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Differentiation of the fungus *Histoplasma capsulatum* into a pathogen of phagocytes

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Abstract

Mammalian body temperature triggers differentiation of the fungal pathogen *Histoplasma capsulatum* into yeast cells. The Drk1 regulatory kinase and an interdependent network of Ryp transcription factors establish the yeast state. Beyond morphology, the differentiation-dependent expression program equips yeasts for invasion and survival within phagosomes. Yeast cells produce α -glucan and the Eng1 endoglucanase which hide yeasts from immune detection. Secretion of yeast phase-specific Sod3 and CatB detoxify phagocyte-derived reactive oxygen molecules. *Histoplasma* cells adapt to iron and zinc limitation in activated macrophages by production of siderophores and the Zrt2 transporter, respectively. Yeasts also respond to inflammation-associated hypoxia. *Histoplasma* pathogenicity thus relies on factors controlled by yeast differentiation as well as environment-dependent responses.

Keywords

fungal pathogenesis; dimorphism; cell wall; ROS; siderophores; zinc; hypoxia

Introduction

Cellular differentiation is fundamental to specialization of cell types for diverse functions in multicellular organisms as well as unicellular microbes. Fungal differentiation includes vegetative forms (hyphae and yeasts) as well as specific morphologies to facilitate mating (e.g., protoperithecia), plant tissue invasion (e.g., appressoria), and airborne dissemination (e.g., conidia). Hyphal or yeast forms are adapted for their particular environments, with some fungi switching between these two basic morphologies. While such fungal species have been called “dimorphic,” dimorphism encapsulates the idea of distinct, differentiated states whereas “pleomorphic” more appropriately describes the co-existence of multiple fungal forms.

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The dimorphism of the fungal pathogen *Histoplasma capsulatum* represents functional differentiation beyond morphology. The hyphal form of *Histoplasma* is well-suited for penetration and colonization of soil environments, nutrient absorption, and production of conidiophores for formation and release of conidia. *Histoplasma* differentiation into pathogenic yeasts is signaled by elevated temperature, typically occurring upon inhalation of conidia by mammals. This differentiation represents a program enabling infection of hosts since locking cells as mycelia at 37°C, either by chemical treatment or by genetic manipulation, renders *Histoplasma* avirulent [1–4]. The life cycle of *Histoplasma* does not require differentiation into yeasts, nor infection of mammalian hosts, further suggesting that yeast differentiation is not simply a response but a program for an alternate lifestyle. The smaller yeast form is more compatible for habitation of the phagosomal compartment, but is also equipped with factors that enable survival and replication within normally inhospitable immune cells. Many of the factors specifically expressed by yeasts represent pre-formed strategies for coping with antifungal defenses of the host rather than extemporaneous responses to encountered stresses. In this review, we highlight findings that detail the regulatory circuitry involved in *Histoplasma* differentiation into yeasts, the expression and function of yeast-phase factors that enable infection of phagocytes, and recent studies on how *Histoplasma* yeast respond, independent of differentiation, to changing conditions in the host during the immune response.

Differentiation and the pathogenic program

Differentiation of *Histoplasma* into yeasts depends on sensing the differentiation cue (i.e., 37°C) and translation of the thermal signal to transcription factors to establish an appropriate state. While differentiation of conidia into yeasts is physiologically more relevant, most studies model this process as a mycelia-to-yeast switch given the difficulties in laboratory production and manipulation of conidia. A genetic screen in the related dimorphic fungus *Blastomyces dermatitidis* identified a hybrid histidine kinase (Drk1) which is required for temperature-induced growth as yeasts. The Drk1 ortholog in *Histoplasma* is similarly required for *Histoplasma* yeast differentiation [2]. Similar genetic screens in *Histoplasma* identified 3 transcription factors: a WOPR-family protein (Ryp1; [3]) and two Velvet-family proteins (Ryp2 and Ryp3; [4]), the homologs of which control fungal morphology in other fungi. A fourth transcription factor (Ryp4) was identified based on yeast-phase expression that depends on the other Ryp factors [5**]. Ryp1 binds to a conserved DNA sequence (“motif A”) upstream of many genes, and Ryp2 and Ryp3 physically interact and bind to a second conserved DNA sequence “motif B” [5**]. The Ryp factors bind upstream of most Ryp-encoding genes and are required for expression of each other [5**] thereby forming an interdependent, self-reinforcing transcriptional regulatory loop common for differentiation switches (Figure 1). ChIP-studies combined with expression profiling further demonstrate that association of multiple Ryp-factors at the promoters of many genes determines their yeast-phase expression, including known virulence factors [5**]. In addition to the Ryp regulators, Veal, the ortholog of Velvet A in *Histoplasma*, also contributes to yeast phase differentiation as depletion of Veal prevents conversion to (but not maintenance of) the yeast phase at 37°C, even though Veal negatively regulates *RYP3* transcription [6]. Yeast differentiation also involves suppression of mycelial phase factors. For example, constitutive

expression of the mycelial phase-enriched Wet1 regulatory protein causes hyphal growth at 37°C [7**]. Identification of these factors controlling the yeast phase regulon provides an important molecular basis for understanding *Histoplasma* differentiation, yet a complete signaling cascade has not been fully established. What factor(s) comprise the phosphate acceptor proteins downstream of Drk1 and how Drk1 is presumably linked to the Ryp regulators remain unanswered questions. Despite the central importance of temperature as the differentiation cue, how elevated temperature is sensed at the molecular level and communicated to the regulating kinase and/or the yeast phase-specification transcription factor network is entirely unknown.

The differentiation triggered by temperature establishes expression of the yeast-phase regulon which includes many of the established virulence determinants of *Histoplasma*. Early expression studies used microarrays [3,8], but since then, two genome-wide RNAseq-based transcriptome studies have been performed to improve gene models and compare the *Histoplasma* pathogenic yeast phase with the avirulent mycelial phase [7**,9*]. These studies examined yeast and mycelial RNA samples from multiple, evolutionarily-divergent clinical isolates to identify a conserved set of yeast-phase genes among the roughly 9000 genes encoded in the genome. Edwards, et al., studied two strains that vary substantially in phenotype (the North America type 2 (NAM 2) and Panama lineages; [10,11]) and showed that strain differences stem largely from regulation of gene expression instead of differing gene content. Comparisons of the RNAseq data identified 275 genes representing a conserved yeast-phase regulon compared to mycelia (at least five-fold enriched; [9*]). Using microarrays, Inglis et al. similarly compared the transcriptional profile of yeast and mycelia but also included conidia [12]. Three-way comparisons with a three-fold cutoff identified 45 conserved yeast-phase enriched transcripts (127 if conidia are not considered) among the 150-or so strain-specific yeast-phase transcripts. Gilmore, et al., again used the NAM 2 and Panama strains and added two *Histoplasma* strains from Africa, resulting in 139 yeast-phase enriched transcripts (three-fold enriched) conserved among these four strains [7**]. Together these transcriptome studies estimate 1.5% to 3% of *Histoplasma* genes constitute the core yeast-phase regulon which includes many of the genes devoted to the pathogenic lifestyle (see below). Although the yeast-phase basis of many established virulence traits has driven yeast-mycelia comparative studies as a discovery approach, *Histoplasma* strains differ in pathogenic mechanisms [10] forcing reexamination of the utility of deriving a conserved yeast phase expression profile.

Production of some yeast-phase gene products has multiple levels of regulation. RNAseq-based gene models identified 187 transcripts with longer 5'UTR regions in either the yeast or mycelial phases [7**] indicating different transcription initiation sites. Combining transcriptome studies with ribosome profiling revealed that 690 genes differ in translational efficiency between yeast and mycelia [7**]. However, the genes exhibiting such phase-dependent characteristics (i.e., enriched transcription, variable mRNA lengths, and different translational efficiency) are not well-correlated with each other making the yeast differentiation role of altered transcription initiation or translation unclear. Transcription is the primary mechanism for governing phase-specific expression of the majority of genes with differential translation contributing some additional regulation.

Yeast-phase effector molecules facilitating pathogenesis

A key feature of the virulence of *Histoplasma* yeasts is their ability to avoid detection by the immune system. To reduce immune cell detection, *Histoplasma* yeasts produce α -glucan, a yeast phase-specific polysaccharide which forms the outward-facing portion of the fungal cell wall [13,14]. This effectively hides the underlying β -glucans from recognition by the host β -glucan receptor (Dectin-1) during infection of phagocytes. Genetic studies show synthesis of α -(1,3)-glucan involves the function of α -glucan synthase (Ags1 [13]), an α -amylase (Amy1 [15]), and UTP-glucose-1-phosphate uridylyltransferase (Ugp1 [15]). Loss of α -glucan due to impairment of its biosynthetic pathway increases immune cell recognition of yeasts, increases production of proinflammatory cytokines, and attenuates *Histoplasma* virulence in mice. Examination of the secreted proteome [16] identified Eng1, a secreted yeast phase-specific glucanase that hydrolyzes β -(1,3)-glucans [17]. Eng1 trims exposed β -glucans from the yeast cell surface, diminishing β -glucan recognition by the Dectin-1 receptor and reducing proinflammatory responses [18**]. Loss of Eng1 attenuates *Histoplasma* virulence in mice but is restored in Dectin-1-deficient mice, confirming the role of Eng1 in reducing β -glucan recognition by Dectin-1 [18**]. Although yeasts also secrete a β -(1,3)-exoglucanase (Exg8), this glycosyl hydrolase has minimal effects on *Histoplasma* virulence [17]. Masking β -glucans and pruning away surface β -glucan fragments constitute two additive mechanisms specified by the yeast-phase regulon to minimize detection of *Histoplasma* yeasts by host phagocytes (Figure 2).

Virulence of *Histoplasma* yeasts also requires counteracting host cell defense mechanisms such as reactive oxygen species (ROS). *Histoplasma* yeasts, but not mycelia, secrete a Cu/Zn-type superoxide dismutase (Sod3) [16], a portion of which associates with the yeast surface [19]. Consequently, Sod3 dismutates extracellular superoxide produced by host macrophages or PMNs, but not cytosolic oxidative stress [19]. Accordingly, extracellular Sod3 superoxide dismutase activity, but not that of intracellular Sod1, is required for survival of *Histoplasma* yeasts against phagocytes and for virulence in respiratory and disseminated models of infection. The restoration of virulence of Sod3-deficient yeasts in cells or animals lacking the ability to produce superoxide confirms the specific role of Sod3 in defense against phagocyte-derived ROS (Figure 2). Superoxide dismutation produces hydrogen peroxide, which is eliminated by *Histoplasma* catalases. Holbrook et al. identified CatB and CatP, which are responsible for extracellular and intracellular catalase activity, respectively [20]. Like Sod3, CatB is produced specifically by yeasts and not mycelia and is secreted to neutralize exogenous ROS [16]. Loss of CatB reduces *Histoplasma* survival in PMNs which is further reduced when the intracellular CatP catalase is lost, indicating a partially redundant role for these catalases in ROS defense [20]. *Histoplasma* virulence in mice is significantly attenuated without CatB and CatP functions. Interestingly, CatP is a peroxisomal catalase (Shen, unpublished) whose putative function is to detoxify peroxide produced through fatty acid catabolism. However, the loss of CatP alone does not attenuate virulence, suggesting minimal endogenous oxidative stress during *Histoplasma* pathogenesis.

The abundantly secreted Cbp1 protein is exclusively produced by yeasts and has typified phase-specific virulence determinants [21]. Although lack of Cbp1 does not impair yeast

growth in liquid culture, it delays yeast proliferation in macrophages and attenuates virulence in pulmonary infection [22,23]. The expression of the *CBP1* gene is only fully active after complete transition into yeast rather than rapid response to elevated temperature, showing Cbp1 production is downstream of the differentiation process [24]. Consistent with its role in pathogenesis, Cbp1 is produced by yeasts within macrophages [24] and is remarkably stable under conditions approximating the phagolysosomal environment [25]. A recent study shows that Cbp1 is important for fungal killing of macrophages which correlates with Cbp1-dependent induction of macrophage genes involved in ER stress and the Trb3 pro-apoptosis kinase [22]. Whether *Histoplasma* yeasts trigger apoptosis of macrophages remains unclear. Macrophage death decreases by 50% in cells lacking the Bak and Bax pro-apoptotic factors but not in cells deficient in pyroptosis- or necrosis-type cell death pathways, suggesting Cbp1-dependent induction of apoptosis could partially account for macrophage killing by yeasts. Infection of macrophages with Cbp1-expressing *Histoplasma* yeasts increases caspase 3/7 activation in macrophage populations, however caspase activity does not localize to cells infected with yeasts [22]. It remains to be determined if Cbp1 induces apoptosis of yeast-infected cells, how intraphagosomal Cbp1 might connect to host ER stress pathways, and if this occurs in vivo or is limited to infection of cultured cells.

***Histoplasma* yeast responses to host immunity**

The production of *Histoplasma* virulence determinants is not entirely specified by yeast differentiation since yeasts must also respond to host environment dynamics due to the development of adaptive immunity (Figure 3). Such immediate responses allow *Histoplasma* to adapt to new constraints to yeast replication. Hypoxia is one such condition that develops as a result of inflammation and granuloma formation. Upregulation of mammalian HIF-1 α , an indicator of oxygen levels below 6%, occurs in *Histoplasma*-infected lungs as early as 7 days post-infection [26*]. Infected liver tissue, particularly at sites of granuloma formation, is markedly hypoxic as demonstrated by pimonidazole labeling, indicating oxygen levels below 1% [26*]. Similar to other fungi, *Histoplasma* yeast responses to hypoxia are mediated by the Srb1 transcription factor, a functional homolog of SREBP, and include upregulation of ergosterol biosynthetic genes [27]. Although Srb1 is not required to survive hypoxic conditions, it is necessary to recover from hypoxia [27]. Accordingly, depletion of Srb1 decreases *Histoplasma* proliferation in cultured BMDMs and reduces the fungal burden in lungs [27]. Given the very low oxygen levels in *Histoplasma*-focused granulomas, it will be interesting to determine if *Histoplasma*'s hypoxia response contributes to latency and reactivation histoplasmosis.

The production of inflammatory cytokines that occurs during cell-mediated immunity also alters the intramacrophage environment by limiting essential metals. For example, IFN γ produced during the adaptive immune response decreases the expression of the transferrin receptor and restricts available iron [28–30]. Although the iron concentration in the *Histoplasma*-containing phagosome has not been determined, studies of iron uptake pathways by *Histoplasma* yeasts indirectly show that phagosomal iron concentrations become limiting. Depletion of the secreted gamma-glutamyl transferase, Ggt1, which catalyzes glutathione-dependent iron reduction, decreases yeast growth in vitro in iron-

deficient media which correlates with reduced yeast proliferation and killing of macrophages [31]. However, depletion of Ggt1 does not impair *Histoplasma* yeast virulence in vivo, at least in the short term (8 days post-infection, Shen Q and Rappleye CA unpublished results). Microarray studies of iron-restricted yeasts identified 7 upregulated genes, 6 of which correspond to a gene cluster for the production of iron-scavenging siderophores [32]. Their expression is regulated by the GATA-transcription factor, Sre1, which represses transcription in iron-replete conditions [33]. Two studies of Sid1, the L-ornithine-N⁵-monooxygenase which is required for siderophore synthesis, confirm the importance of siderophores in combating iron limitation [32,34]. Depletion of Sid1 decreases *Histoplasma* yeast growth in iron-limited media and proliferation in macrophages. Loss of siderophores in the NAM 2 strain decreases lung fungal burdens by 10-fold at 7 days post-infection [34]. However, loss of siderophore production does not attenuate virulence of the Panama strain until day 15 post-infection, a time corresponding to high IFN γ production [32]. The existence of the Fet3/Ftr1 iron reduction/uptake system in the Panama lineage but not the North American 2 lineage at least partially explains why Panama strains do not require siderophores until after the onset of adaptive immunity [34]. Characterization of the Vma1 subunit of the yeast vacuolar ATPase H⁺ transporter provides further evidence of the role of siderophores in *Histoplasma* iron acquisition [35]. Together these results indicate that intramacrophage iron levels vary during the course of infection with cell-mediated immunity causing the greatest restriction of available iron, thereby imposing the need for siderophore-based iron acquisition for continued *Histoplasma* proliferation.

Histoplasma yeasts must also contend with zinc sequestration in macrophages that become activated by GM-CSF. Zinc is essential for *Histoplasma* yeast proliferation in vitro as well as in macrophages [36]. GM-CSF treatment of macrophages increases total cellular zinc, but it also impairs *Histoplasma* zinc assimilation [37**]. This results from GM-CSF-induced expression of cytoplasmic zinc-binding metallothioneins and redistribution of zinc into Golgi compartments away from intraphagosomal *Histoplasma* yeasts. When faced with decreasing zinc concentrations, *Histoplasma* yeasts increase transcription of Zrt2, a zinc transporter that can functionally substitute for both high and low affinity zinc transporters in *Saccharomyces* [38]. Zrt2 is not required for *Histoplasma* yeasts proliferation in non-activated macrophages and during early infection (i.e., day 3), but becomes necessary beginning at day 5 post-infection as the adaptive immune response develops [38]. Thus, at different time points during infection, zinc availability in the *Histoplasma*-containing phagosome becomes more limited due to GM-CSF activation of host cells, after which continued *Histoplasma* proliferation requires expression of the Zrt2 transporter.

Conclusions

The pathogenesis of *Histoplasma* involves factors specified by differentiation into yeasts as well as responses to changes in the macrophage involvement. The expression program specified by yeast differentiation, prepares yeasts to evade host defenses. Without such ready-made mechanisms in place when host defenses are encountered, yeasts face rapid, often lethal, consequences. Not all facets of infection are constant, particularly as the host environment changes with inflammation and the development of cell-mediated immunity. For less-immediately detrimental situations posed by such dynamics, such as micronutrient

availability and hypoxia, time permits yeasts to sense and respond appropriately to allow for continued yeast proliferation.

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* of special interest

** of outstanding interest

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Highlights

Yeast-phase gene expression is primarily transcriptionally regulated

Interdependent Ryp proteins establish the temperature-dependent yeast-phase program

Yeast-phase products minimize host detection of cell wall β -glucans

Secreted yeast-phase factors detoxify phagocyte-derived reactive oxygen

A zinc transporter and siderophores combat micronutrient limitation in the phagosome

Figure 1. Differentiation of *Histoplasma* into the pathogenic yeast state

Mammalian body temperature (37°C) acts as a differentiation cue to establish the yeast phase program. Differentiation requires the Drk1 hybrid histidine kinase and four Ryp transcription factors that comprise an interdependent, self-reinforcing transcriptional regulatory loop. The Ryp factors control expression of the yeast-phase regulon, which includes factors and characteristics important for *Histoplasma* virulence. For many of these factors, yeast-phase expression is specified by combinations of Ryp factors binding to the respective promoter regions (black text).

Figure 2. Mechanistically defined yeast-phase factors that facilitate evasion and neutralization of phagocyte defenses

Histoplasma yeast minimize phagocyte detection of cell wall β -glucans by masking the β -glucans beneath a layer of α -glucans, thereby preventing recognition by the host β -glucan receptor, Dectin-1. Secretion of the Eng1 β -endoglucanase further reduces potential Dectin-1 detection by pruning away any surface-exposed β -glucans. *Histoplasma* secretion of the Sod3 superoxide dismutase and the CatB catalase enable yeasts to eliminate antifungal reactive oxygen molecules produced by phagocytic host cells (through the phagocyte NADPH-oxidase (Phox) complex).

Figure 3. *Histoplasma* yeast responses to alterations of the host environment

Histoplasma yeasts have access to sufficient oxygen, iron, and zinc within the phagosome after infection of resident macrophages. Inflammation and granuloma formation causes hypoxic conditions to which *Histoplasma* yeast responds via the Srb1 regulator. Cytokine-activation of macrophages upon development of an adaptive immune response causes depletion of micronutrient availability in the phagosome. IFN γ reduces phagosome availability of iron necessitating secretion of siderophores (red) by *Histoplasma* yeasts. GM-CSF activated macrophage sequester zinc from yeasts by redistributing zinc out of the phagosome into Golgi organelles and to cytoplasmic metallothioneins (MTs; dark blue). Faced with limited zinc, *Histoplasma* yeasts produce the Zrt2 zinc transporter (yellow) for zinc acquisition.