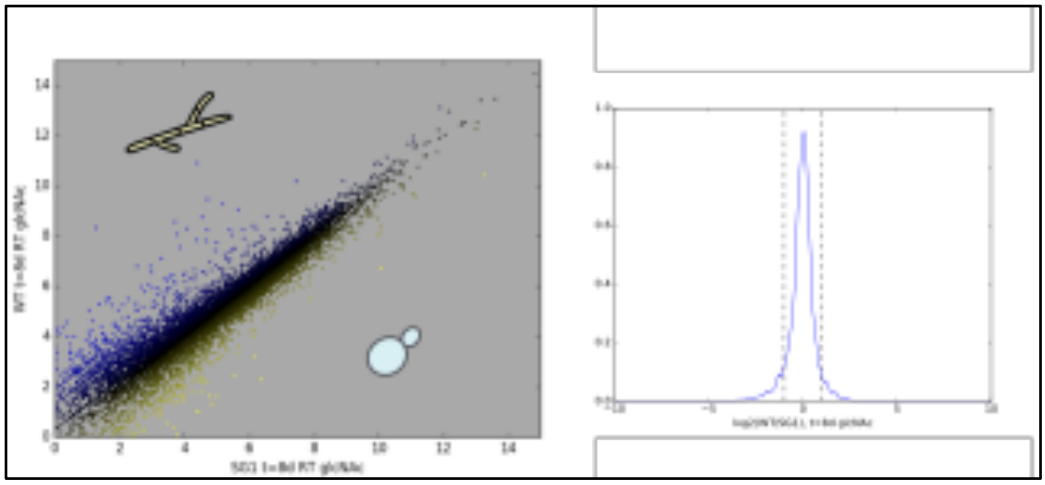


Figure 3-7. Transcript abundance comparison: wildtype hyphae vs *msb2* room temp. yeast



Genes of known morphology or temperature-dependence are identified as *MSB2*-dependent

We looked into transcripts that failed to express to wildtype levels in the mutant at room temperature. Since this gene set represents genes turned on at room temperature in wildtype but not expressed, with at least a 2-fold difference, in the *msb2* mutant we cross-referenced with what we previously have established as hyphal-specific genes in *H. capsulatum*. This identified our hyphal-specific genes that are differentially expressed at room temperature in an *MSB2*-dependent manner.

Two genes *flbA* and *flbC* are hyphal-specific genes that are upregulated in wildtype *H. capsulatum* at room temperature but are not in *msb2*. They have orthologs in *Apergillus* where their function has been elucidated as regulators of conidiation (Wieser, Lee, Fondon, & Adams, 1994).

MS8 and *MS95* have been shown to be highly hyphal-enriched in *H. capsulatum* through transcriptional profiling and ribosomal footprint analysis (Tian & Shearer, 2002). That result is replicated in these data showing that *MS8* and *MS95* are upregulated as early as 2 days at room temperature and that expression pattern is absent in the *msb2* mutant. Their function and how it pertains to hyphal growth remains unclear but their expression is reliably specific for growth at room temperature.

Two genes that have also been characterized as highly upregulated at room temperature however, their function has been studied in *H. capsulatum* are *TYR1* and *TYR3*. They are both tyrosinases and these data agree with their hyphal-specific characterization. Additionally they cluster together supporting that their transcriptional pattern over the time course at room temperature is very similar.

Lastly, two genes that caught my interest were *STU1* and *HOG2*. *STU1* is an APSES transcription factor that has orthologs in other fungi. In *C. albicans*, its ortholog, *EFG1*, has been thoroughly characterized as a promoter of filamentation (Stoldt, Sonneborn, Leuker, & Ernst, 1997). Additionally, the Aspergillus ortholog, *StuA*, has been well studied for its role in morphological development (Miller, Toennis, Adams, & Miller, 1991; Miller, Wu, & Miller, 1992). These results support a role in filamentous regulation of *H. capsulatum STU1* that is explored further in the next chapter.

The other gene, *HOG2*, was initially identified through phylogenetic analysis of mitogen-activated protein (MAP) kinases of fungi. It is a recent paralog of *HOG1* and seems to only appear in dimorphs. Since the *MSB2* orthologs in *S. cerevisiae* and *C. albicans* function in the HOG pathway (Alonso-Monge et al., 1999; Tatebayashi et al., 2007), this gene hit was one we decided to follow. Additionally, the kinase cascades are very common modules for signaling

from extracellular stimuli to a downstream transcriptional response. With this in mind, I performed a more rigorous molecular analysis on these potential regulators of filamentation that will be presented in Chapter 4.

Table 3-1. Genes failed to be induced in *msb2* at room temperature

Gene	Description
flbA	*Aspergillus* ortholog is a developmental regulator of conidiation
flbC	*Aspergillus* ortholog is a developmental regulator of conidiation
MS8	highly expressed at room temperature
MS95	highly expressed at room temperature
TYR1	tyrosinase
TYR3	tyrosinase
STU1	APSES transcription factor; *C. albicans* ortholog, *EFG1,* is a promoter of filamentation
HOG2	MAP kinase; paralog of *HOG1*

Summary

While initially I hypothesized that *MSB2* is required for the room temperature gene expression signature, these data support that in fact there is a relatively small set of genes that are *MSB2*-dependent. These are now considered potential regulators of hyphal growth and I used multiple criteria when deciding which ones to follow up on for my thesis. After an extensive literature search, it was interesting to find that various genes fell into similar categories of interest. These categories included regulators of morphology in other fungi and known highly expressed mold genes in *H. capsulatum.* These findings were encouraging because of the nature of the experimental setup to identify genes involved in morphological regulation. In the next chapter I show the follow up analysis of *STU1*, a transcription factor that has orthologs in other fungi, and *HOG2,* a mitogen-activated protein kinase recently identified in dimorphic fungi.

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CHAPTER FOUR

Genetic analysis of *HOG2*, *STU1* and a morphology-dependent signaling pathway

Introduction

Setting up the transcriptional profiling experiment over the course of a morphological transition helped identify a small set of genes that are potential regulators of filamentous growth. From that list, the genes I followed up on are *HOG2*, a recent paralog of the MAP kinase *HOG1*, and the APSES transcription factor *STU1*. Here I show the molecular analysis of these genes in order to further understand what role they play in filamentation. These genes were chosen for follow up experiments due to a few reasons. *STU1* has been extensively studied in other fungi such as *C. albicans* and *Aspergillus sp*. and its role as a regulator of filamentation in these fungal species motivated a closer look at its function in *H. capsulatum*. Since it is a transcription factor, I looked for what could be relaying the message between Msb2, which is a transmembrane protein, and the nucleus where Stu1 would localize. This led me to look into *HOG2* which is a MAP kinase. The phosphorylation cascades are a common module used for signal transduction between extracellular environmental cues and a transcriptional response so it made sense to look further into the role *HOG2* plays in this pathway. Here I show the results from experiments designed to identify a pathway involved in morphological regulation.

Materials and Methods

Strains

Histoplasma capsulatum G217BΔura5 was used for the insertional mutagenesis screen which was performed using the *Agrobacterium tumficiens* strain LBA1100 containing the plasmid pRH5b as described (Marion, Rappleye, Engle, & Goldman, 2006; Sullivan, Rooney, & Klein, 2002; Zemska & Rappleye, 2012). All strain manipulations were done in the Histoplasma capsulatum G217BΔura5 background. Sub-cloning was performed in *E. coli* DH5α. Strains used in the characterization of *MSB2* can be found in Table 4-1.

Construction of strains

Knockdown strains were produced using the RNAi method as previously described (Rappleye, Engle, & Goldman, 2004). Multiple 500bp regions of each gene were chosen as RNAi targets. Transformants were screened for knockdown efficiency through qPCR of target gene compared to an empty control vector (pSB200). Overexpression strains were constructed by amplifying the coding region of the gene and cloning into an expression vector with a strong promoter (pACT1, pGAPDH). The control vectors for these strains are pTM4 for pACT1 and pSB275 for pGAPDH. Cell morphology of transformants was screen through growth at appropriate temperature. Primers used in this section are show in Table 4-2.

RNA extraction

Total RNA was extraction from fungal cells using a trizol-based RNA extraction protocol. Frozen, resuspended pellets of cells were incubated at room temperature for 5 minutes to thaw. The lysate was bead beat with 300-500ul zirconia beads for 2 minutes. Place on ice immediately

after the 2 minutes of beating. Add 200ul of chloroform, vortex 15 seconds, incubate at RT for 5 minutes, and centrifuge at 12000g for 20 minutes at 4C. The aqueous phase contains the RNA. Transfer this phase to a new tube, add 600ul of 100% ethanol, quickly vortex and load onto an Epoch RNA column. Wash filter with 300ul of 3M NaOAC, pH 7.5 and centrifuge at max speed for 30 seconds. Wash filters with 800ul of wash buffer (10mM TrisCl, pH7.5, 80% EtOH). Centrifuge at max speed for 30 seconds. Spin columns at max speed to remove excess ethanol. Treat each column with 40ul Purelink DNase/buffer mix for 20 minutes at RT. Put 300ul 1:1 mixture of guanidinium lysis butter and 100% EtOH and spin at max speed for 30 seconds. Wash filters with 3M NaOAC, pH 7.5 and centrifuge at max speed for 30 seconds. Wash filters with 800ul wash buffer and spin at max speed. Spin at max speed for 1 minute to dry columns. Elute with 50ul RNase-Free H_20. RNA quality was determined with a High Sensitivity DNA bioanalyzer chip from Agilent.

Expression level by qRT-PCR

Relative gene expression was observed using qRT-PCR. RNA extraction was performed as stated above and cDNA was prepared by priming 2ug DNase treated total RNA with oligodT/pdN9 and 10uM dNTPs for 5 minutes at 65°C. Samples were brought down to room temperature and 1ul RNaseOUT Recombinant Ribonuclease Inhibitor from ThermoFisher and 0.5ul Thermo Scientific Maxima H Minus Reverse Transcriptase. Negative controls include samples without the addition of reverse transcriptase. Samples +RT were diluted 1:50 and samples –RT were diluted 1:10. qPCR reactions were set up using 96-well plates with Roche FastStart Universal SYBR Green Master (Rox) and 1.5uM primers (found in Table 4-2) and

plates were read using Mx3000P machine (Stratagene) and analyzed using MxPro software (Stratagene).

Imaging

Imaging was performed on a Zeiss AxioCam MRM microscope DIC at 100X magnification.

Results

Expression of a transcription factor, *STU1,* is sufficient for filamentation

Orthologs of the transcription factor *STU1* have been thoroughly studied in species across the ascomycetes. The *Candida albicans* ortholog, *EFG1,* has been observed as a promoter of filamentation (Stoldt, Sonneborn, Leuker, & Ernst, 1997) and various sequencing strategies have identified binding sites upstream of genes it transcriptionally regulates (Hernday et al., 2013; Leng, Lee, Wu, & Brown, 2001; Pujol, Srikantha, Park, Daniels, & Soll, 2016; Sohn, Urban, Brunner, & Rupp, 2003). The ortholog in the more closely related filamentous *Aspergilli* species, *StuA*, has been extensively studied as required for the asexual cycle (Miller, Toennis, Adams, & Miller, 1991; Miller, Wu, & Miller, 1992) thus establishing precedent to investigate the role of *STU1* in filamentation of *H. capsulatum*. Overexpression of *STU1* in wildtype *H. capsulatum* results in a filamentous phenotype at 37°C (Figure 4-1). When *STU1* was overexpressed in the *msb2* mutant background, the filamentous phenotype at room temperature was rescued as well as filamentation was observed at 37°C (Figure 4-2). These data show *STU1* is sufficient for filamentation at 37°C. Additionally, the RNA-seq analysis showed the *msb2* mutant failed to induce *STU1* transcription to wildtype expression levels at room temperature (Figure 4-3). To confirm *MSB2* is required for *STU1* expression, *STU1* transcript level was measured by qRT-PCR in the *msb2* knockdown strain at room temperature and 37°C (Figure 4-4). The data show *STU1* expression is significantly ($p<0.001$) depleted in the *msb2* knockdown strain at room temperature. In a strain complemented with a wildtype copy of *MSB2*, *STU1* expression is restored to wildtype levels at room temperature (Figure 4-5). We also utilized our *MSB2* overexpression strain to observe *STU1* transcript levels in a mutant that is filamentous at 37°C.

We found that after growth at 37°C, while the wildtype strain remains yeast, the *MSB2* overexpression strain is hyphal and by qRT-PCR we confirmed that *STU1* is inappropriately upregulated in this strain (Figure 4-6).

These data support the requirement of *MSB2* for *STU1* expression at room temperature, which is sufficient for filamentation. With *MSB2* as a putative transmembrane signaling protein and *STU1* a transcription factor, we then turned our attention to identifying signaling components that could function between these two filament promoting elements.

Figure 4-1. *STU1* overexpression phenotype at 37°C

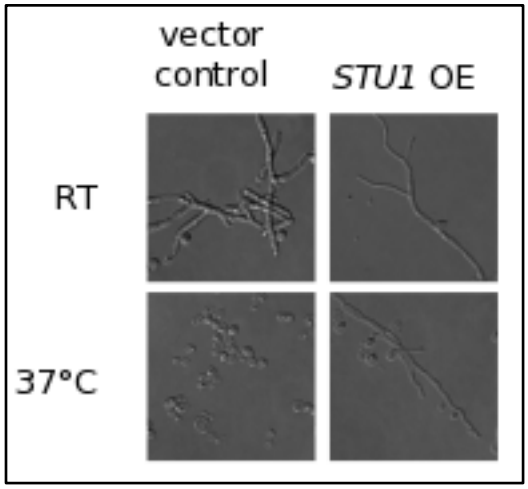


Figure 4-2. *STU1* overexpression phenotype in *msb2* background at room temperature and 37°C

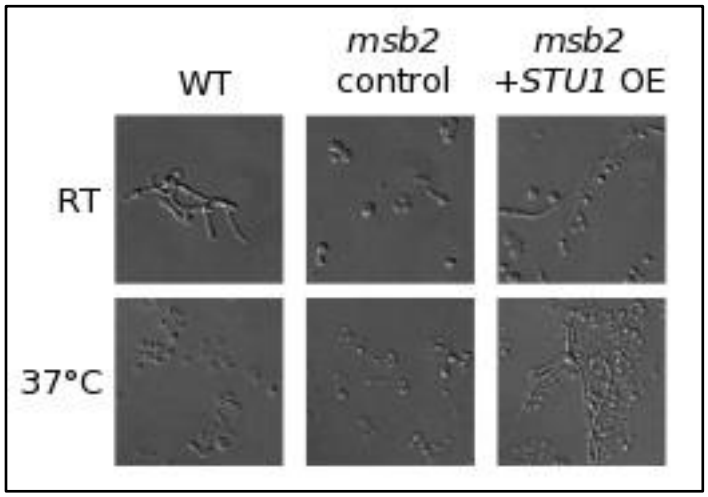


Figure 4-3. *STU1* transcript level at room temperature in RNAseq data

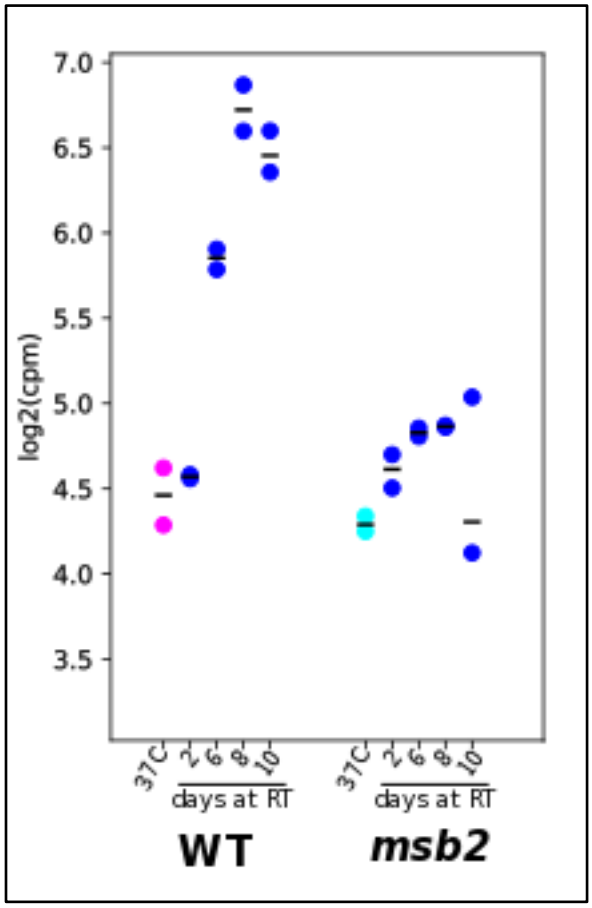


Figure 4-4. *STU1* transcript level in *msb2* RNAi strain at room temperature and 37°C by qRT-PCR

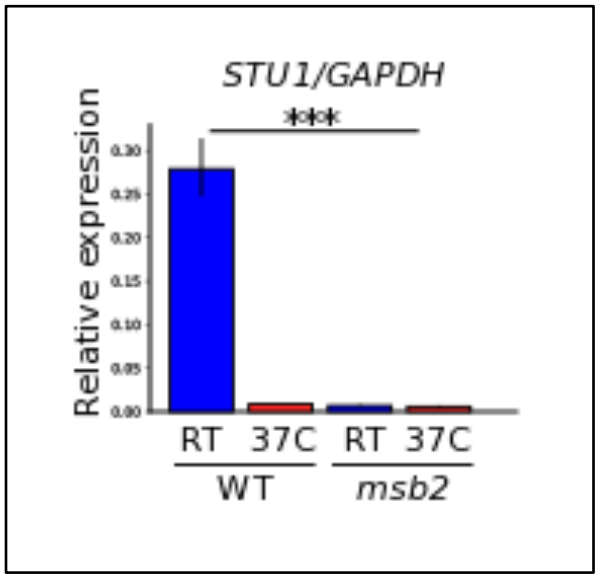


Figure 4-5. *STU1* transcript level in *MSB2* complemented strain at room temperature

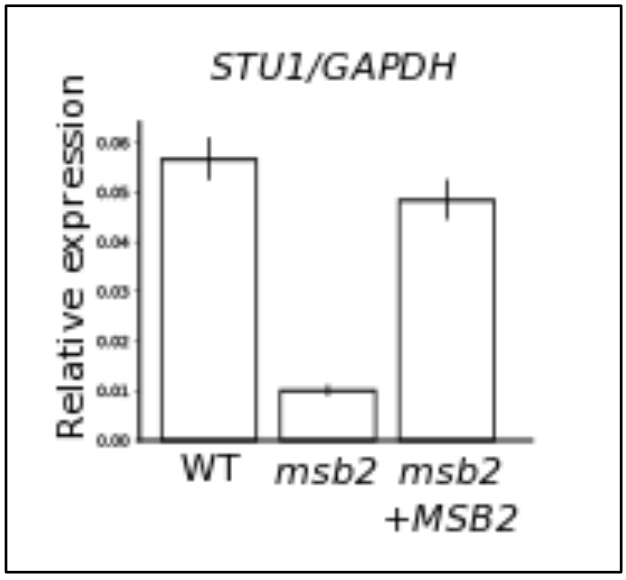
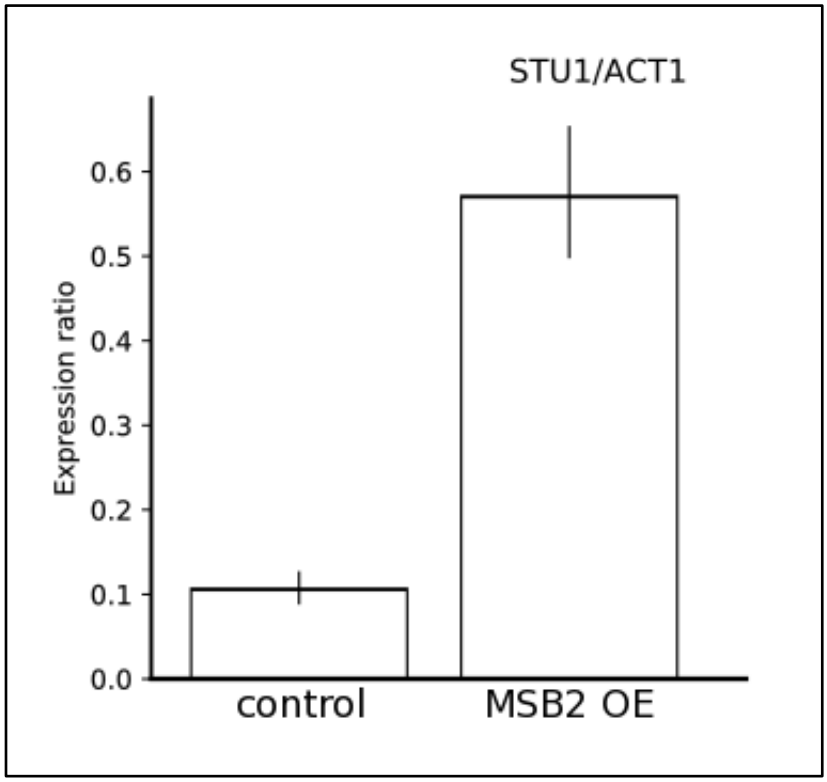


Figure 4-6. *STU1* transcript level in the *MSB2* overexpression strain at 37°C



The MAP kinase *HOG2* is an *MSB2*-dependent signaling element

The systematic truncations of the various domains of *MSB2* have allowed for functional regions to be identified (Swidergall, van Wijlick, & Ernst, 2015). This has supported the role of *MSB2* as a transmembrane signaling molecule and more specifically, as one of signaling proteins in the *Saccharomyces cerevisiae* high osmolarity glycerol (HOG) pathway (Tanaka et al., 2014; Tatebayashi et al., 2007; Yamamoto, Tatebayashi, & Saito, 2015; Zuzuarregui, Li, Friedmann, Ammerer, & Alepuz, 2015). The mitogen-activated protein kinase (MAPK) involved in this pathway is *HOG1* (Brewster & Gustin, 1994), which undergoes phosphorylation and translocation to the nucleus where the signal continues in the form of transcriptional regulation.

It was of interest to us to investigate how *MSB2* signals downstream from the membrane and MAP kinase cascades have been well characterized as signaling pathways activated by transmembrane elements. Using phylogenetic analysis, we identified 4 MAP kinases in *H. capsulatum* closely related to other fungal MAP kinases (Figure 4-7). We looked at the gene expression of these 4 genes in the transcriptional profiling dataset to determine if any of them are dependent on *MSB2* expression. While *SLT2*, *HMK1*, and *HOG1* exhibited transcript levels in the *msb2* mutant strain comparable to wildtype, *HOG2* had more than a 2-fold decrease in expression compared to wildtype at all timepoints at room temperature (Figure 4-8) and expression is restored to wildtype levels in the *MSB2* complement strain. This showed *HOG2* is dependent on *MSB2* expression and indicated a potential signaling component required for hyphal growth. A *HOG2* RNAi strain was constructed and when grown at room temperature the isolates with efficient knockdown levels were yeast-locked indicating that *HOG2* expression is required for filamentation at room temperature (Figure 4-9). Additionally, when *STU1* expression level was probed by qRT-PCR, it was dramatically decreased compared to *STU1* expression in wildtype at room temperature (Figure 4-10). Finally, using the *MSB2* overexpression strain, we found that *HOG2* is transcriptionally upregulated in the strain that is filamentous at 37°C suggested that the transmembrane protein drives expression of the MAP kinase in this signaling pathway (Figure 4-11). Taken all together we conclude that *MSB2* is the most upstream element, required for *HOG2* and *STU1* expression, and *HOG2* lies upstream of *STU1*.

Figure 4-7. Phylogenetic analysis of fungal mitogen-activated protein (MAP) kinases

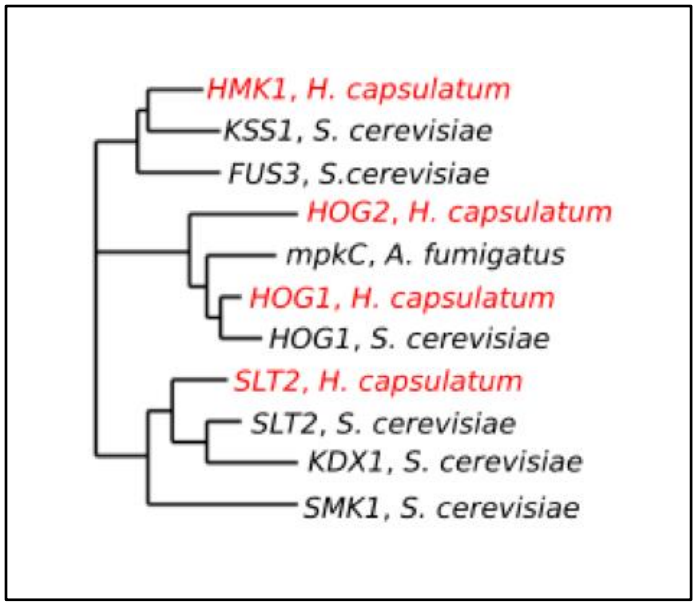


Figure 4-8. Heatmap showing expression of the 4 MAPKs at room temperature

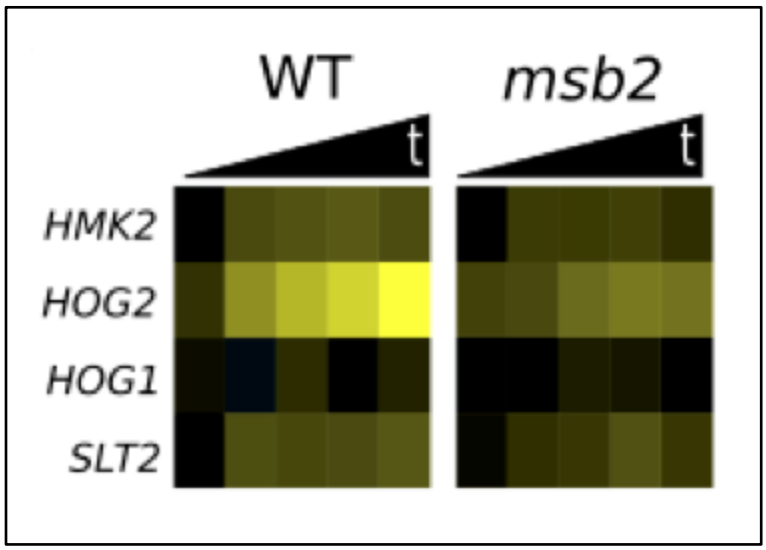


Figure 4-9. ***HOG2*** **RNAi knockdown transcript level and cell morphology at room temperature**

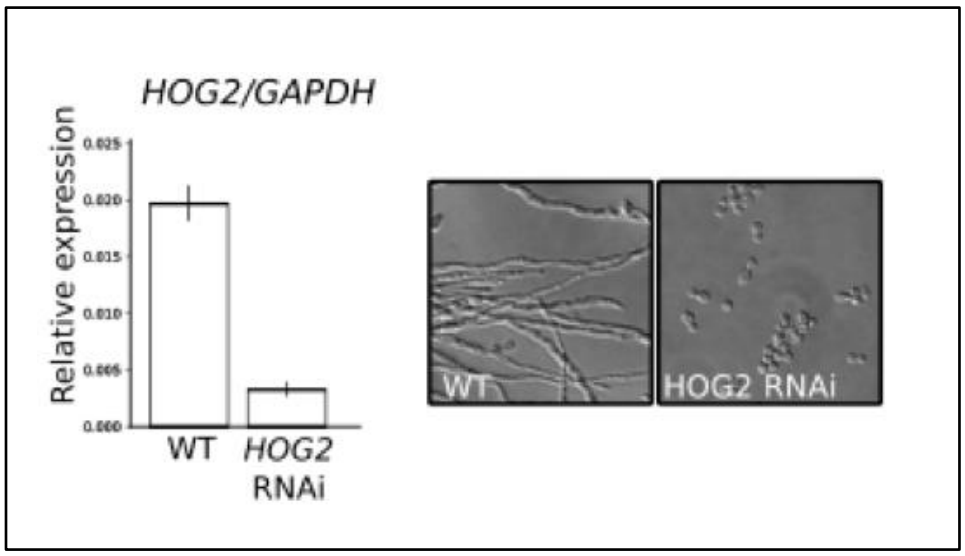


Figure 4-10. ***STU1*** **transcript level in the** ***HOG2*** **knockdown strain**

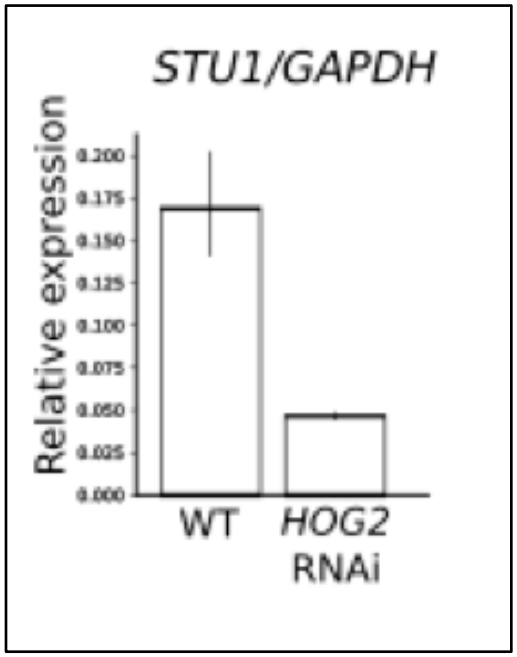


Figure 4-11. *HOG2* expression in *MSB2* overexpression strain

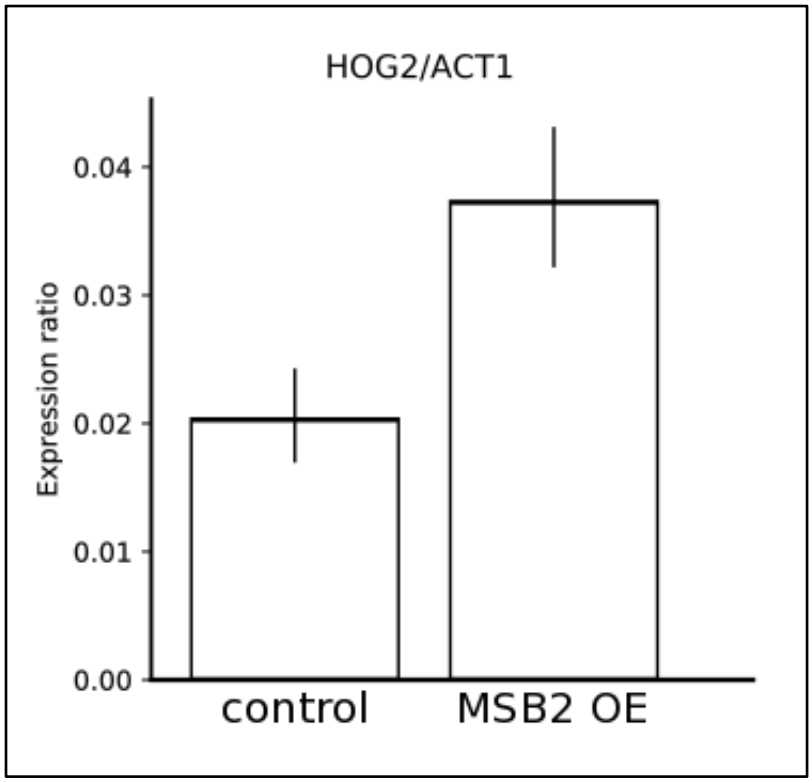


Table 4-1: Strains used to investigate the HOG pathway

STU1 OE	G217B*ura5*⁻ pACT1:*STU1* + *URA5*
HOG2 RNAi	G217B*ura5*⁻ *hog2*::RNAi + *URA5*
MSB2 RNAi	G217B*ura5*⁻ *msb2*::RNAi + *URA5*
MSB2 OE	G217B*ura5*⁻ pGAPDH:*MSB2* + *URA5*

Table 4-2. Primers used for cloning *STU1* and *HOG2*

qPCR primer for HOG2 RNAi knockdown validation	
forward	GACCTGTGCCGGAGAGCGTAT
reverse	GTCATGCCTTGGTGCTGTGAT
qPCR primer for STU1 expression levels	
forward	TGGTCAAATACCACCACCTG
reverse	CGCAACACAAACACCCTTAG
Construction of HOG2 RNAi	
forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAGCATTTCCTCAGCTCCTC
reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTGGATGGTTTCAAGTCGCAAT

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CHAPTER FIVE

Conclusions and Future Directions

Conclusions

Through extensive molecular analysis I have shown that *MSB2* acts as a regulator of filamentous growth. The gene was initially identified through a insertional mutagenesis screen for mutants that failed to transition to the hyphal stage at room temperature. Through inverse PCR, Southern blot, and whole genome sequencing, I showed that the disrupted gene in the yeast-locked mutant was *MSB2*. To confirm that that it was indeed MSB2 that was responsible for the yeast-locked phenotype at room temperature, I constructed an RNAi strain to knockdown the gene. After successful knockdown, the phenotype was observed at room temperature. Compared to the vector control that filamented, the knockdown strain remained yeast. Additionally, I successfully complemented the mutant. I constructed a strain expressing a wildtype copy of *MSB2* in the mutant and it rescued the filamentation phenotype at room temperature. Finally, overexpressing *MSB2* from a strong, phase-neutral promoter, I showed that driving *MSB2* expression at 37°C resulted in filamentation. These data support my conclusion that *MSB2* promotes filamentation at room temperature.

I then tried to understand how *MSB2* is responsible for hyphal growth. The initial test was to see if simply the *RYP1-4* genes, which are regulators of yeast phase growth, are inappropriately expressed at room temperature, driving the yeast program. Through qPCR analysis I showed that all for of these yeast-promoting genes were in fact upregulated at room temperature in the mutant.

This led to my transcriptional analysis of *msb2* compared to the wildtype strain. The thought process was that if the RYP genes are what is driving the yeast morphology at room temperature, than the transcriptional profile of the mutant at room temperature will look like wildtype yeast. Through extensive analysis I showed that, in fact, that is not the case.

Surprisingly, while the morphology of the mutant looks like yeast at room temperature, its transcriptional profile actually looks almost identical to hyphae. I only identified a small subset of differing genes that we conclude are potential regulators of filamentation. Additionally, since the majority of the genes respond the same during the temperature change, I conclude that *MSB2* is not responsible for temperature sensing and the small set of Msb2-depedent genes may dictate cell shape.

From that gene set, I chose to further look at two *MSB2*-dependent genes that could be regulators of filamentation. One was *STU1*, which is a known transcription factor involved in development in other fungi. Here I showed that *STU1* is sufficient for filamentation at 37°C in both wildtype and in the *msb2* mutant. From that, I conclude that *STU1* plays a role in filamentation in *H. capsulatum*. Additionally, a paper recently published showed that knockdown of *STU1* results in a hyphal-defective strain, showing it is necessary for filamentation (Longo, Ray, Puccia, & Rappleye, 2018). This result supports my finding that *STU1* is a regulator of hyphal growth. The same analysis was performed on a *HOG2* knockdown strain that resulted in yeast-locked phenotype. To confirm that MSB2 drives the expression of HOG2 and STU1, I showed that overexpression of MSB2 not only produced hyphae at 37°C but also upregulated the expression of *HOG2* and *STU1*.

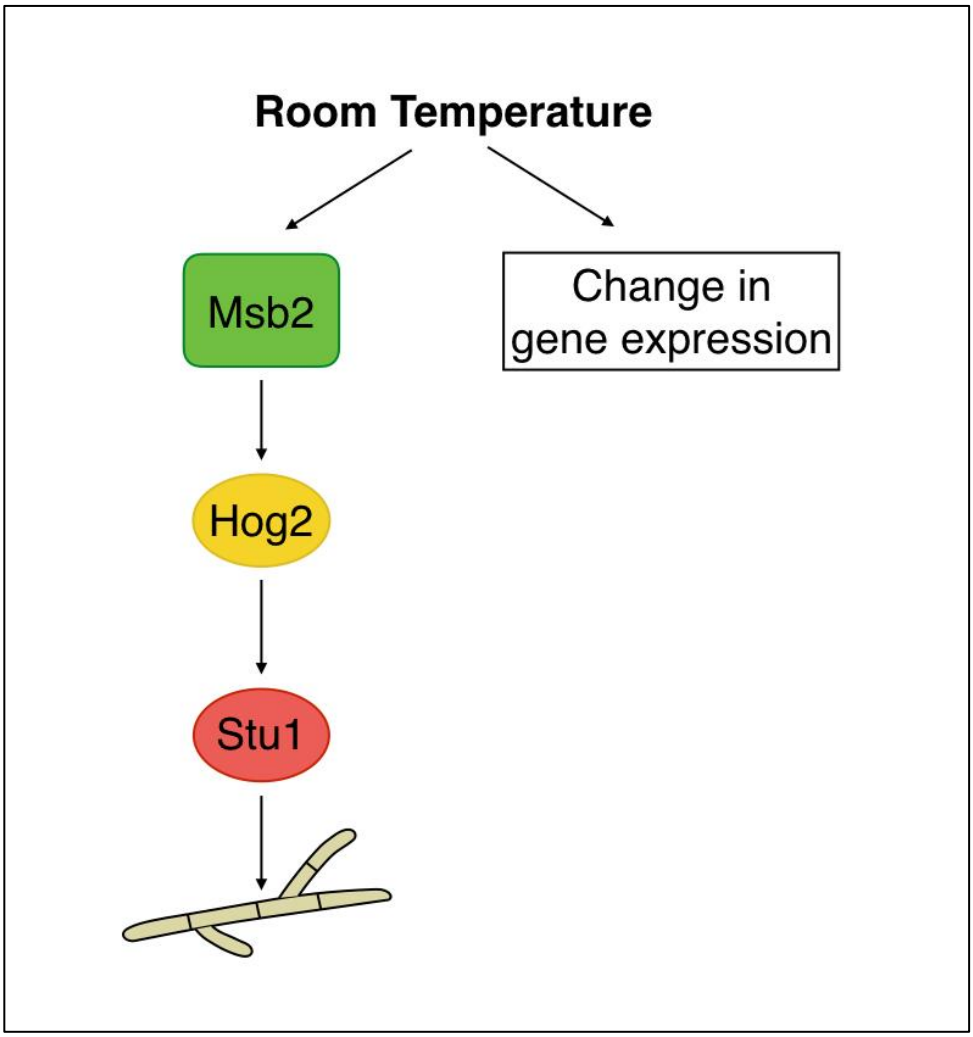
Since *MSB2* is a transmembrane protein, *HOG2* is a MAP kinase, and *STU1* is a transcription factor, using the data I have shown here, I have put together a model for the genetic elements involved in filamentation (Figure 5-1). While it remains unclear how the sensing occurs, I can conclude that *MSB2* is required for hyphal growth and drives the activation of a signaling pathway that results in the morphological response. Filamentation is only one outcome of growth at room temperature; a large gene expression restructuring is also coupled to

temperature. Since the temperature-dependent gene expression signature remains mostly unchanged in the *msb2* mutant, I can conclude that the transcriptional response is independent from the *MSB2*-mediated morphological response.

Taken together, these data concisely show a specific pathway involved in the morphological regulation. However, the transcriptional profiling also provided data used to identify *MSB2*-dependent, phase-specific genes. When looking across other experiments and then to other fungi, this group of genes is highly predictive, suggesting there is a conserved phase-specific regulon under the control of *MSB2*. This is an interesting result as many times it is difficult to compare across datasets but now we have evidence of conserved sets of hyphal and yeast-specific genes.

The intricate regulation of morphology in *H. capsulatum* is far from being completely explained but my work shown here has filled in a space previously vacant in the field. In the process, I produced a very large transcriptional profiling dataset that will continue to be analyzed for future work in the lab. Morphology is a central part of the *H. capsulatum* life cycle and the more data we have to explore it, the closer we will get to understanding it.

Figure 5-1. *MSB2*, *HOG2*, and *STU1* are regulators of filamentous growth



References:

Longo, L. V. G., Ray, S. C., Puccia, R., & Rappleye, C. A. (2018). Characterization of the APSES-family transcriptional regulators of Histoplasma capsulatum. *FEMS Yeast Res*. doi:10.1093/femsyr/foy087

Future directions

How do *MSB2* and *RYP1-4* regulate morphology in response to temperature?

I have definitely shown that the *msb2* mutant is yeast-locked and previous work has shown that mutating any of the *RYPs* results in filament-locked strains. I am now working to make double mutants to further understand the relationship between the *MSB2* and *RYP1-4* pathways. This will help elucidate the genetic regulation of the temperature-dependent morphological states.

How does Msb2 respond to temperature?

While I have shown that the gene *MSB2* is responsible for the morphology change, it remains completely unknown how the protein senses the environmental temperature and initiates the signaling pathway to produce the morphology result. Since its orthologs have large extracellular domains, it would be interesting to see if Msb2 undergoes a conformational change in response to temperature. Additionally, it is of interest to find what the intracellular domain interacts with if it indeed is part of a signaling pathway.

Which pathway does *HOG2* function in?

Phosphorylation cascades are a common module in intracellular signaling and it is not uncommon that kinases are involved in perpetuating a signal initiated by a transmembrane protein. *HOG2* is a kinase novel to this study so much is left to understand. Since it is a recent paralog of *HOG1* and seems to only be found in thermal dimorphs, it is tempting to examine its role in dimorphic regulation alongside *HOG1*.

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