Title
Identification and Characterization of Two Conserved Factors Controlling Temperature-Regulated Development and Spore Formation in *Histoplasma capsulatum*

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Publication Date
2008-07-24

Peer reviewed|Thesis/dissertation

Identification and Characterization of Two Conserved Factors Controlling Temperature-Regulated Development and Spore Formation in *Histoplasma capsulatum*

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOCHEMISTRY AND MOLECULAR BIOLOGY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

DEDICATION

For my family

ACKNOWLEDGEMENTS

My journey through graduate school was like a long and winding road. The destination always seemed so far away as it was hard to see around the many bends. I was able to keep going on this difficult journey with the constant support, guidance, and companionship of the many people that helped me along the way.

I would like to thank **Anita Sil** for making my graduate career possible. As my graduate advisor, she taught me many important life lessons. Her caring and support are unrivaled by any mortal. I highly value the relationship and the mutual respect we have as it has taken a long time to build.

I am grateful to **Van Nguyen** for his experimental help and providing the ground work for my project. During his time in the lab, he always had a playful disposition, but was so subtle about it that it was hard to know the difference when he was pulling your leg and when he was being serious.

I am eternally indebted to **Diane Inglis** for all of her experimental advice and emotional support. She really is a force of nature who inspires all who know her.

I need to thank **Charlotte Berkes** for wonderful conversations and incredibly useful advice. Her warm mentoring disposition made me feel lucky to have her as a part of my graduate school experience.

I also thank all of the past and present **Sil lab members** who have listened to my ideas and have offered valuable advice about my project as well as providing amazing friendship.

I am thankful to the members of my Thesis Committee, **Jeff Cox** and **Hiten Madhani**, for helping me focus my project and offering incredible support and valuable wisdom.

I am very blessed to have had a classmate like **Rebecca Zordan**. She is a beautiful person who is full of kindness and joy. There are very few people in the world like her and I am grateful for her friendship.

I am eternally grateful for all the love and encouragement that **my family** has given me since the beginning without which I would not be who I am today.

I consider myself lucky to have had the opportunity to have a high school chemistry teacher like **Lori Lancaster**. She inspires students to love learning and to accomplish great things.

I am thankful for the incredible research opportunity I was given by **Mike Gustin** as an undergraduate student at Rice University. Despite it being the first day of orientation week, he took a chance and gave me the opportunity to have a wonderful 4-year research experience.

I am indebted for the inspiration that I have received from **K. Beth Beason**. As an undergraduate, she opened my eyes to the endless possibilities that await me and helped me develop a love of teaching.

Most importantly I am forever grateful to **Dale Webster** for his support and love.

Identification and Characterization of Two Conserved Factors Controlling Temperature-Regulated Development and Spore Formation in *Histoplasma capsulatum*

Rachael Hanby Webster

ABSTRACT

The human fungal pathogen *Histoplasma capsulatum* grows in a sporulating filamentous form in the soil and, after inhalation of infectious spores, converts to a pathogenic yeast form inside host macrophages in response to temperature. Here we report the identification of two new genes (*RYP2* and *RYP3*) required for yeast-phase growth. Ryp2 and Ryp3 are homologous to each other and to the Velvet A family of regulatory proteins in *Aspergillus* species and other filamentous fungi. Wild-type *H. capsulatum* grows as filaments at room temperature and yeast cells at 37°C, but *ryp2* and *ryp3* mutants constitutively grow as filaments independent of temperature. *RYP2* and *RYP3* transcripts accumulate to higher levels at 37°C than at room temperature. This differential expression is similar to the previously identified *RYP1* transcript, which encodes a transcriptional regulator required for the yeast-phase expression program. Ryp1 associates with the upstream region of *RYP2*, and each of the three *RYP* genes is required for the differential expression of the others at 37°C. In addition to responding to the elevated temperature of the mammalian host, *RYP2* and *RYP3* are essential for viable spore production and regulation of sporulation at room temperature. This regulatory

function is strikingly similar to the role of the *Aspergillus* Velvet A protein family in spore development in response to light, with the notable distinction that the *H. capsulatum* circuit responds to temperature.

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Introduction

Introduction

Fungi must be able to respond to environmental stimuli and regulate cellular development for optimal growth and survival. Upon interaction with host cells, dimorphic fungal pathogens must be able to regulate critical developmental pathways for the establishment of the pathogenic form of the organism. My thesis work will describe the identification and characterization of two regulators of cellular development in the thermal dimorph *Histoplasma capsulatum* that are conserved with a family of genes in *Aspergillus nidulans* known to regulate cellular development in response to environmental stimuli (1).

Dimorphism in Fungal Pathogens

Dimorphic fungal pathogens (*Histoplasma capsulatum, Blastomyces dermatitidis, Sporothrix schenckii, Paracoccioides brasiliensis,* and *Coccidioides spp.*) have the ability to grow in and switch reversibly between two distinct morphologic forms: the infectious filamentous form and the pathogenic budding yeast-like growth form (or spherules in the case of *Coccidioides*) (2). Although the conversion into the yeast form is thought to be required for host infection, infection of a host is not necessary for the ultimate growth and survival of these organisms (3, 4). Because host infection is not an essential part of the life cycle for these fungi, the capability to drastically alter cellular growth form must have evolved to serve an important but unknown function in the soil environment.

There is much speculation to explain why species of dimorphic fungi have retained the ability to change their cellular growth form when it is not ultimately required for survival. One current theory is that within the soil, dimorphic fungi come into contact

with phagocytic microorganisms like amoeba and that this interaction has contributed to the fungi's ability to alter its growth form as a means of survival (5). Phagocytic microorganisms engulf fungal conidia or filaments with the goal of digesting them for nutrients (5). Dimorphic fungi may have evolved the mechanism that allows the fungi to drastically change their cell shape into a budding yeast-like growth form to successfully colonize and reproduce after phagocytosis by the amoeba thereby ensuring survival (5). This theory suggests that virulence traits of the dimorphic fungi are incidental to their ability to colonize phagocytic microbes in the soil.

Morphological transition

Dimorphic fungi can sense and respond to a wide variety of external stimuli that lead to a change in cell shape. Environmental temperature, nutrient availability, the oxidation-reduction potential, and the level of cAMP can all have an effect on the morphologic growth form of dimorphic fungi (2).

Temperature is a key regulator of morphological transition in dimorphic fungi. Without exposure to high temperature (37°C), cells are unable to convert into the yeast-like growth form. Upon inhalation by the host and exposure to mammalian body temperature or when grown at 37°C in the lab, the filamentous cells will switch and begin to grow in the yeast form (6). This transition is not a quick process as cell shape changes are only detectable in a portion of the cell population after a growing period of at least 24 hours on solid media (6, 7). As stated above, this shape shifting is completely reversible and these yeast cells can be induced to switch back into filamentous cells by lowering the environmental temperature to 22-25°C (8). While the conversion of yeast cells into

filaments is not synchronous, it is a slightly faster process with the entire population switching by 48 hours of growth on solid media (2).

When yeast cells grown at 37°C deplete the available nutrients in the media, the cells will spontaneously shift into filamentous growth (9). Additionally, filaments are the only cell shape detected when cells are grown to starvation at room temperature, indicating the filamentous form is the default morphological structure for dimorphic fungal growth when environmental nutrients are lacking (9). The yeast form of growth is only achievable for these fungi when a particular nutrient threshold is attained regardless of environmental temperature.

In addition to high temperature and the general availability of nutrients, compounds containing SH groups like cysteine are also required for the complete conversion of filaments into yeast-like cells (10). The SH-containing compounds are thought to be necessary to lower the oxidation-reduction potential to levels suitable for yeast-like growth (11). Experiments have shown that cells grown at 37°C in the absence of cysteine are unable to grow in the yeast form unless the oxidation-reduction potential of the medium was artificially lowered (12). The cysteine requirement for morphological transition is separate from the cells nutrient needs for cysteine necessary for normal cellular growth (13). A current theory is that cysteine or other SH-containing compounds are required for conversion into the yeast form as a result of the inability of yeast-like cells to reduce the levels of sulfur-containing compounds that build up in the media.

Cyclic nucleotides are also known to be important molecules in the regulation of morphologic transition between filamentous and yeast-form growth in dimorphic fungi. The addition of cAMP (cyclic adenosine 3’ ,5’-monophosphate) or compounds that

raise the cellular levels of cAMP in the culture media have been shown to induce the morphologic conversion of yeast cells into filaments at 37°C (14). Filamentous cells have been found to contain intracellular levels of cAMP five-times greater than that of yeast-like cells (15). During the transition of yeast cells into filaments at room temperature, the steady rise of cellular cAMP has been detected (15). When filaments were converted into yeast-like cells at 37°C, the intracellular level of cAMP was shown to steadily decrease over time as the cells shifted their cell shape (15). When exogenous cAMP or cAMP phosphodiesterase inhibitors were added to the culture media of yeast cells grown at 37°C, cells transitioned into the filamentous growth form (14). The exact mechanism by which cAMP is regulating cell shape in dimorphic fungi is unknown. One hypothesis is the genetic pathway which controls morphologic conversion includes a cAMP-dependent protein kinase that activates the genetic expression program which leads to filamentous growth. Therefore, low levels of intracellular cAMP may inactivate the filamentous growth expression program and thus would be essential to permit the transition into the yeast form.

Morphology and Virulence

Dimorphic fungi are naturally occurring worldwide. In the environment the fungi are found growing in the soil as long filamentous cells that produce conidia (vegetative spores). When the conidia or filaments are aerosolized by soil disruption, the small infectious particles are inhaled into the lungs of the mammalian host. Within the lungs, the fungal cells convert into a budding yeast-like growth form that is able to survive and replicate within host macrophages. This cellular morphology conversion into the yeast

form is thought to be vital for virulence (3, 4). Although the few experiments that have been done support this theory, the definitive experiment has not been conducted to prove whether or not this is the case.

The first experiment conducted to determine whether yeast cells are required for virulence was performed by treating *H. capsulatum* filaments with the SH-blocking chemical p-chloromercuryphenylsulfonic acid (PCMS) (3, 14). After exposure, the filaments never converted into the yeast form even after PCMS was removed from the media (3, 14). The treatment of *H. capsulatum* with PCMS is a method that permanently and irreversibly forces the cells into filamentous growth. The mechanism by which PCMS blocks growth in the yeast form is unknown. One hypothesis is that the chemical is an SH-blocking agent that permanently alters the oxidation-reduction potential of key SH groups on the fungal cell wall so that even without the continued presence of the compound, the cells remain in the filamentous state. The experiment conducted showed that after host infection, *H. capsulatum* PCMS-treated filaments did not convert into yeast-like cells inside the host as determined by histopathological examination of the infected lungs (3). When compared to wild-type, the filament-only strain was also found to be far less virulent in murine infections (3). This lead to the conclusion that dimorphic fungi must be able to switch into the yeast-like growth form to successfully colonize host cells. However, this experiment does not fully establish that yeast-form growth is required for virulence due to the toxic effects and unknown function of PCMS (14). The filaments tested in the experiments were treated with a chemical that is itself toxic to cells and functions by an unknown mechanism to drastically and permanently alter the cellular growth form of the organism. For these reasons, it is difficult to know whether exposure

to PCMS is leading to a viability defect of the filaments or if the PCMS treatment is causing other changes to the fungal cells that lead to decreased virulence.

To avoid the need for chemical treatments, an experiment utilizing genetic mutants of *B. dermatitidis* and *H. capsulatum* was performed to test the link between yeast-form growth and virulence of dimorphic fungi (4). The mutants used in the study were defective in the histidine kinase Drk1 and unable to grow in the yeast form regardless of environmental conditions (4). The study determined that mice infected with fungal strains lacking Drk1 were attenuated for growth in the host compared to wild-type strains (4). This virulence defect was characterized by assessing the survival of infected hosts as well as fungal colonization in host lungs. The infections performed in this experiment were conducted using spores produced by the wild-type and mutant strains (4). It was not determined whether the mutant spores had a viability defect under normal growth conditions in the absence of a host. Therefore, this experiment does not definitively prove the link between yeast-form growth and virulence.

Although the experiments described above have multiple caveats, the evidence suggests that dimorphic fungal pathogens must be able to grow in the yeast form to trigger the conventional disease process.

Regulators of Morphology in Dimorphic Fungi

Even though the ability to switch morphologic growth forms appears to be essential for virulence in dimorphic fungi, there is very little known about the cellular mechanism that regulates this transition. At the beginning of this thesis work, only two

genes had been identified to be required for yeast-form growth in dimorphic fungal pathogens.

As discussed previously, Drk1 is a functional histidine kinase that is required for yeast growth in both *B. dermatitidis* and *H. capsulatum* (4). It was determined to also play a role in cell wall integrity, to be required for the production of spores, and to be necessary for the expression of virulence (yeast-form specific) genes, in addition to potentially being essential for virulence (4). Cells defective in *DRK1* showed altered cell wall composition and were sensitive to cell wall-binding chemicals (4). *drk1* mutant strains produced about 10% fewer spores than wild-type, indicating a role for Drk1 in promoting sporulation (4). Expression analysis of three yeast-form virulence genes was performed in the *drk1* strain and it was found that Drk1 is necessary for the expression of all three genes (4). Drk1 can functionally complement the *S. cerevisiae* histidine kinase mutant *sln1*, suggesting the protein encodes histidine kinase activity (4). In *S. cerevisiae*, Sln1p regulates a range of genetic pathways: a mitogen-activated protein kinase (MAPK) cascade activated by sensing osmotic stress, an oxidative stress-response pathway, and cell wall biosynthesis (16, 17). Because histidine kinases play an important role in sensing external stimuli that lead to cellular changes in many fungi, Drk1 may have evolved a role to be functioning as the sensor for the environmental signals (temperature, nutrient availability, or oxidative-reduction potential) that lead to the transition into the yeast form in dimorphic fungi. As a result of this study, the first regulator of morphological transition was clearly identified and characterized in two different dimorphic fungal pathogens.

The other gene identified to regulate morphology in dimorphic fungi is Ryp1. Ryp1 was found to be a transcriptional regulator required for yeast-phase growth of *H. capsulatum* (18). In strains lacking *RYP1*, the cells were trapped in the filamentous growth form regardless of temperature (18). This result indicated this gene is required for the switch into the yeast form. *RYP1* transcript and Ryp1 protein levels were higher in wild-type yeast-form cells when compared to wild-type filaments, suggesting this gene is either promoting the activation of the yeast-phase genetic program at 37°C or inhibiting the filamentous-phase program at 37°C (18). To determine the role of Ryp1 at 37°C, whole-genome expression profiling of wild-type and *ryp1* cells was performed (18). Through a series of experiments, it was found that Ryp1 is required for the activation of the majority of genes expressed in the yeast form (18). Furthermore, chromatin immunoprecipitation experiments showed Ryp1 was able to associate with its own promoter as well as with the promoter of a target gene predominantly expressed in the yeast form, suggesting Ryp1 is likely to promote the expression of yeast-specific genes in a direct manner as a transcriptional regulator (18).

Interestingly, the homolog to Ryp1 in *Candida albicans,* Wor1, is also a presumptive transcriptional regulator (19-21). *C. albicans* cells have the ability to growth as either white round cells or as mating-competent elongated opaque cells. Wor1 was identified to regulate the transition between the two distinct cell types by being both necessary and sufficient for cell fate regulation (19-21). This morphologic regulator is thought to trigger the transition into the heritable opaque phase by direct transcriptional regulation (19). These findings indicate that the conserved regulatory proteins in *H. capsulatum* and *C. albicans* may be playing similar roles in a regulatory pathway that has

been reconfigured to sense different external stimuli and ultimately respond by regulating cell growth form depending on the environment and life-style of the fungi.

Regulators of Cell Development in Filamentous Fungi

Based on phylogenetic data, constitutively filamentous and dimorphic fungi are closely related. Both classes of fungi grow as filaments in the soil and even though dimorphs have the ability to completely change cellular growth form when exposed to a temperature shift, the regulation of many genes and genetic pathways are highly conserved between them. This is a fortunate circumstance since constitutively filamentous fungi have been genetically tractable organisms in laboratory studies for a significantly longer period of time than dimorphs.

Among the known developmental regulators in filamentous fungi, one of the first identified was Velvet A (VeA) (22). While VeA has been implicated in spore and hyphal development in an array of filamentous fungi, its role in *A. nidulans* has been best characterized (23-26). *A. nidulans* VeA is localized to the nucleus and required to promote sexual spore development as well as to inhibit the formation of asexual spore structures when there is no light present (25, 27, 28). In the presence of light, VeA is confined to the cytoplasm, which allows asexual spores to form (27, 28). Under light conditions, the VeA protein has been shown to interact with both the red and blue light sensor proteins (FphA and LreA/B, respectively) by co-immunoprecipitation (27, 29). In addition, VeA has been recently shown to interact with a putative methyl transferase LaeA, which is thought to regulate gene expression (30). Based on the interaction data, the VeA protein may be a transcriptional regulator that is directly inhibited by the light

sensor proteins and sequestered outside of the nucleus in the presence of light. When the light stimulus is removed, the light sensors may release VeA, which allows for migration of VeA back into the nucleus and activation of sexual development.

A recent study has discovered that VeA also physically interacts with a related protein VelB by co-immunoprecipitation (30). The study determined VelB was required for the appropriate regulation of sexual and asexual spore production similar to VeA (30). Unlike VeA however, VelB is not sufficient to promote the development of sexual spore structures as overexpression of *velB* did not result in the formation of inappropriate sexual spores (30). Also unlike VeA, which is localized to the nucleus depending on light conditions, and LaeA, which is only found in the nucleus, the cellular localization of VelB is not affected by light (it is consistently found in both the nucleus and the cytoplasm) (30). Cellular development in *A. nidulans* may be controlled by a multi-protein complex composed of VeA, VelB, and LaeA that is found in the nucleus to regulate gene expression of downstream genes by epigenetic control through chromatin remodeling (30).

Another Velvet A family member, *A. nidulans* VosA, has been shown to be required for appropriate asexual sporulation and spore viability (31). VosA was originally identified as a negative regulator of asexual sporulation: when *vosA* is over-expressed, cells lose their ability to produce asexual spores under normal conditions (31). The *vosA*Δ strain was found to inappropriately make asexual spores under aerating culture conditions (31). Taken together these data indicate the requirement of VosA for appropriate asexual sporulation. Under normal sporulation conditions, the *vosA*Δ mutant produced spores that had an extreme viability defect (31). This loss of viability was attributed to the inability

of spores to accumulate trehalose (31). Trehalose is a sugar that is necessary for normal cellular development as well as protection against environmental stresses. Without trehalose, the spores lyse after final maturation and dispersal (31). Because VosA was found to be expressed in asexual spore structures and localized to the nucleus in the spores (31), the protein may function as a transcription factor that is required to repress spore development genes.

Although there are interesting implications in cell regulation for these members of the Velvet A protein family in *A. nidulans* and other filamentous fungi, there are no known biochemical motifs present in the protein sequences. Thus the molecular mechanism by which these proteins act remains a mystery. Based on the small amount of data so far, it is likely that VeA, VelB, and VosA are fungal specific transcriptional regulators that activate or repress genes involved in cellular development. The information gained from analyzing the genetic pathways that control cellular development in filamentous fungi can be utilized to potentially shed light on the mysterious regulation of cellular growth form in response to temperature in the closely related dimorphic fungal pathogens.

Conclusion

H. capsulatum is a primary dimorphic fungal pathogen endemic to the Mississippi and Ohio River Valleys (32). Although *H. capsulatum* is thought to be the leading cause of fungal respiratory disease in healthy individuals in the U.S., very little is understood about how *H. capsulatum* regulates the morphologic switch into the yeast form, which is required for virulence (32). To build upon our limited knowledge of morphological

regulators in dimorphic fungi, I decided to identify novel genes that are required for yeast-phase growth (*RYP* genes) in *H. capsulatum*. By discovering and characterizing these new players in the genetic pathway that controls the transition between the filamentous and yeast growth forms, we will gain insight into the regulatory network that is responsible for the establishment and maintenance of this dramatic change in cellular development.

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Chapter Two:

Conserved factors Ryp2 and Ryp3 control cell morphology and infectious spore formation in the fungal pathogen *Histoplasma capsulatum*

Introduction

Developmental programs are triggered in response to environmental stimuli in a vast array of organisms. The systemic dimorphic fungal pathogens, which grow as filaments in the soil, initiate a dramatically different developmental program during colonization of mammalian hosts (1). An outstanding question in fungal evolution is how these pathogens, which include *Histoplasma capsulatum*, modified ancestral pathways to develop morphology and pathogenesis programs that foster disease. Here we describe the identification and characterization of two temperature-responsive regulators of morphology in *H. capsulatum.* Of note, these factors are homologous to a regulator in the filamentous fungus *Aspergillus nidulans* that controls cellular development in response to light (2).

H. capsulatum is a primary fungal pathogen endemic to the Mississippi and Ohio River Valleys. It grows in the soil as filamentous cells that produce vegetative spores. When the spores or filaments are aerosolized, the particles can be inhaled into the lungs of a mammalian host, where the fungal cells convert into a budding-yeast form that is able to survive and replicate within host macrophages. The ability of cells to adopt the yeast growth program is thought to be critical for *H. capsulatum* virulence (3, 4). Interestingly, the morphologic switch from the filamentous form to the yeast form can be recapitulated in culture by changing the temperature (room temperature growth triggers the soil form of the organism whereas growth at 37°C triggers the host form of the organism). We recently identified Ryp1, a key transcriptional regulator required for yeast-phase growth at 37°C (5). Ryp1 is required for the vast majority of the yeast

expression profile, but little else is known about the regulatory network that governs how temperature activates growth in the yeast phase.

In this study, we use a genetic screen to identify key regulators of morphological development in *H. capsulatum*. We identified two genes (*RYP2* and *RYP3*) that are required for yeast-phase growth at 37°C. We show that both *RYP2* and *RYP3* transcripts are more highly expressed in yeast cells at 37°C than in filaments grown at room temperature. Furthermore, we show that regulation of *RYP1, RYP2* and *RYP3* is interdependent, suggesting that each is required for the expression and function of the others.

Notably, Ryp2 and Ryp3 are homologous to the Velvet A (VeA) family of regulators (which includes VeA, VelB, and VosA) from the filamentous fungus *Aspergillus nidulans*. Although *Aspergillus* species do not change their morphology in response to temperature, these fungi do exhibit VeA-dependent regulation of developmental programs, such as the production of spores, in response to environmental conditions such as light (6, 7). Additionally, the VeA orthologs in the filamentous fungi *Fusarium verticillioides, Acremonium chrysogenum* and *Neurospora crassa* regulate hyphal morphology and spore formation (8-10). Taken together with this study, these data indicate that the Velvet proteins are conserved fungal factors that regulate distinct developmental pathways in response to diverse environmental stimuli.

Materials and Methods

Strains and Growth Conditions

H. capsulatum G217B (ATCC 26032) and G217B*ura5*Δ (WU15), both generously provided by William Goldman (Washington University, St. Louis, MO), were grown in the yeast or mycelial form by changing the temperature of the culture environment.

Yeast-form cultures: G217B and G217B*ura* were grown in *Histoplasma* macrophage medium (HMM) liquid or on HMM agarose plates (11). G217B*ura5*Δ cultures were supplemented with 0.2 mg/mL uracil (Sigma-Aldrich). Broth cultures were grown at 37°C with 5% CO2 on an orbital shaker at 120-150 rpm. Plates were grown in a humidified incubator at 37°C with 5% CO2. Cells were thawed fresh from frozen stock and passaged on HMM agarose plates every 1-2 weeks for up to 2 months.

Mycelial-form cultures: H. capsulatum yeast cultures were converted into filamentous mycelial cultures by inoculating Sabouraud Dextrose (Difco) agar plates with yeast cells and growing at room temperature (22-26°C) for at least 2 weeks. The mycelial cells were then inoculated into liquid HMM broth and grown for 2-4 weeks at room temperature on an orbital shaker at 120 rpm to establish robust mycelial cultures. Cultures were passaged every 1-2 weeks at 1:20 dilution. *ryp2*, *ryp3*, and *ryp1* mutant strains were grown on either HMM or 3M (12) agar plates in a humidified incubator at 37°C. Liquid cultures of the filamentous mutants were grown in either HMM broth or HMM supplemented with 200ug/mL hygromycin B (Roche) at 37°C with 5% CO2 on an orbital shaker at 120 rpm. Cultures were passaged every 1-2 weeks at 1:20 dilution.

H. capsulatum Insertional Mutagenesis

H. capsulatum strain G217B was transformed with the *Agrobacterium tumefaciens* strain LBA1100 (the kind gift of Bruce Klein (University of Wisconsin, Madison) with permission from Paul Hooykas (Leiden University, Leiden, The Netherlands)) carrying the plasmid pRH5b (5) or pBTS4 (13). The co-culturing of *H. capsulatum* with *A. tumefaciens* was done as previously described (13) and transformants were selected on HMM agarose plates supplemented with 200 µg/mL hygromycin B and 200 µg/mL cefotaxime. Approximately 40,000 insertion mutants were screened visually at 37°C to identify a total of 15 filamentous strains. The locations of the insertions were mapped by digesting mutant genomic DNA with Hind*III* and performing inverse PCR (14). The primers OAS907 (5'-gtaagcgcccactccacatc-3') and OAS908 (5'-gttgcgcagcctgaatggcg-3') were used to amplify the left border of mutants generated with either plasmid. To map the right border of mutants generated with pBTS4, the primers OAS750 (5'-ggctccttcaacgttgcggt-3') and OAS910 (5'-gcttccggctcgtatgttgtg-3') were used. To map the right border of mutants generated with pRH5b, the primers OAS1322 (5'-gggcgacacggaaatgttgaatactc-3') and OAS750 were utilized. Two mutants had insertions in the ORF of *RYP2* (M9 and M15) and one mutant had a 20kb deletion at the site of insertion that contained the *RYP3* gene as well as 2 other predicted genes (F14) (Fig. 1).

Genomic DNA Preparation and Southern Analysis

Genomic DNA from *H. capsulatum* wild-type and mutant cells was isolated and subjected to Southern analysis as previously described (5).

RNA Interference against *RYP2* and *RYP3*

Integrated RNAi Constructs

H. capsulatum strain G217B was co-cultured with *A. tumefaciens* strain LBA1100 containing either the vector control pVN69 (5), pRH15, or pRH17 (Fig. 2A). All plasmids are derived from pFANTAi4 (15). pRH15 contains a 487bp hairpin repeat of the large exon in the *RYP2* coding sequence (Fig. 2B) generated by PCR amplification of *H. capsulatum* G217B genomic DNA with OAS1482 (5'-ggggacaagtttgtacaaaaaagcaggctcacattgaatcgagcggaacca-3') and OAS1483 (5'-ggggaccactttgtacaagaaagctgggtagattcgcccatctgtgacat-3'). pRH17 contains a 498bp hairpin repeat targeting one of the long exons in the *RYP3* coding sequence (Fig. 2B) which was PCR-amplified using OAS1484 (5'-ggggacaagtttgtacaaaaaagcaggctcacgacacctcattctacgtcc-3') and OAS1485 (5'-ggggaccactttgtacaagaaagctgggtaaacgagccttcagtgcgaac-3'). Integrated RNAi strains were used to perform the experiments described in Figures 6, 8, and 9.

Episomal RNAi Constructs

H. capsulatum strain G217B*ura5*Δ was transformed by electroporation as described previously (16) with the vector control pCR186 (a kind gift of Chad Rappleye (Ohio State University) derived from pCR138 (17)), pRH27, or pRH29 (Fig. 2A). pRH27 contains the same 487bp of the *RYP2* coding sequence as pRH15. The hairpin repeat segments were generated by PCR amplification of *H. capsulatum* G217B genomic DNA with tailed primers OAS1507 (5'-ggcgcgcccattgaatcgagcggaacca-3') and OAS1508 (5'-ctcgaggattcgcccatctgtgacat-3') and tailed primers OAS1509 (5'-

actagtcattgaatcgagcggaacca-3') and OAS1510 (5'-accggtgattcgcccatgtgtgacat-3') followed by the insertion of each piece into the AscI-XhoI sites and the SpeI-AgeI sites of the pCR186 backbone, respectively. pRH29 was constructed in a similar manner with the 498bp of *RYP3*, as in pRH17, as the hairpin repeat segments. The fragments were PCR amplified by the AscI and XhoI tailed primers OAS1499 (5'-ggcgcgcccgacacccattctacgtcc-3') and OAS1500 (5'-ctcgagaacgagccttcagtgcgaac-3') and SpeI and AgeI tailed primers OAS1501 (5'-actagtcgacacctcattctacgtcc-3') and OAS1502 (5'-accggtaacgagccttcagtgcgaac-3'). Transformants were selected on HMM agarose plates grown at 37°C for 2 weeks. Episomal RNAi strains were used to perform the experiments shown in Figure 3. To screen for cells that lost the episomal RNAi plasmids, these strains were grown on HMM agarose plates supplemented with 100ug/mL uracil and incubated at 37°C with 5% CO_2 for 2 weeks. Colonies were patched onto HMM agarose plates in the absence of uracil supplementation to identify cells that no longer contained the *URA5* marker present on the episomal plasmid. Genomic DNA was obtained and screened by PCR using primers that recognize the episomal plasmid to confirm that plasmid sequences were not present (PCR was performed with OAS249 (5'-ggcgattaagttgg-3') and OAS1488 (5'-cacatgaagcagcacgactt-3').

Microscopy

All images were obtained using a Zeiss Axiovert 200 microscope with either a DIC or Phase objective.

RNA Preparation and Northern Analysis

Total RNA from *H. capsulatum* wild-type and mutant cells was isolated and subjected to Northern analysis as previously described (5). Probes were amplified from G217B genomic DNA with the following primers: *RYP2* – OAS1964 (5'-ccctccacctattatccaac-3') and OAS1976 (5'-agaactgtacgggcttcctt-3'); *RYP3* – OAS1977 (5'-gaacttcgtcaacgttggca-3') and OAS1978 (5'-gaaaaaggtgaagggaatgac-3'); and *GAPDH* – OAS1120 (5'-accaacaggcctacatgctc-3') and OAS1121 (5'-tactgctcgctgttgattgc-3').

Mapping of *RYP2*, *RYP3*, and *VEA1* Transcripts

Total RNA was isolated from *H. capsulatum* G217B and treated with DNase*I* (Promega). To identify the 5' and 3' ends of the *VEA1*, *RYP2*, and *RYP3* transcripts, the FirstChoice RLM-RACE Kit (Ambion) was used per the manufacturer's instructions. For 5' RACE, the following primers were used: *RYP2* – OAS1970 (5'- accttgaaagccatgtggac-3') and OAS1971 (5'- aacgacttcatctctcgagc-3'); *RYP3* – OAS1972 (5'- tatgcggtttgcgggtatcc-3') and OAS1973 (5'- gacgtagaatgaggtgtcga-3') and *VEA1* – OAS1974 (5'-cttgggctccttcattcct-3') and OAS1975 (5'- cctgtcaggacaggccatga-3'). For 3' RACE, the following primers were used: *RYP2* – OAS1958 (5'-aaggctatcctactgctgga-3') and OAS1959 (5'-atgtcacagatgggcgaatc-3'); *RYP3* – OAS1960 (5'-tcaatgttggttcggcacct-3') and OAS1961 (5'-gacagtcacatggctccaaa-3'); and *VEA1* – OAS1962 (5'-tacgaccaatcgaagtcctc-3') and OAS1963 (5'-cacctccagcagcaatagca-3'). The transcript coding sequences were amplified from reverse transcribed cDNA using the following primer pairs: *RYP2* – OAS1964 (5'- ccctccacctattatccaac-3') and OAS1965 (5'-gattcgcccatctgtgacat-3'); *RYP3* – OAS1966 (5'- ccatggatcacctccagcat-3') and OAS1967

(5’- tttggagccatgtgactgtc-3’); and *VEA1* – OAS1968 (5’-gagactgagcataccatgtc-3’) and OAS1969 (5’-tgctattgctgctggaggtg-3’). All amplified products were cloned using the TOPO-TA system (Stratagene) and sequenced with M13-forward and M13-reverse primers.

Quantitative RTPCR (qRTPCR)

Total RNA from *H. capsulatum* G217B and control, *RYP2*, and *RYP3* RNAi strains was isolated and subjected to qRTPCR as previously described (16). The transcripts were amplified with the following primers: *RYP2* – OAS1942 (5’-cggctcgagagatgaagtcgtt-3’) and OAS1943 (5’-aagtgtacgggcttccttccg-3’); *RYP3* – OAS1944 (5’-ccaaaggccaagatggagaagg-3’) and OAS1945 (5’-ggaaatgagaggaaggggggaaaga-3’); *RYP1* – OAS1057 (5’-acccttgcagcttacaacct-3’) and OAS1058 (5’-tccgtccatcgcttaatacc-3’); and *TEF1* – OAS1687 (5’-tcgatgccattgacgccattgaac-3’) and OAS1689 (5’-gagaacttgcaagcaagttgggca-3’). All intensity signals from the *RYP* genes were normalized to the *TEF1* expression levels.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed as previously described (5). PCR primers were designed at approximately 500 bp intervals across the 6 kb intergenic region immediately upstream of the *RYP2* ORF (Table 2). PCR primers were designed at approximately 150 bp intervals across the 1.2 kb intergenic region immediately upstream of the *RYP3* ORF (Table 2). *TEF1* was used as the reference ORF (Table 2).

Spore Viability

Spore Harvest

Spores were obtained from the control, *RYP2*, and *RYP3* RNAi strains by first growing a dense culture in HMM liquid for 2 weeks at either room temperature (for the control) or 37°C (for the RNAi mutants). To initiate spore formation using standard conditions, 1 mL of culture was inoculated onto 15 cm Sabouraud agar plates. The plates were allowed to grow in a closed container with minimal exposure to light at room temperature for 4 weeks. Plates were flooded with PBS and spores were harvested by gentle agitation with a glass rod. The spore suspensions were filtered through glass wool to minimize contaminating filaments and collected by centrifugation at 2000xg for 10 minutes at 4°C. Spores were stored at 4°C.

Viability Testing

Spores from the control, *RYP2*, and *RYP3* RNAi strains were stained with lactophenol blue and quantified immediately after harvesting. BHI agar plates supplemented with 10% sheep's blood were inoculated with spores at regular intervals over the course of 7 days, and incubated at 30°C. The percent viability was calculated as a ratio of the number of CFUs growing on the BHI plates to the number of spores inoculated initially. The percent viability of the each strain was normalized to the viability of the control strain on Day 0.

Host Cell Interactions

Macrophage Infections

Murine bone marrow derived macrophages (BMDMs) were isolated and differentiated as described previously (16). The macrophages were seeded into wells of 24-well tissue culture plates containing coverslips for use in staining. The BMDMs were infected with spores from the control, *RYP2*, or *RYP3* RNAi strains at an MOI of 0.1. The infected macrophage plates were spun for 5 minutes at 500xg at room temperature. After 2 hour incubation, the macrophages were washed twice with BMM (16) and then incubated in fresh BMM. At 48 and 72 hours post infection, the BMM was aspirated and the macrophages were fixed in 3.7% formaldehyde for 1 minute. The macrophages were washed twice with water and stored in PBS at 4°C.

Infection Staining

The *H. capsulatum* cells and macrophage monolayer were stained with Periodic Acid-Schiff base (PAS) (Sigma). The cells were incubated with periodic acid for 5 minutes and then washed 5 times with water. Schiff base was added to the cells and incubated for 5-10 minutes. Cells were then washed for 5 minutes under continuously running water. The macrophage nuclei were visualized by staining with methyl green (Vector Labs) for 5 minutes and then washed for 1 minute under running water. The coverslips were removed from the wells and mounted onto glass slides with Permount (Fisher).

Homology Analysis

The protein sequences used to generate the phylogenetic tree and alignments are as follows. Hc: *H. capsulatum* Vea1 EU543494, Ryp2 EU543495, Ryp3 EU543496; Bd: *B. dermatitidis* Vea1, Ryp2, and Ryp3 were identified by TBLASTN search of the Hc protein sequences against the *B. dermatitidis* genome assembly version 3.0 (http://genome.wustl.edu/); Pb: *P. brasiliensis* Vea1, Ryp2, and Ryp3 were identified by TBLASTN search of the Hc protein sequences against the version 1 of the Pb03 genome assembly (http://www.broad.mit.edu/annotation/fgi/); Ci: *C. immitis* Vea1 CIMG_06878.2, Ryp2 CIMG_01530.2, Ryp3 CIMG_09962.2 (http://www.broad.mit.edu/annotation/fgi/); An: *A. nidulans* VeA AF109316, VosA DQ856465, VelB EF540815; Af: *A. fumigatus* VeA XM747526, VosA EF544392, VelB Afu1g01970 (http://www.broad.mit.edu/annotation/fgi/); Mg: *M. grisea* VeA MGG08556.4, VosA MGG00617.4, VelB MGG01620.4 (http://www.broad.mit.edu/annotation/fgi/); Nc: *N. crassa* VE-1 NCU01731.2, VosA NCU05964.3, VelB NCU02775.3 (http://www.broad.mit.edu/annotation/fgi/); Fv: *F. verticillioides* VeA DQ274059, VelB FVEG_01498.3 (http://www.broad.mit.edu/annotation/fgi/); and Ac: *A. chrysogenum* VeA AM410093. The sequences were aligned with clustalX and the phylogenetic tree was generated using NJ Plot (18).

Results

Identification of *RYP2* and *RYP3* as regulators of cell fate.

A genetic screen was used to identify genes required for yeast-form growth at 37°C. Insertional mutagenesis was performed on wild-type *H. capsulatum* G217B using *A. tumefaciens*-mediated transformation (13). Whereas wild-type cells growing in the yeast form give rise to shiny, smooth colonies, morphology mutants that are trapped in the filamentous form grow as dull, fuzzy colonies. Of the 40,000 insertion mutants assessed at 37°C, 15 constitutively filamentous mutants were identified and further analyzed. Inverse PCR and Southern analysis were used to identify two mutants (M9 and M15, Fig. 1) with independent insertions in the same ORF, which we named *RYP2* (required for yeast-phase growth), and one mutant (F14, Fig. 1) with a 20kb deleted region. The chromosomal deletion in F14 eliminated three ORFs, one of which was homologous to the *RYP2* ORF, one of which had no significant BLAST homology, and one of which represented the remnants of a retrotransposon. We hypothesized that the *RYP2* homolog might be responsible for the F14 mutant phenotype, and named the corresponding ORF *RYP3.*

Microscopic analyses were performed to compare the morphology of wild-type and mutant cells at 37°C (Fig. 3). Whereas wild-type cells grew in the yeast form, the *ryp2* and *ryp3* mutants failed to produce yeasts and instead grew as filaments, indicating that the mutants do not sense or respond appropriately to temperature. The mutant filaments displayed terminal bulbous structures of unknown significance. In addition to the prominent 37°C morphology defects, the *ryp2* and *ryp3* mutants also displayed a phenotype at room temperature. Whereas wild-type strains do not produce many spores

under submerged, shaking culture conditions at room temperature, both the *ryp2* and *ryp3* mutants showed robust, inappropriate sporulation under these conditions (Fig. 3). These phenotypes were reminiscent of the *ryp1* mutant (5), and suggest that *RYP2* and *RYP3* regulate developmental processes at both room temperature and 37°C.

Because the original *ryp3* insertion mutant contained a large chromosomal deletion (Fig. 1), it was critical to test whether the mutant phenotype was linked to absence of the *RYP3* gene. No markers were available for complementation of the mutant phenotype, and we were unable to obtain gene disruptions of *RYP2* or *RYP3* due to the inefficient nature of this technology in *H. capsulatum*. We generated episomal plasmids that expressed RNA interference (RNAi) constructs for each gene (Fig. 2) and transformed these plasmids into *H. capsulatum* yeast cells. Targeted knockdown of *RYP2* or *RYP3* by RNAi recapitulated the mutant phenotypes (Fig. 3B). Upon loss of the episomal RNAi plasmids, the wild-type phenotype was restored (Fig. 3B).

Ryp2 and Ryp3 are homologous to VeA.

Ryp2 and Ryp3 are both homologous to Velvet A (VeA), a regulator of asexual and sexual development in *A. nidulans* (2). Specifically, Ryp2 is the ortholog of the VeA family member VosA (which regulates the production and viability of asexual spores (7)), and Ryp3 is orthologous to VelB (which is required for sexual spore formation (19)). In addition to Ryp2 and Ryp3, *H. capsulatum* has a third homolog of unknown function which we named Vea1 since it is most closely related to VeA. The three-member VeA family is highly conserved among other dimorphic and constitutively

filamentous fungi (Fig. 4), but is not present in strictly yeast-phase species such as *Saccharomyces cerevisiae* (2).

Because *H. capsulatum* genes tend to contain multiple small introns, it was necessary to sequence the Vea1, Ryp2, and Ryp3 cDNAs to determine the spliced coding sequences and predicted translation products (GenBank accession numbers EU543494-EU543496). Protein alignments revealed three highly conserved blocks of amino acids along with regions of charged residues and proline-rich stretches (Fig. 5). Sequence motifs that would imply a particular biochemical function were not observed in Vea1, Ryp2, or Ryp3.

RYP2 and _RYP3_ are differentially expressed in response to temperature.

To determine whether the expression levels of *RYP2* or *RYP3* were regulated in response to temperature and/or cell morphology, we used quantitative reverse transcriptase polymerase chain reaction (qRTPCR) and Northern blot analyses to evaluate *RYP2* and *RYP3* transcripts in wild-type cells grown at either 37°C (yeast cells) or room temperature (filaments). Expression of *RYP2* was approximately 15-fold higher in wild-type yeast cells compared to filaments, whereas expression of *RYP3* was approximately 5-fold higher in yeast vs. filaments (Fig. 6A and B). Nonetheless, both genes showed expression at room temperature, consistent with a role at this temperature in addition to 37°C (Fig. 6C).

Expression of *RYP* genes is interdependent.

We were interested in understanding the basis of the enriched expression of *RYP2* and *RYP3* at 37°C in yeast cells. *RYP1* is a transcriptional regulator required for yeast-phase growth at 37°C in *H. capsulatum* (5). Because the *ryp1* mutant strain displayed similar phenotypes to *ryp2* and *ryp3*, we investigated whether *RYP1* was required for differential expression of *RYP2* and *3*. In parallel, we analyzed the expression of each *RYP* gene in the absence of the others. The level of each *RYP* transcript was analyzed in the control strain, stably integrated *RYP2* or *RYP3* RNAi strains, and the *ryp1* mutant strain (Fig. 6D-F). Surprisingly, each *RYP* gene was required for the high level of expression of the other *RYP* genes at 37°C. This decreased *RYP* gene expression in each of the mutants could be a secondary consequence of their filamentous growth (Fig. 6A-C, (5)). However, in that case, the expression level of the *RYP* genes in the mutants at 37°C would be equivalent to that of wild-type filaments. Instead, in each of the *ryp* mutants, the expression level of all of the *RYP* genes at 37°C was significantly lower than that of wild-type filaments (Table 1). For example, in the *ryp2* mutant growing filamentously at 37°C *RYP3* transcript levels were, on average, 7-fold lower than in wild-type filaments. Similarly, in the *ryp3* mutant, levels of *RYP2* transcript were approximately 24-fold lower than in wild-type filaments. A similar, but less dramatic, trend was observed for the *RYP1* transcript, which was 2-3-fold lower in the *ryp2* and *ryp3* mutants compared to wild-type filaments. These data imply that loss of *RYP* gene expression is not simply a secondary consequence of filamentous growth, and that regulation of each of the *RYP* genes is interdependent.

Ryp1 associates with the *RYP2* promoter

Since Ryp1 is a transcriptional regulator that associates with DNA (5), we tested whether it associates with the upstream regions of either *RYP2* or *RYP3*. Using chromatin immunoprecipitation (ChIP) and quantitative polymerase chain reaction (qPCR) analysis, the occupancy of Ryp1 at discrete locations upstream of the *RYP2* or *RYP3* ORF was compared to the occupancy of Ryp1 at a reference control ORF. The entire intergenic region between either *RYP2* or *RYP3* and the most proximal upstream ORF was examined. Interestingly, Ryp1 was associated with DNA upstream of the *RYP2* ORF, but not upstream of the *RYP3* ORF, at 37°C (Fig. 7). Enhanced association was observed at approximately 500, 4500, and 5500 bp upstream of the *RYP2* ATG; the long-range association events at -4500 and -5500 were reminiscent of the binding characteristics of Wor1, the *Candida albicans* Ryp1 ortholog (20, 21). Similar association events were obtained using ChIP-on-chip studies aimed at determining all Ryp1 target loci (M. Gutierrez, M. Voorhies, and A. Sil, unpublished observations). These results indicate that Ryp1 may directly regulate *RYP2,* but not *RYP3,* expression.

Production of viable spores requires *RYP2* and *RYP3*.

The observation that deregulated spore production occurs in *ryp2* and *ryp3* mutants at room temperature (Fig. 3) suggested that Ryp2 and Ryp3 may control sporulation. In addition to inhibiting spore production under submerged, shaking conditions, we wanted to determine whether Ryp2 and Ryp3 were required for the formation of normal spores under conditions that promote sporulation. Control and integrated *RYP2* and *RYP3* RNAi strains were allowed to produce spores under standard

conditions. Spores from each strain were isolated and microscopic analysis revealed that the control strain produced both small smooth and larger tuberculate spores, whereas the *ryp2* and *ryp3* mutant strains predominately produced large tuberculate spores (data not shown). Although most of the control spores appeared full of cellular material, the majority of the mutant spores were in varying stages of lysis (Fig. 8A). The viability of the spores was tested at regular intervals for 7 days after harvesting. Both the *ryp2* and *ryp3* mutant spores displayed severe viability defects (at least a 10-fold decrease) when compared to control spores (Fig. 8B). These data indicate that, like *A. nidulans* VosA, Ryp2 and Ryp3 are required for the production of normal viable spores (7).

The switch to yeast-form growth in the presence of host cells requires *RYP2* and *RYP3*

Temperature is a key signal that stimulates *H. capsulatum* yeast-form growth in the host, but the existence of other uncharacterized host-dependent signals has been suggested (22, 23). To determine if host factors produced by macrophages could induce a morphologic change in the *ryp* mutants, murine bone marrow derived macrophages were infected with spores from control or the integrated *RYP2* or *RYP3* RNAi strains and observed over a period of 72 hours. Although the majority of *ryp2* and *ryp3* spores were inviable and did not germinate, it was possible to assess and quantify the cellular morphology of 150 germinating spores associated with macrophages for each strain. As expected, the control spores predominantly germinated to give rise to yeast cells (71 +/- 3%) when associated with macrophages (Fig. 9), while the remainder gave rise to filaments. In the *ryp2* or *ryp3*-infected macrophage populations, spores germinated to

give rise only to filaments, and yeast cells were never observed (Fig. 9). These data suggest that the ability of macrophages to promote yeast-phase growth is dependent on *RYP2* and *RYP3*.

Discussion

Functional genomic comparisons and molecular studies have revealed that transcriptional networks can undergo dramatic rewiring during evolution (24-29). An outstanding question in fungal biology is how the systemic dimorphic fungal pathogens, which initiate a host-colonization program in response to temperature, have evolved temperature-dependent regulatory circuits from the ancestral state (30). Here we describe the identification of two conserved fungal proteins, Ryp2 and Ryp3, which are required for *H. capsulatum* to grow in the parasitic yeast form in response to host temperature. In the ascomycete lineage, Ryp2 and Ryp3 are highly conserved in systemic dimorphic and filamentous fungi (Fig. 4), but clear homologs are not present in fungi that grow exclusively as yeast, such as the Saccharomycetales (2). In filamentous fungi, homologs of Ryp2 and Ryp3 control spore formation and viability (2). Interestingly, we also show that in addition to their role in yeast-form growth, Ryp2 and Ryp3 are required for appropriate spore production and viability during *H. capsulatum* filamentous growth. These data indicate that Ryp2 and Ryp3 have distinct functions under different environmental conditions. Furthermore, it is likely that the systemic dimorphic fungi have adapted a conserved module that controls sporulation in filamentous fungi for use in regulation of yeast-phase growth at 37°C (Fig. 10).

Molecular studies in filamentous fungi including *Aspergillus*, *Fusarium*, and *Neurospora* have defined the roles of Ryp2 and Ryp3 homologs in the regulation of cellular development (2, 6-10, 19). These proteins are members of the Velvet A family, which include VeA, VelB, and VosA. The role of VeA and VelB in spore and hyphal development has been best characterized in *A. nidulans* where they are required to

promote sexual spore development as well as to inhibit the formation of asexual spore structures (Fig. 10) (2, 19). *A. nidulans* VosA has been shown to be required for appropriate asexual sporulation and spore viability (Fig. 10) (7). The first breakthrough regarding the biochemical functions of these proteins comes from a recent study showing that VeA binds VelB as well as the putative methyl transferase LaeA, which is thought to regulate gene expression (19). Our data are consistent with an analogous role for Ryp2 and Ryp3 in the regulation of yeast-phase and spore transcriptional programs.

We observed that Ryp2 and Ryp3 are required for the differential expression of the transcriptional regulator Ryp1 at 37°C. Ryp1 is required for the majority of the yeast-phase specific gene expression program (5), including the higher levels of expression of Ryp2 and Ryp3 at 37°C. Indeed, we determined that the enhanced expression of all three *RYP* genes at 37°C is interdependent; when accumulation of any single *RYP* transcript is disrupted, the other two *RYP* transcripts are no longer highly expressed. These data may help explain the apparent lack of functional redundancy between *RYP2* and *RYP3*, since disruption of either results in failure to transcribe both genes. Interestingly, Ryp1 associates with the upstream regulatory region of *RYP2* but not *RYP3*, suggesting that Ryp1 may directly regulate *RYP2*, but not *RYP3*. Taken together, these data suggest that Ryp1, 2, and 3 could function in a complex to regulate the transcription of yeast-specific genes, analogous to the complex formed by VeA, VelB, and LaeA. Additionally, *ryp2* and *ryp3* mutants undergo inappropriate sporulation, much like that observed with *ryp1* mutants (5), suggesting that the three Ryp proteins may share a role in regulation of sporulation at room temperature. Of note, though Ryp1 is conserved across all fungal orders, Ryp2 and Ryp3 are found only in the filamentous and systemic dimorphic fungi,

indicating that transcriptional regulation by Ryp1 orthologs in other fungi, such as the *C. albicans* Wor1, does not involve proteins homologous to Ryp2 or Ryp3.

Since *RYP1* is required for expression of virulence genes, and as *RYP2* and *RYP3* are required for Ryp1 accumulation at 37°C, it is likely that *RYP2* and *RYP3* are also required for the expression of virulence genes. It would be interesting to directly assess the role of *RYP2* and *RYP3* in virulence. However, conventional mouse models of *Histoplasma* pathogenesis require infection with discrete fungal particles, such as spores or yeast cells but not filaments. Mutants lacking *ryp2* or *ryp3* cannot grow in the yeast form, and the severe viability defect of *ryp2* and *ryp3* mutant spores made it impossible to utilize these cells in a mouse infection. Future development of conditional alleles of *RYP2* and *RYP3* will help determine the role of these genes in pathogenesis.

Temperature is the best characterized signal known to stimulate yeast-form growth in *H. capsulatum*. The existence of host-specific signals other than high temperature is implied by the observation that conversion of *H. capsulatum* cells to the yeast form occurs faster within host cells than in culture (31). Since phagocytosis of *ryp2* or *ryp3* mutant spores by macrophages fails to trigger yeast-form growth, either *ryp2* and *ryp3* mutants are refractory to temperature-independent host signals, or these signals are not sufficient to shift morphology in the absence of the temperature- and *RYP*-dependent program. In sum, these data indicate that Ryp2 and Ryp3 are essential regulators of yeast-form growth. In addition, the requirement of these regulators to produce viable asexual spores, which are thought to be the most common infectious particle during the natural course of infection, highlights the important role of these regulators in *H. capsulatum* biology and pathogenesis.

Table 1: Relative Gene Expression by qRTPCR

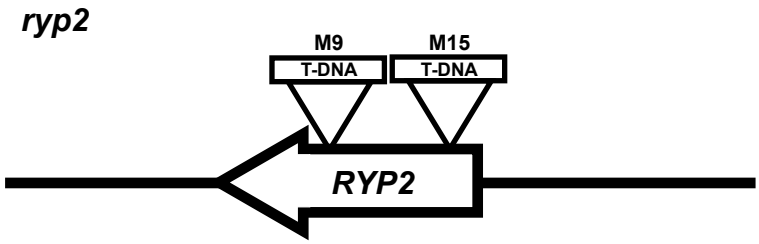
	RYP2		*RYP3*		*RYP1*	
Sample	**Relative Expression**	**Standard Deviation**	**Relative Expression**	**Standard Deviation**	**Relative Expression**	**Standard Deviation**
RT Control Filaments	1	0.341	1	0.414	1	0.353
RT *ryp2-1*	0.268	0.061	0.330	0.145	1.532	0.680
RT *ryp2-2*	0.280	0.064	0.563	0.255	2.012	0.736
RT *ryp3-1*	0.513	0.135	0.214	0.159	5.351	1.606
RT *ryp3-2*	0.165	0.050	0.219	0.100	0.791	0.408
RT *ryp1*	2.274	1.365	0.344	0.163	nd*	nd*
37°C Control Yeast	36.979	11.010	3.301	1.089	23.244	6.320
37°C *ryp2-1*	0.107	0.040	0.164	0.090	0.752	0.366
37°C *ryp2-2*	0.054	0.018	0.121	0.048	0.322	0.149
37°C *ryp3-1*	0.066	0.022	0.049	0.025	0.178	0.088
37°C *ryp3-2*	0.028	0.008	0.079	0.034	0.424	0.194
37°C *ryp1*	0.468	0.206	0.229	0.112	nd*	nd*

*not determined

Table 2: Primers used for ChIP analysis

RYP2		*RYP3*		*TEF1*	
Primer	**Sequence 5' to 3'**	**Primer**	**Sequence 5' to 3'**	**Primer**	**Sequence 5' to 3'**
OAS1738	tttggttcgtggggtaattt	OAS1852	tcaacttcaccgtgtcctacc	OAS1768	catcaagcccggtatggtc
OAS1739	agtacacagagttgggcttgc	OAS1853	acagcgttcctgaccttgtt	OAS1769	agggtaaccagcctggagtt
OAS1740	tccaagatctgtgaagcaggt	OAS1854	catcacagcagccgcatc		
OAS1741	caatgagatcccacagtcca	OAS1855	gccggatgaaacaaaacaac		
OAS1742	agcgatcatgtaaagggaaca	OAS1856	tccaaacctcccagactttc		
OAS1743	cgctaaatacttctggctggt	OAS1857	caagtacaggccctttcgtc		
OAS1744	cctcggctggaaagtaacaa	OAS1858	aggcccgttttcttcttgag		
OAS1745	gcaatggatgacaaacgaaa	OAS1859	aaagagaaagagaaacacctgacg		
OAS1746	agatggaaaattgggaatcg	OAS1860	agatctgtgccgtgcaagt		
OAS1747	ttccaaatatcaagtcgacagc	OAS1861	gtgcaaaacgggagaaaaag		
OAS1748	acaatgggcccgttttatta	OAS1862	gatatatccattcgttgcagttg		
OAS1749	ctcatgaggtcattaacattcctaga	OAS1863	tgatataccctatccgaggaa		
OAS1750	tgatgcaccgataggtttga	OAS1864	gcgttcaccggatatgattt		
OAS1751	ttgcacctactagcgccttt	OAS1865	tatcctgcgttcttcgcttc		
OAS1752	agcacaagagcgaaaaccat	OAS1866	gtccggacaaatcaggtgag		
OAS1753	atccaactcaagtcgtcgaaa	OAS1867	tcccccgaaaagatatggat		
OAS1754	cattgtgcttacggctttca	OAS1868	gatggttaacggctttattatttgtt		
OAS1755	agagccattcgaagttgagg	OAS1869	tctaagtgtcaaatcacacaacacc		
OAS1756	gtacgacgcccttgtttctc				
OAS1757	tctcaagctctccggtgatt				
OAS2088	cataggtcgaatttgggaaca				
OAS2089	ggatcgaggaaagtggaatg				
OAS2090	tggcttcttagataggacctgct				
OAS2091	ggttaacgaggctggttcata				

Figure 1: Schematic representation depicting the position of the T-DNA insertion events in the *ryp2* and *ryp3* mutants obtained from the screen. Two independent T-DNA insertions mutants, M9 and M15, were located within the *RYP2* ORF. F14 contained a 20kb deletion that eliminated the *RYP3* ORF as well as two unrelated genes, one of which represents the remnant of a retrotransposon.



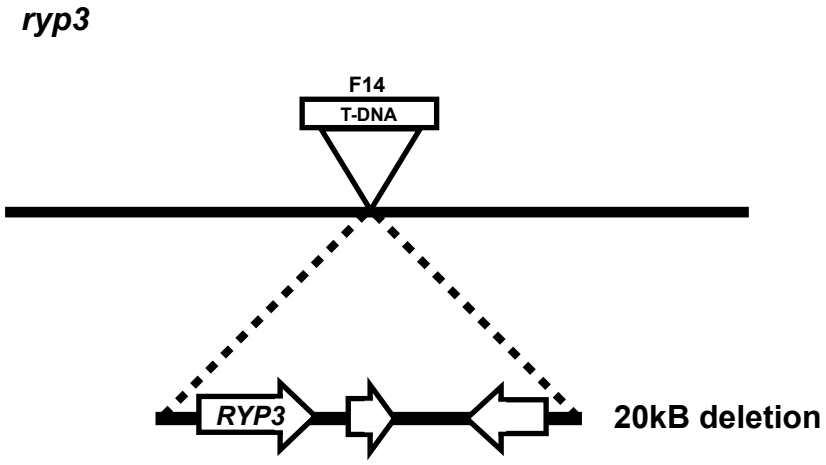
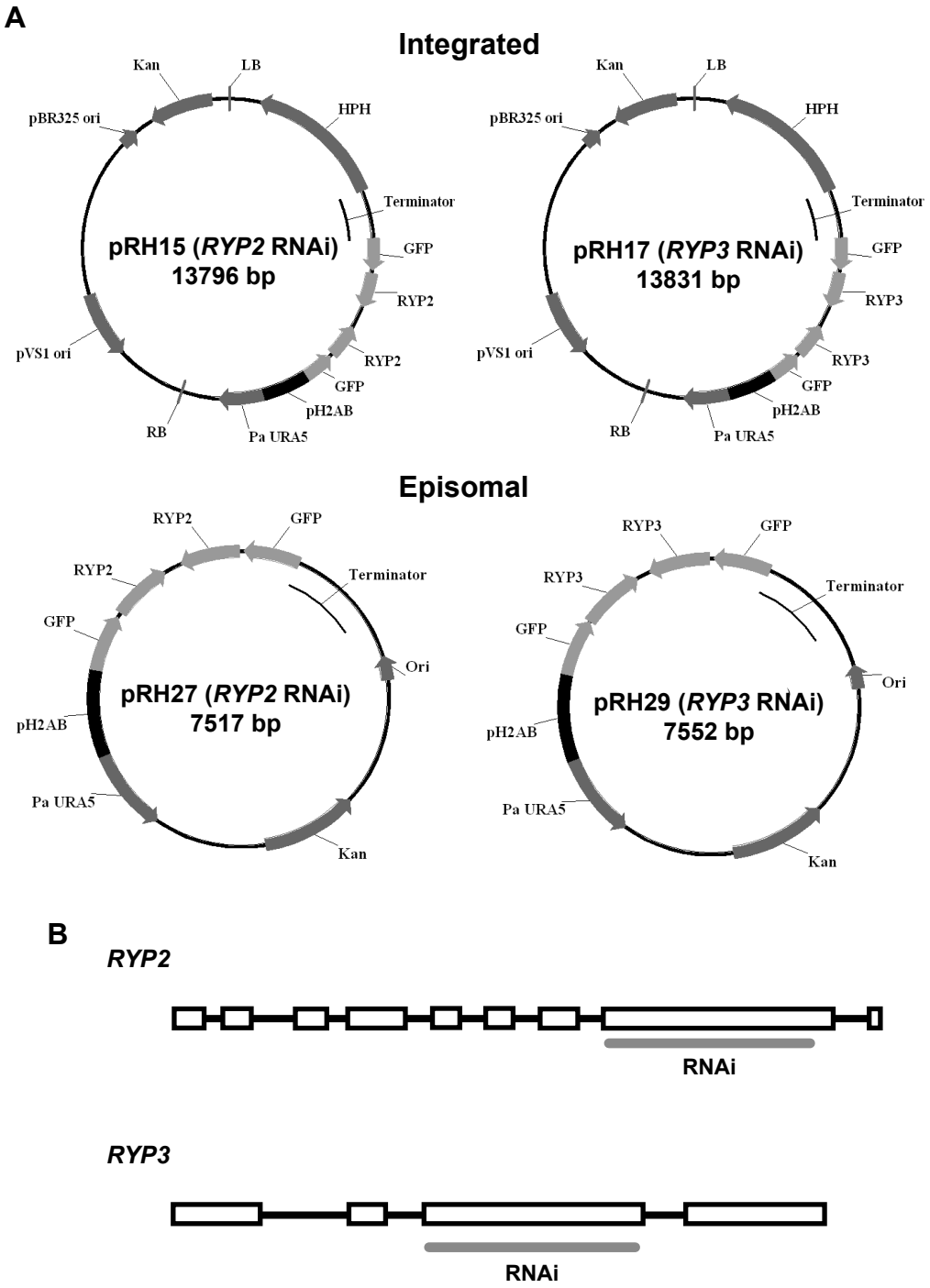


Figure 2: Diagrams of the constructs used to produce the RNAi strains utilized in this study. (A) Schematics of the episomal RNAi plasmids pRH15 and pRH17 based on pCR186. pRH27 and pRH29 are the integrating RNAi plasmids based on the pFANTAi4 backbone. See Materials and Methods for details. (B) Diagram of the 5' to 3' exon (rectangle) and intron (line) structure of *RYP2* and *RYP3*. Gray bar indicates the approximately 500bp sequence used for RNAi.



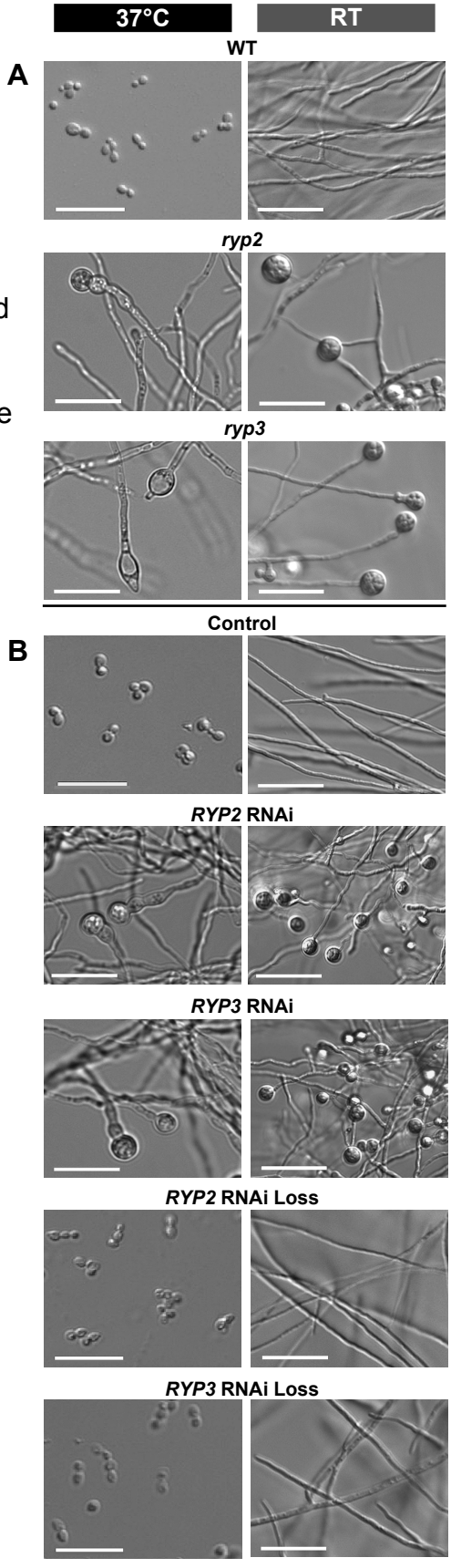


Figure 3: *RYP2* and *RYP3* are required for yeast phase growth at 37°C and regulating sporulation at room temperature.
(A) Microscopic analysis of wild-type (G217B), a representative *ryp2* insertion mutant (M9), and the *ryp3* insertion mutant (F14) identified by the screen. The cells were grown in aerated liquid cultures at either 37°C or room temperature (RT). (B) Microscopic analysis of the control, *RYP2* or *RYP3* RNA interference (RNAi) strains, and RNAi loss strains that no longer contain the RNAi constructs, grown under the same conditions as in (A). Strains shown are representative of at least 4 independent isolates.

Figure 4: Ryp2 and Ryp3 are homologous to VeA and are part of a family of conserved proteins in both dimorphic and filamentous fungi. The phylogenetic tree shows the relationship of the three VeA homologs (Vea1, Ryp2, and Ryp3) in an array of fungal species (*H. capsulatum* (Hc), *B. dermatititis* (Bd), *P. brasiliensis* (Pb), *C. immitis* (Ci), *A. nidulans* (An), *A. fumigatus* (Af), *M. grisea* (Mg), *N. crassa* (Nc), *F. verticillioides* (Fv), and *A. chrysogenum* (Ac)). Bootstrapping values for 1000 iterations are shown. * indicates predicted protein.

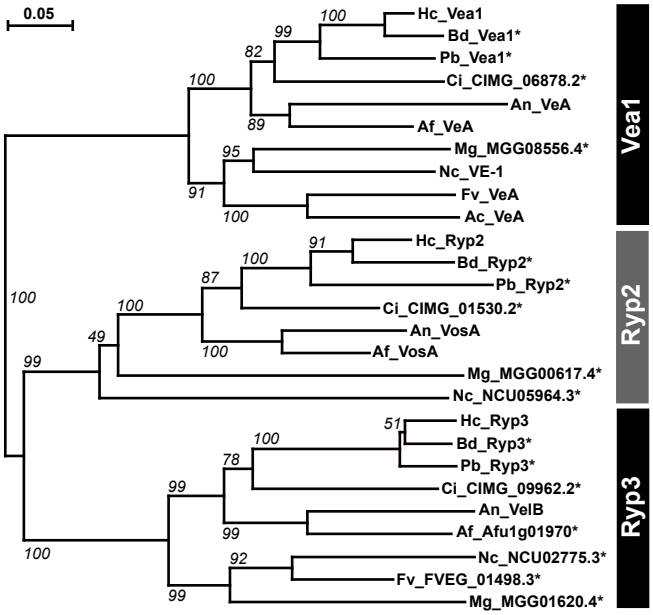


Figure 5: Ryp2, Ryp3, and VeA are highly conserved. (A) Diagram representing the protein domain structure of the VeA family members in *H. capsulatum*. Blocks 1, 2, and 3 are shown in more detail in (B). (B) Alignments of the highly conserved regions of the VeA family members in *H. capsulatum* and *A. nidulans*. Putative NLS and NES in *A. nidulans* VeA are indicated.

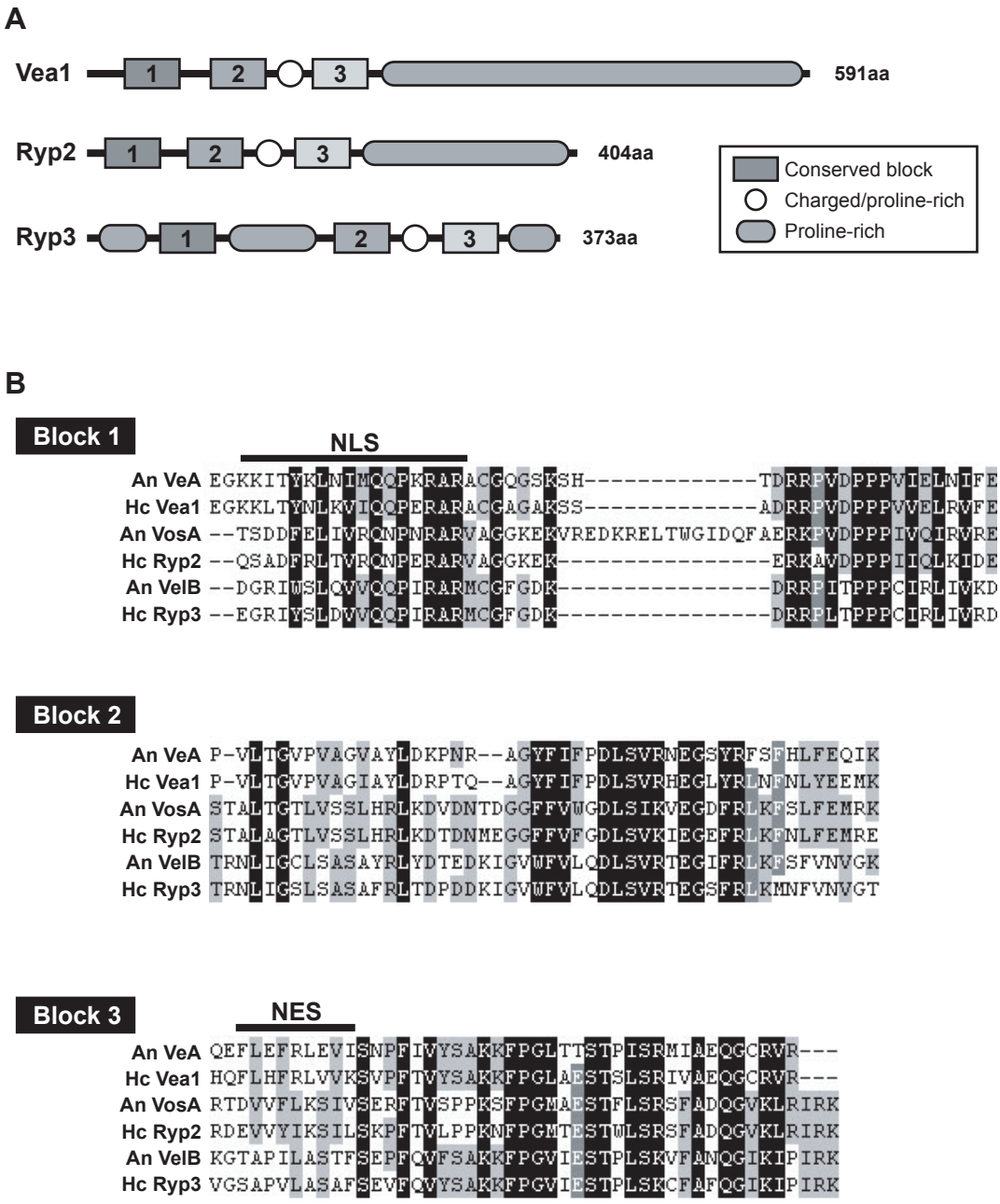


Figure 6: *RYP2* and *RYP3* expression is regulated in response to temperature and by other *RYP* genes. All strains were grown in aerated conditions at either 37°C or RT as in Figure 1. (A) and (B) Quantification of the relative expression of *RYP2* and *RYP3* in wild-type cells as measured by qRTPCR signal normalized to levels of *TEF1*. (C) Northern blot analysis of *RYP2*, *RYP3*, and *GAPDH* transcripts in wild-type cells. (D) and (E) Relative expression levels of *RYP2* and *RYP3* in the control strain, two independent *RYP2* or *RYP3* RNAi isolates, and the *ryp1* mutant measured by qRTPCR signal normalized to levels of *TEF1*. (F) Relative *RYP1* expression in the control and *RYP2* or *RYP3* RNAi isolates measured by qRTPCR signal normalized to levels of *TEF1*.

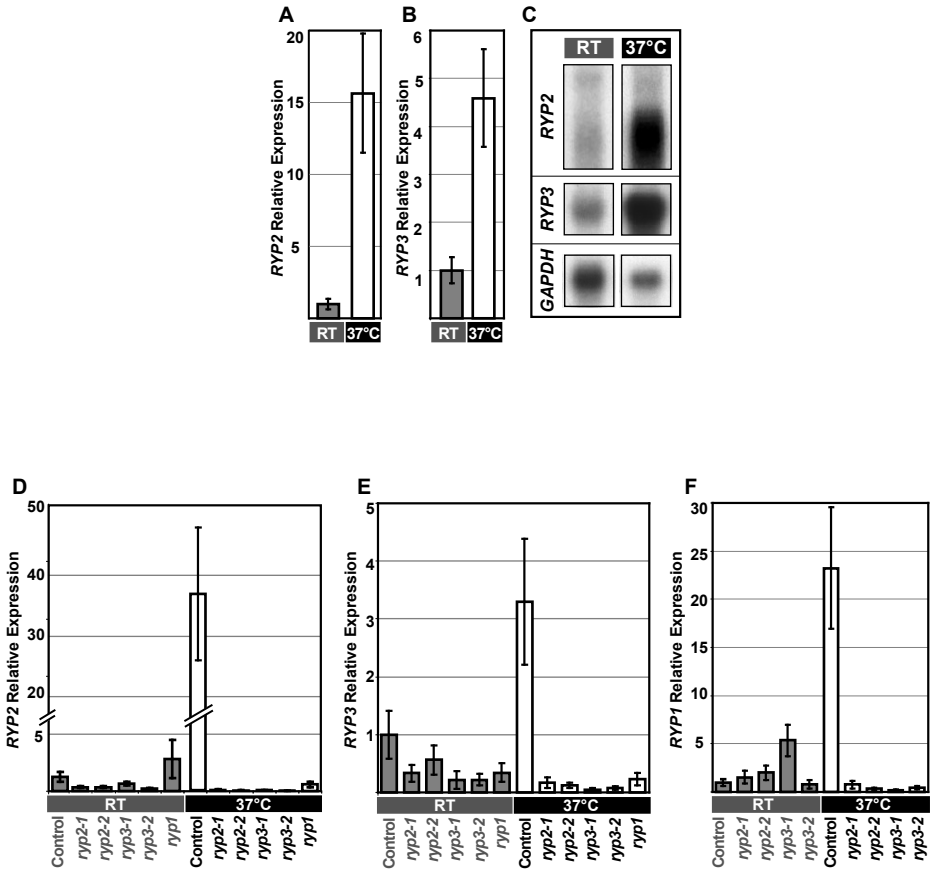


Figure 7: The transcriptional regulator Ryp1 associates with the region upstream of *RYP2*, but not *RYP3*. ChIP was performed with anti-Ryp1 antibodies in wild-type cells grown at 37°C or at RT and in *ryp1* cells grown at 37°C. Ryp1 ChIP enrichment was measured by qPCR at 500 bp intervals across the 6 kb intergenic region immediately upstream of the *RYP2* ORF (A) or at 150 bp intervals across the 1.2 kb intergenic region immediately upstream of the *RYP3* ORF (B). The enrichment values shown are for each position upstream of the ORFs relative to the reference gene *TEF1*.

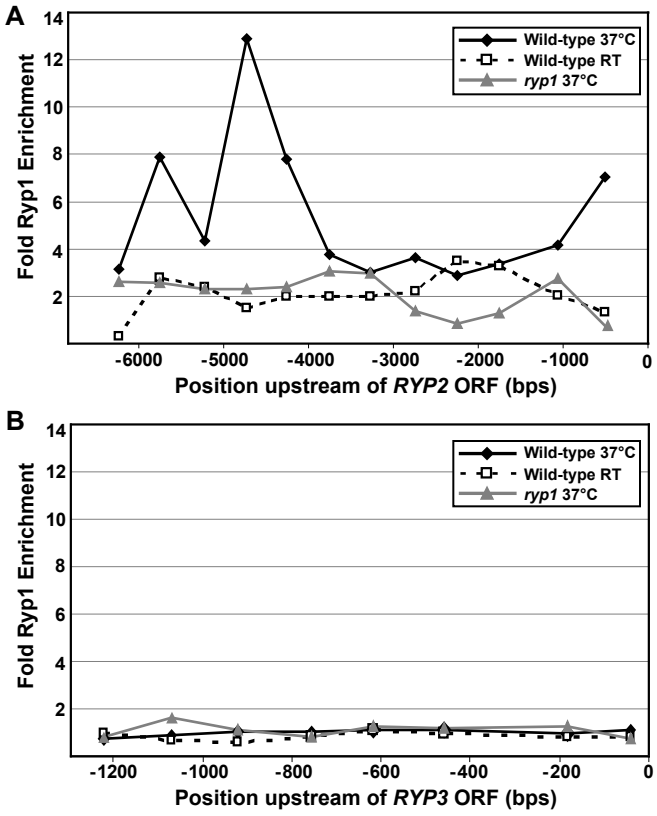


Figure 8: *RYP2* and *RYP3* are required for the production of viable spores. Cells carrying control, *RYP2*, or *RYP3* RNAi constructs were grown on sporulation plates for at least 4 weeks at RT. Spores were harvested in PBS and quantified. (A) Representative microscopic images of the spore populations observed from the control and *ryp* (*ryp2* and *ryp3*) mutant strains. (B) The spores were stored at 4°C for 7 days and plated to calculate viable CFUs at Day 0, 1, 3, 5, and 7. The viability data were similar for the entire time course and only the Day 0 time point is shown. The percent viable spores were calculated as described in the Materials and Methods. The plot shown is representative of two independent experiments with two *RYP2* or *RYP3* RNAi isolates.

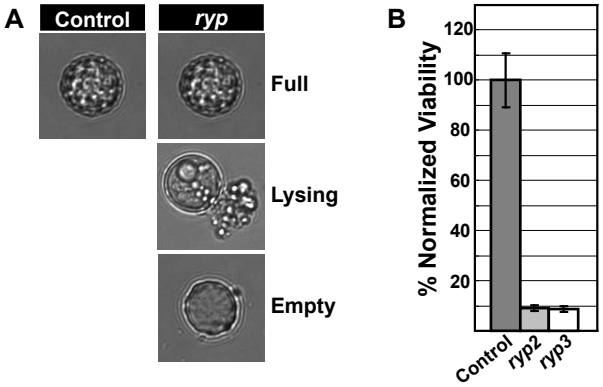


Figure 9: Induction of yeast-phase growth in macrophages depends on *RYP2* and *RYP3*. Bone marrow-derived macrophages were infected with spores from the control strain, *RYP2*, or *RYP3* RNAi isolates at an MOI of 0.1 for 72 hours and stained at the 48-hour timepoint with PAS and methyl green. Two representative images are shown for each infection. The infections were performed in triplicate with 2 independent *RYP2* or *RYP3* RNAi isolates. Black arrowheads point to representative spores, the open arrowhead points to a cluster of yeast cells, and the white arrowhead points to a representative filament.

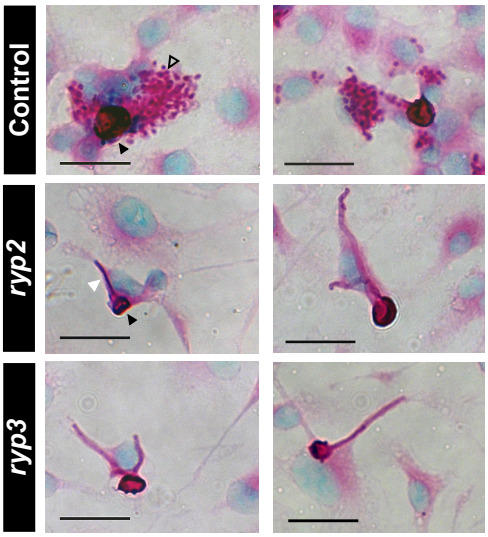
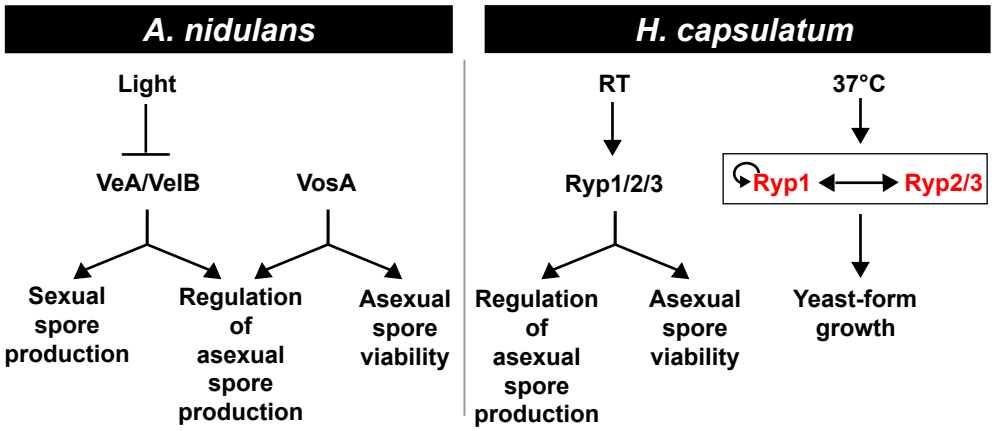


Figure 10: Regulatory diagram comparing homologous developmental regulators in *A. nidulans* and *H. capsulatum*. Although the inputs (light and temperature) into these regulatory pathways are different, the outputs are remarkably similar (regulation of cellular development). Light inhibits *A. nidulans* VeA accumulation in the nucleus, resulting in the inhibition of sexual spore development and the production of asexual spores. VeA has been shown to bind VelB (the Ryp3 ortholog), and both are required for sexual spore formation in the absence of light. VosA, the Ryp2 ortholog, is required to produce viable asexual spores and to inhibit inappropriate asexual sporulation. In *H. capsulatum*, Ryp2, Ryp3, and the transcriptional regulator Ryp1 function at room temperature (RT) to produce viable asexual spores and to inhibit inappropriate asexual sporulation. At 37°C, each of these Ryp transcripts accumulates to higher levels than at room temperature, as indicated by the red color. This transcript accumulation is interdependent, as described in the text and indicated by the bidirectional arrow. Additionally, Ryp1 associates with the Ryp2 promoter, implying that Ryp1 directly regulates Ryp2 accumulation. We showed previously that Ryp1 associates with its own promoter at 37°C, suggesting the presence of a positive feedback loop as indicated by the circular arrow.



Acknowledgements

We thank Van Nguyen and Diane Inglis for invaluable experimental assistance and advice; Mark Voorhies for exceptional assistance with sequence analysis; William Goldman (Washington University, St. Louis), Chad Rappleye (Ohio State University), Bruce Klein (University of Wisconsin, Madison), Thomas Sullivan (University of Wisconsin, Madison), and Paul Hooykaas (Leiden University, Leiden, The Netherlands) for the generous gifts of strains, plasmids, and/or protocols; and Jeff Cox, Hiten Madhani, Rebecca Zordan, Amy Kistler, Aaron Hernday, and members of the Sil lab for helpful comments on the manuscript. This work was supported by the National Institutes of Health grants 5T32AI060537-04 (to R.H.W.), R01AI066224 and PO1AI063302 (to A.S.), the Sandler Program in Basic Sciences, and the Howard Hughes Medical Institute Biomedical Research Support Program Grant 5300246 (to the University of California School of Medicine, San Francisco).

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Chapter Three:

Conclusion

Cellular response to environmental stimuli is required for optimal survival

Cells must be able to sense stimuli and regulate their development in response to the environment. Depending on the environmental conditions, cells need to control important developmental processes like sporulation or change their cellular growth form for optimal survival. In constitutively filamentous fungal species like *A. nidulans*, cells can grow either as vegetative filaments or as part of multi-cellular sporulation structures which produce spores that are required for maximal dispersal and reproduction. Once vegetative filaments reach developmental competence, the conversion between vegetative growth and spore production requires the cells to be exposed to environmental sporulation induction factors such as nutrient starvation, an air interface, or light, which leads to the activation of a complex genetic regulatory pathway (1-3). A complex genetic regulatory network is also activated in systemic dimorphic fungal pathogens like *H. capsulatum* during host infections leading to a change in morphology (4, 5). *H. capsulatum* cells sense environmental temperature and drastically alter their cellular growth form from long filamentous cells found in the soil to budding yeast-like cells inside the host. This change in growth pattern is necessary for the expression of virulence genes (6). Because sensing environmental stimuli is critical for cellular growth and survival, it is necessary to understand in detail how cells are able to sense external signals and respond appropriately. In addition, it is important to understand the genes involved in sensing and responding to environmental stimuli as we can explore the evolution of the required regulatory mechanisms. Although the input stimuli and output responses may be different for various fungi, the regulatory signal transduction network responsible can be quite conserved across species (7-12). In this body of work, I explored the morphologic

regulation of a fungal pathogen to elucidate the link between a key developmental transition and pathogenesis.

RYP2 and *RYP3* play vital roles in cellular development and morphologic maintenance in *H. capsulatum*

In this study, we set out to understand how *H. capsulatum* cells sense temperature and alter their cellular growth form by identifying genes necessary for the morphologic conversion of the dimorphic fungal pathogen *H. capsulatum* into the pathogenic yeast form. As a result of our forward genetic screen, two genes were determined to be required for yeast-phase growth at 37°C which we named *RYP2* and *RYP3* (*RYP*: required for yeast-phase growth). The *ryp2* and *ryp3* mutant strains are unable to convert into the yeast form regardless of temperature. Upon molecular characterization, we found that both transcripts accumulate to higher levels in yeast cells at 37°C when compared to room temperature filaments. Together these results indicate *RYP2* and *RYP3* are playing critical roles in *H. capsulatum* to maintain the cellular yeast form. We also found that *RYP2* and *RYP3* are expressed at low levels at room temperature, suggesting that these factors may have other functions. Accordingly, we discovered *ryp2* and *ryp3* mutants inappropriately produce spores under aerating culture conditions at room temperature, suggesting that the sporulation program is deregulated in the mutants. Moreover, when allowed to sporulate under normal sporulation conditions at room temperature, the mutant spores were found to have an extreme viability defect. These data indicate that *RYP2* and *RYP3* are genes that play crucial roles in cellular development and morphologic

maintenance of *H. capsulatum* and that these genes have distinct functions under different environmental conditions.

Ryp2 and Ryp3 are homologous to developmental regulators in filamentous fungi

Genetic networks that conduct environmental signals to regulate development can be highly conserved across organisms (7-9). Interestingly, Ryp2 and Ryp3 are related to members of the Velvet A family of regulatory proteins (VeA, VelB, and VosA) which have been shown to regulate cellular development in filamentous fungi (13-19). Although little is known about the biochemical function of these genes in fungi, the conservation across filamentous fungal species suggest these genes are playing a critical role in cellular development and gene regulation in response to environmental stimuli. The homology between the Velvet A family members in *A. nidulans* and *H. capsulatum* demonstrates how exact protein mechanisms can be co-opted to function as part of different sensory and output networks. Whereas the protein family members act to regulate sexual and asexual sporulation in filamentous fungi, the proteins control cellular morphology as well as sporulation in *H. capsulatum*. The Velvet A family of proteins is well conserved among the dimorphic fungi indicating a likely regulatory function in related organisms. However, to date, there have been no studies characterizing the roles of Ryp2 or Ryp3 orthologs in other dimorphic fungi.

***RYP* genes work cooperatively to regulate *H. capsulatum* yeast-form growth**

Ryp2 and Ryp3 have been shown to be required for the growth of *H. capsulatum* in the yeast form. Although the exact function of these proteins is unknown,

transcriptional data in this study have given hints about how the yeast-form growth transcription program is regulated. Previously, Ryp1 was identified to be a transcriptional regulator of yeast-form growth in *H. capsulatum* and like *RYP2* and *RYP3*, the *RYP1* transcript is predominately expressed in the yeast form at 37°C versus the filamentous form at room temperature (20). This study determined that the high expression of all 3 *RYP* genes at 37°C is dependent on one another. When one *RYP* gene transcript is lost, the other *RYP* transcripts are no longer highly expressed. Additionally, Ryp1 associates with the regulatory region of *RYP2*, but not *RYP3*, as shown by chromatin immunoprecipitation. This suggests that *RYP2*, but not *RYP3*, is directly regulated by Ryp1. Based on the homology and localization data in *A. nidulans*, Ryp2 and Ryp3 proteins may be fungal specific transcription factors acting with Ryp1 to control the complex regulation of yeast-form growth in *H. capsulatum*.

RYP2 and *RYP3* are implicated to have a role in virulence

As Ryp1 was identified to be essential for the expression of genes required for virulence, and *RYP2* and *RYP3* regulate *RYP1*, it is implied that *RYP2* and *RYP3* are thus required for virulence (20). However, this was not directly tested in this study as a result of the mutant spore viability defect. Instead, we ascertained that the switch to yeast-form growth of *H. capsulatum* in the presence of host cells requires *RYP2* and *RYP3*. When host macrophages were infected with the *ryp* mutants, only filamentous germinates were observed to be growing associated with macrophages indicating that no host factors can override the temperature-controlled switch regulated by Ryp2 and Ryp3.

Evolution of a conserved regulatory pathway

Systemic dimorphic fungi are more closely related to constitutively filamentous fungi than budding yeast ascomycetes by phylogenic comparisons. This study suggests that dimorphic fungi have utilized regulatory proteins found only in filamentous fungi that are implicated in development of filaments and spores (Velvet A family) and evolved new roles for these proteins in a completely different circuit that controls the yeast-form growth program in response to temperature in dimorphic fungi. Remarkably, the circuit that controls yeast-form growth in *H. capsulatum* combines components that are found only in filamentous and dimorphic fungi (e.g. VeA family) with proteins present in all fungi (e.g. Ryp1). Overall, this study has demonstrated how proteins within conserved genetic pathways can evolve to be involved in the regulation of different external inputs and cellular outputs depending on the environmental conditions. We have implicated the VeA family of proteins found in filamentous fungi and dimorphic fungi to be crucial for regulating cellular development and cell survival.

Future Directions

This initial discovery and basic characterization of the importance of Ryp2 and Ryp3 in regulating cellular development in *H. capsulatum* has lead to new diverse lines of investigation and many intriguing questions.

Function

Are Ryp2 and Ryp3 global regulators of cellular development in dimorphic fungi?

Based on homology data, I have determined the VeA family of proteins is present in all dimorphic fungal species sequenced to date. This piece of information leads to the question of whether or not Ryp2 and Ryp3 function to control cell shape and development in the other dimorphic fungal pathogens. If Ryp2 and Ryp3 orthologs are found to play a role in cellular development, it would mean that the temperature-controlled genetic pathway is highly conserved among dimorphic fungi and that Ryp2 and Ryp3 are global regulators of cellular development.

Are Ryp2 and Ryp3 master regulators of *H. capsulatum* morphology?

Based on experimental evidence provided in this study, Ryp2 and Ryp3 have been shown to be required to maintain yeast-form growth in *H. capsulatum* at 37°C. It is unknown if Ryp2 or Ryp3 are sufficient to maintain the yeast form during growth at filament-inducing room temperature conditions or whether the proteins are required for the establishment of the pathogenic yeast form. If Ryp2 and Ryp3 are necessary and sufficient to both establish and maintain the yeast-form of *H. capsulatum* growth, it

would lead to the conclusion that these proteins play a key regulatory role in the genetic pathway that induces and determines cell shape.

How do Ryp2 and Ryp3 regulate *H. capsulatum* morphology?

Ryp2 and Ryp3 are vital players in the genetic pathway to ultimately control the morphologic state of *H. capsulatum*. It is unclear how these proteins function to regulate this key developmental process as the proteins do not share homology with any known biochemical motifs. Based on data gathered from orthologs in other fungi, it is likely that these proteins may be transcription factors that activate or repress downstream genes that lead to changes in cell shape. Understanding the actual mechanism of how these proteins control this fundamental process, is crucial to elucidating how *H. capsulatum* ultimately causes disease.

What role do Ryp2 and Ryp3 play in controlling spore viability?

Spores produced by the *H. capsulatum ryp2* and *ryp3* mutants have an extreme viability defect. This is significant because VosA, the Ryp2 homolog in *A. nidulans*, is also required for spore viability (15). *vosA*Δ mutants fail to accumulate the sugar trehalose inside spores (15). Since trehalose is important for cell survival, it is likely that the loss of viability in the *ryp2* and *ryp3* mutant spores can be attributed to the lack of this essential osmotic stabilizer.

What is the function of Vea1 in *H. capsulatum*?

The VeA family of proteins in *H. capsulatum* consists of Ryp2, Ryp3, and Vea1. The function of Ryp2 and Ryp3 controlling morphological development has been characterized in this study. It is unknown whether Vea1 also plays a role in morphological development. In the event that Vea1 is not required for morphologic switching, Vea1 is highly likely to be an important regulatory protein in other processes since the homolog in filamentous fungi, VeA, has been shown to be a critical genetic regulator of secondary metabolite production in filamentous fungi (13). This suggests Vea1 may be playing a similar role in *H. capsulatum* regulating secondary metabolite synthesis. In addition, although it is unclear whether *H. capsulatum* is affected by light, VeA is inhibited by light in filamentous fungi so it would be interesting to determine if light affects key phenotypes of *H. capsulatum* and if Vea1 plays a role in those pathways.

Regulation

How do Ryp2 and Ryp3 regulate the genetic program that leads to yeast-form growth?

Ryp2 and Ryp3 are proteins that are key players in the genetic pathway that controls cell shape in *H. capsulatum*. Besides Ryp1, other components of this pathway have not been identified. Characterizing genes that are downstream of Ryp2 and Ryp3 in this regulatory network will help us understand the complex pathway that leads to the change in morphologic state. Based on the interdependent nature of *RYP* gene expression discovered during this study and results obtained by characterizing the *RYP* homologs in other organisms, it is possible that the Ryp proteins could be acting either alone or as a

complex to function as a transcriptional regulator that may be directly activating or repressing particular genes that ultimately control the expression program determining yeast-form growth and regulation of sporulation. Future experiments will determine if the Ryp proteins physically interact and whether Ryp2 or Ryp3 physically associate with the target DNAs.

How are the *RYP* genes regulated?

Interestingly, this study has shown that the expression of all three *RYP* genes is interdependent. The proteins required for the activation of the known *RYP* genes are unknown at the present time. To fully understand how temperature regulates cellular development, upstream components of this system must be identified. In addition, Ryp1 was determined to associate with the upstream region of *RYP2*, but not *RYP3*, implying Ryp1 is responsible for the expression of *RYP2*. Although *RYP3* is a key player required for *RYP1* and *RYP2* expression, its regulation unclear. Future experiments may determine that Ryp2 may associate with the upstream region of *RYP3*. It is also possible that Ryp2 and/or Ryp3 associate with the upstream region of *RYP1*. These results would help us to understand the complex interdependent transcriptional regulation of these genes.

Virulence

Are Ryp2 and Ryp3 required for virulence in a host infection?

This study has provided evidence that temperature and association with host macrophages do not induce the *ryp2* and *ryp3* mutants to grow into the yeast form. Although previous experiments suggest that dimorphic pathogens are required to convert

into the yeast form for survival in the host, definitive experiments have not been conducted to prove whether or not this is the case. It would be an important contribution to the field to determine if the *ryp2* or *ryp3* mutants could not cause disease in the host as a result of the inability to change growth form. To conduct these experiments with the *ryp2* and *ryp3* mutant strains, infections need to be performed with either mutant filaments or with conditional mutant strains. The results of these experiments would ultimately show that conversion into the yeast form is absolutely essential for survival in the host.

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