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Transcriptional control of sexual development in *Cryptococcus neoformans*

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Abstract

Developmental processes are essential for the normal life cycles of many pathogenic fungi, and they can facilitate survival in challenging environments, including the human host. Sexual development of the human fungal pathogen *Cryptococcus neoformans* not only produces infectious particles (spores) but has also enabled the evolution of new disease-related traits such as drug resistance. Transcription factor networks are essential to the development and pathogenesis of *C. neoformans*, and a variety of sequence-specific DNA-binding proteins control both key developmental transitions and virulence by regulating the expression of their target genes. In this review we discuss the roles of known transcription factors that harbor important connections to both development and virulence. Recent studies of these transcription factors have identified a common theme in which metabolic, stress, and other responses that are required for sexual development appear to have been co-opted for survival in the human host, thus facilitating pathogenesis. Future work elucidating the connection between development and pathogenesis will provide vital insights into the evolution of complex traits in eukaryotes as well as mechanisms that may be used to combat fungal pathogens.

Keywords

Transcription factor network; fungal sexual development; fungal pathogenesis; evolution

Fungal survival and pathogenesis depend on diverse developmental responses

Developmental events are central to the biology of both higher eukaryotes and microorganisms, including pathogenic fungi. In these processes new cell types are forged from precursors, resulting in astonishing complexity and the appearance of novel structures (Estella *et al.*, 2012; Pittet *et al.*, 2014). Frequently, developmental cells and structures

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exhibit unique traits that facilitate the ability of an organism to survive new environments. For pathogenic fungi, that new and harsh environment can be the human host, an ecological niche that includes a variety of immunological mechanisms that have evolved to inhibit microbiological growth. Researchers have leveraged relatively simple fungal systems for decades to understand not only the evolution and appearance of broad, complex processes like sexual development but also the emergence of pathogenesis.

Developmental processes in the three most common fungal pathogens (*Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans*) are intimately associated with disease. In *C. albicans*, the fourth most common cause of blood stream infections in United States hospitals, cell-type switching between white and opaque cells connects development to pathogenesis (Edmond *et al.*, 1999). Opaque cells are able to mate and undergo development, and they can cause cutaneous disease (Kvaal *et al.*, 1999; Miller and Johnson, 2002). White cells, in contrast, mate at an exceptionally low rate and can cause potentially life-threatening systemic disease, especially in immunocompromised individuals. Recent studies investigating possible mechanisms for these differences in disease presentation show that white and opaque cells interact with immune phagocytes in unique ways. Depending on the kind of phagocyte and the local environment, *C. albicans* cells are either phagocytosed and killed (white) or they are ignored (opaque) (Sasse *et al.*, 2013). This suggests that the evolution of the requirement for phenotypic switching facilitated the appearance of a novel trait: survival in the antimicrobial environment of a human host.

Aspergillus fumigatus is another common human fungal pathogen that causes both invasive disease in immunocompromised patients and allergic disease in immunocompetent individuals (Denning, 2001; S.J. Lin *et al.*, 2001). Sexual development has only relatively recently been described in *A. fumigatus*, although the presence of recombinant populations and mating type genes was known previously (Paoletti *et al.*, 2005; O’Gorman *et al.*, 2009). While the connection between virulence and development in *A. fumigatus* is still under active investigation, ascospores (the end products of sexual development in *A. fumigatus*) are known to be highly heat resistant relative to conidia (the end products of asexual development), and may aid in the survival and dispersion of the organism. In addition, it has been hypothesized that drug resistance in an *A. fumigatus* population in Europe arose from a relatively small number of individuals that then spread the trait to drug susceptible mating partners via sexual recombination (Camps *et al.*, 2012).

One system that lends itself well to the study of both development and pathogenesis is that of the basidiomycete fungus *Cryptococcus neoformans*. The study of sexual development in *C. neoformans* is facilitated by the fact that it is a genetically tractable system with well-developed molecular tools. *C. neoformans* is also a cause of meningoencephalitis, primarily in immunocompromised individuals. Cryptococcosis is responsible for the deaths of approximately 600,000 people annually worldwide, mostly among those with AIDS in Sub-Saharan Africa (B. Park *et al.*, 2009).

C. neoformans in the environment is found in association with tree bark, soil, and bird droppings. It is a haploid, budding yeast that is able to undergo a multicellular sexual development process that results in the production of spores, and population studies of

isolated strains show that recombination can occur in the environment, including in Sub-Saharan Africa (Litvintseva *et al.*, 2003). Spores are likely one of the infectious particles inhaled by humans; they are small (1–3 μm in size), able to survive harsh stresses (such as high temperature, desiccation, and reactive oxygen species), and cause disease in a mouse model of cryptococcal infection (Botts *et al.*, 2009; Giles *et al.*, 2009). As well as producing infectious particles, sexual development in *C. neoformans* has been shown to facilitate the evolution of drug resistance (Ni *et al.*, 2013). Therefore, studies of the sexual cycle of *C. neoformans* are valuable not only for the insights they provide into fungal development and evolution but also for their contributions to our knowledge of a process that is intimately connected to the virulence of a human pathogen.

One strategy used by pathogenic fungi like *C. neoformans* to control both sexual development and virulence is the implementation of genetic networks constructed from diverse sets of transcription factors. Developmental networks are frequently controlled by a relatively large number of transcription factors that act as regulatory hubs and coordinate the expression of target genes (Kim *et al.*, 2008; MacArthur *et al.*, 2009; Nobile *et al.*, 2012). Regulatory hubs are frequently members of protein families that have shared DNA-binding motifs that include the Zinc Finger, Hox/Homeotic, and High-Mobility Group (HMG) domains, and they are the building blocks on which selection occurs to yield the resulting network. In this review we will focus on the transcription factor networks that control sexual development in *C. neoformans* and their connections to virulence as a way to better understand the mechanisms that contribute to fungal disease.

***Cryptococcus neoformans* undergoes robust sexual development**

Sexual development in *C. neoformans* has been shown to occur via two mechanisms: same-sex (α only) and opposite-sex ($\mathbf{a} \times \alpha$) development. While both have been shown to occur under laboratory conditions, opposite-sex development is generally more robust (Lin *et al.*, 2005). Two stable mating types ($\mathbf{a}$ and α) are present in *C. neoformans* and are specified by an approximately 120kb *MAT* locus that contains ~23 genes. These genes are involved in pheromone production and sensing, the establishment of sex-specific cell identities, and other developmental pathways (Kwon-Chung and Bennett, 1978; Lengeler *et al.*, 2000; Lengeler *et al.*, 2002; Fraser *et al.*, 2004). Sexual development between $\mathbf{a}$ and α cells can be defined by five stages: mate detection, cell fusion, dikaryotic filamentation, basidium formation, and spore biogenesis.

The process begins when pheromones from one haploid mating partner are sensed by the pheromone receptor of the other mating partner, setting in motion a series of events leading to cell fusion (mating). After mating, the resulting fusant begins growing as a dikaryon, a filament in which both nuclei are maintained and replicated individually. A hallmark of dikaryon formation and maintenance in basidiomycetes is the appearance and use of clamp cells to properly transport replicated nuclei across septa and into daughter filaments (Casselton and Olesnick, 1998). *C. neoformans* is no different, and fused clamp cells are readily visible on the septa of mature filaments.

In response to unknown signals, the growing dikaryotic filament terminates with the production of a fruiting body known as a basidium. Nuclear fusion and meiosis occur inside the basidium, and the four meiotic products undergo repeated mitosis before being packaged as spores and budding off the fruiting body in four long chains (Idnurm, 2010). Aerial chains can then provide a platform for spores to disperse in the environment, and models suggest that aerial spore dispersal is a mechanism for *C. neoformans* to colonize new (and possibly more hospitable) locations. *C. neoformans* spores are able to survive multiple environmental stresses that include high temperatures, desiccation, and reactive oxygen species (Botts *et al.*, 2009).

Spores can also be phagocytosed by macrophages in the absence of opsonin, whereas yeast cells must generally be opsonized for efficient phagocytosis to occur (Giles *et al.*, 2009). The efficiency with which *C. neoformans* is phagocytosed by macrophages is positively correlated with a decrease in human patient survival, and as such the ability of spores to be phagocytosed more readily than yeast could be a factor in spore-mediated disease (Sabiiti *et al.*, 2014). Once spores have found a suitable environment in which to grow, they undergo germination by shedding their spore coat and resuming the process of mitotic reproduction (vegetative growth) as haploid yeast.

Only within the past two decades have the molecular events controlling development been discovered, but the previously-described morphological features of *C. neoformans* development have been known since 1975 (Kwon-Chung, 1975; Erke, 1976; Kwon-Chung and Bennett, 1978; Hull and Heitman, 2002; Idnurm *et al.*, 2005). During that time, much has been learned regarding the nature of the pheromone/pheromone receptor system and, more recently, the transcription network regulating filamentation and later developmental events. These findings have underscored the value of not only studying the developmental pathways in *C. neoformans* as a way to better understand the evolution of transcription factor networks, but they have also revealed connections between sexual development and pathogenesis that may be applicable to other disease-causing fungi.

Development begins with cell fusion and a switch to filamentous growth

Upon encountering a mating partner, pheromone production is up-regulated in both mating types. The secreted MF α pheromone from **a** cells binds to its receptor on the surface of **α** cells (Ste3 α), and conversely, the MF α pheromone binds to the Ste3 α receptor on **a** cells (Hull and Heitman, 2002; Chung *et al.*, 2002). After pheromone binding to these G protein-coupled receptors, a MAP Kinase cascade occurs that eventually results in the activation of the atypical pheromone response transcription factor Mat2 (X. Lin *et al.*, 2010; Kruzel *et al.*, 2012). Pheromone Response Factors (PRFs) in fungi integrate signals such as nitrogen limitation and the presence of a mating partner and convert them into a transcriptional response (Sugimoto *et al.*, 1991; Hartmann *et al.*, 1996; Zarnack *et al.*, 2008). Once PRFs become activated, they regulate genes required for developmental events such as cell fusion. Activation of Mat2 results in its binding to the Pheromone Response Element (PRE), a 16 nucleotide *cis*-regulatory element found upstream of known development genes and regulators, and an increase in the expression of its target genes (X. Lin *et al.*, 2010; Kruzel *et al.*, 2012). The *C. neoformans* PRE is similar to those used by other fungi; however, Mat2 is

highly diverged at the amino acid level, and this lack of regulator conservation in the context of binding site preservation suggests that the transcription network regulating the pheromone response has been rewired (Dolan *et al.*, 1989; Sugimoto *et al.*, 1991; Zarnack *et al.*, 2008; Sahni *et al.*, 2009).

In addition to Mat2, two other sequence-specific High Mobility Group Domain proteins exist in *C. neoformans* (Hgr1 and Hgr2) and both more closely resemble PRFs from other systems than Mat2 (Kruzel, 2011; Mead, 2015; Mead *et al.*, unpublished data). While neither protein is the PRF in *C. neoformans*, both play roles in sexual development. *HGR1* is required for wild type levels of filamentation and spore formation; that is, crosses between *hgr1* strains exhibit fewer, shorter filaments than wild type crosses, fewer numbers of spores, and a lower frequency of spore germination (Kruzel, 2011; Mead, 2015; Mead *et al.*, unpublished data). In addition, *HGR1* interacts genetically with the filament regulators *STE12a* and *STE12α* (Kruzel, 2011; Mead *et al.*, unpublished data). *HGR2* is required for wild type levels of spore production and normal frequencies of spore germination (Mead, 2015; Mead *et al.*, unpublished data). Deletion of both *HGR1* and *HGR2* results in a nearly complete loss of spore production (Mead, 2015; Mead *et al.*, unpublished data).

MAT2, *HGR1*, and *HGR2* form an interconnected network where each gene is required for proper expression of the other two (Mead, 2015; Mead *et al.*, unpublished data). This high level of interdependency suggests that rewiring of the ancestral network occurred, and it resulted in Mat2 assuming the role of a PRF (Kruzel *et al.* 2012). Because *HGR1* more closely resembles PRFs from other fungal systems, it is likely that the ancestral PRF in *C. neoformans* was *HGR1*, but at some point in the evolutionary history of *C. neoformans*, Mat2 became the functional PRF. This shift in the function of Mat2 could occur because its targets (including *HGR1* and *HGR2*) were likely under the control of multiple regulators which facilitated the proper expression of Mat2 target genes after they lost their Mat2-dependence. This high level of connectedness is a frequently observed attribute of transcription networks and can aid in the evolution of new traits such as pathogenesis (Nobile *et al.*, 2012; Monteiro, 2012; Sorrells and Johnson, 2015).

Two of the direct transcriptional targets of Mat2 are genes that encode the homeodomain transcription factors *Sex Inducer 2a* and *Sex Inducer 1α* (*Sxi2a* and *Sxi1α*). Each haploid mating partner encodes only one Sxi protein: *Sxi2a* is found in **a** cells while *Sxi1α* is only found in **α** cells, and they are homologs of the cell identity factors **a1/α2** and **bE/bW** from *S. cerevisiae* and *U. maydis*, respectively (Johnson, 1995; Brefort *et al.*, 2009). Following the paradigm of homeodomain protein-mediated regulation of fungal sexual development, *Sxi2a* and *Sxi1α* form a heterodimeric complex, and both proteins are necessary for development to occur (Hull *et al.*, 2002; Hull *et al.*, 2005; Kües and Casselton, 1992). Interestingly, the Sxi complex is also sufficient to drive sexual development; when either Sxi factor is expressed in cells of the opposite mating type (*Sxi2a* in **α** cells or *Sxi1α* in **a** cells), full sexual development can occur in the absence of a mating partner (Hull *et al.*, 2002; Hull *et al.*, 2005). This shows that the addition of only one transcription factor is sufficient to initiate and complete the series of events that lead to filamentation and sporulation.

Targets of the Sex Inducer Proteins regulate development and pathogenesis

The Sxi proteins regulate genes directly via the Sxi Binding Site (SBS) and indirectly through an unknown mechanism that could include RNA regulation (Mead *et al.*, 2015). The SBS is a 16 base pair sequence that is found upstream of 50 Sxi-regulated genes and consists of two half sites that correspond to each individual protein's binding site. Both proteins must be contacting DNA for the complex to exert transcriptional regulation, and the degree of regulation correlates with the conservation of that binding site relative to the cognate sequence (Mead *et al.*, 2015).

Direct targets of the Sxi proteins include known developmental genes that harbor roles in multiple stages of the process. *CLP1* is a homolog of a gene required for clamp cell formation in *Coprinopsis cinerea*, and in *C. neoformans* it is a Sxi target essential for filament formation (Inada *et al.*, 2001; Ekena *et al.*, 2008). Another Sxi target with known developmental functions is *CPR2*, a constitutively active G protein-coupled receptor that is similar to the canonical pheromone receptors *STE3a* and *STE3b*. Crosses between *cpr2* strains result in clamp cells that are not fused and aberrantly long (Hsueh *et al.*, 2009). Models suggest that *CPR2* competes with the canonical pheromone receptors for pheromone binding, thus lowering the overall levels of free pheromone and decreasing the odds of deviant clamp (or haploid) cell fusion.

A gene containing an argonaute domain (*AGO2*) was also identified as a Sxi direct target, and its null mutant was shown to have a defect in spore production (X. Wang *et al.*, 2010). *AGO2* is also required for the process of Sex-Induced Silencing (SIS), an RNAi-mediated form of silencing that protects the genome from the high retrotransposon activity observed during sexual development (Janbon *et al.*, 2010; X. Wang *et al.*, 2013). Interestingly, SIS is absent in a sibling species of *C. neoformans* (*Cryptococcus gattii*) that caused an outbreak of cryptococcosis on Vancouver Island, B.C., suggesting that relatively high genomic instability could facilitate the acquisition of mutations that lead to increased virulence (D'Souza *et al.*, 2010). Together, these data suggest that sexual development actively maintains a delicate balance between promoting mutation and controlling rampant, deleterious genome instability.

Finally, the last direct Sxi target with known developmental roles, is a gene encoding the RNA binding protein Vad1. Vad1 is responsible for proper pheromone and virulence factor regulation (Panepinto *et al.*, 2005; Y.-D. Park *et al.*, 2010). Vad1 regulates MF α pheromone genes directly, and *vad1* cells exhibit enhanced filamentation when crossed with a wild type mating partner (Y.-D. Park *et al.*, 2010). *VAD1* regulation of MF α genes acts as part of a "futile" cycle in which pheromone genes are expressed at very high levels, but the resulting RNA messages are kept at lower levels via Vad1 binding and *VAD1*-dependent degradation. Futile cycles are used in biology as a mechanism to quickly react to changing environments, such as a newly encountered mating partner (Y.-D. Park and Williamson, 2012). *VAD1*-mediated regulation, coupled with regulation of *CPR2*, suggests that an important role of Sxi direct targets is to decrease the amount of pheromone surrounding cells that have already fused and begun sexual development. A cell cycle arrest likely occurs in *C.*

neoformans cells once mating pheromone has been sensed (as is observed in *U. maydis*), and if spurious amounts of pheromone are constantly present even after mating has occurred, the cell cycle block would likely not allow for nascent filament elongation and continued development (Ekena *et al.*, 2008; Heimel, Scherer, Schuler, *et al.*, 2010).

Along with functioning during development, *VAD1* is also involved in *C. neoformans* virulence and stress responses. In an insertional mutagenesis screen of *C. neoformans*, a disruption in *VAD1* was recovered that exhibited decreased laccase production, a canonical virulence trait (Salas *et al.*, 1996; Panepinto *et al.*, 2005). *VAD1* is expressed in *C. neoformans* during human cryptococcosis, and cells lacking the gene are unable to cause wild type levels of disease in a mouse model of cryptococcal infection. Specifically, *VAD1* aids in the evasion of and resistance to the host immune response, likely by initiating a robust and varied stress response (Qiu *et al.*, 2013).

Targets of the Sxi proteins other than *VAD1* also play roles in metabolism and virulence. These include the known virulence factors *LAC1* and *MPK1* and genes for catabolic processes such as the TCA cycle, protein degradation, and β -oxidation (Kraus *et al.*, 2003). It does not appear that the Sxi proteins play roles in virulence *in vivo*; however, their direct regulation of virulence factors and metabolic “stress response” genes (i.e. those involved in catabolism) suggests that *C. neoformans* has co-opted response factors that may have originally evolved to facilitate survival under sub-optimal environmental conditions during sexual development for survival in the metabolically stressful human host (Hull *et al.*, 2004; Kronstad *et al.*, 2012; Mead *et al.*, 2015).

ZNF2 is a regulator of development with diverse targets

In addition to the Sxi complex, other transcription factors are required for filamentation in *C. neoformans*, and some are also connected to pathogenesis. The gene *ZNF2* encodes a zinc finger domain-containing transcriptional regulator that is required for both same-sex and opposite-sex development (X. Lin *et al.*, 2010). It is a homolog of *RBF1* from *Ustilago maydis*, a regulator of sexual development controlled directly by bE and bW (homologs of Sxi2a and Sxi1a) (Heimel, Scherer, Vranes, *et al.*, 2010). In *C. neoformans*, however, *ZNF2* is not a regulated target of the Sxi proteins and does not harbor an SBS in its promoter. Instead, *ZNF2* is an indirect target of *MAT2* and appears to act both downstream and in parallel to it (Kruzel *et al.*, 2012; Mead *et al.*, 2015). When *ZNF2* is overexpressed in *C. neoformans*, filamentation occurs constitutively, and the filaments are avirulent in a mouse model, again illustrating a connection between morphology and disease (L. Wang *et al.*, 2012).

Targets of *ZNF2* produce many putative secreted factors and the first characterized basidiomycete adhesion, Cfl1. *CFL1* is regulated by *ZNF2*, and its product is required for both adhesion and development (L. Wang *et al.*, 2012). Specifically, *CFL1* regulates the initiation of filamentation but is dispensable for filament extension and basidium formation. In addition, Cfl1 is excreted and acts as a signaling molecule to nearby cells - inducing filamentation in crosses grown on what is normally a development-repressing medium (L. Wang *et al.*, 2013). This is surprising given that signaling molecules in eukaryotes are

generally very small (e.g. unprocessed *MFa1* has a mass of approximately 4 kDa) and Cfl1 is a protein with a mass of over 20 kDa. While *CFL1* possesses interesting roles in both development and cell-cell signaling, it appears dispensable for virulence as cells over-expressing *CFL1* are less virulent in mice (L. Wang *et al.*, 2012).

Another *ZNF2* target that contributes to filament formation is the Pumilio domain-containing protein Pum1 (L. Wang *et al.*, 2014). Pumilio proteins are posttranscriptional regulators that bind to specific sequences in the 3' UTRs of their target mRNAs, and they control diverse processes in a wide variety of eukaryotes (Quenault *et al.*, 2011). In *C. neoformans* sexual development, *PUM1* is important for post fusion events and regulates those processes through a diverse set of target genes like the filament-specific factor *FAS1* and the meiosis-related *DMC1*. Similar to what was observed for *ZNF2* (and its target *CFL1*); overexpression of *PUM1* results in an attenuation of virulence.

While *MAT2* is a key regulator of development (along with its downstream targets *ZNF2* and *SXI2a/SXI1a*), a parallel pathway that does not depend on *MAT2* has been discovered that contains the zinc finger transcription factor *ZNF3*. *ZNF3* is required for same-sex development, and when filamentation occurs in *znf3* strains it is severely delayed (Feretzi and Heitman, 2013). In addition, targets of *ZNF3* include genes involved in the pheromone response, sex-induced silencing, and meiosis, therefore implicating this factor as a key connection between early development events and terminal processes, including spore production.

Sexual development concludes with spore formation, and the vegetative lifestyle resumes after spore germination

Following filamentation, a fruiting body is formed (the basidium) in which the two nuclei that were previously maintained in the filament finally undergo karyogamy. The resulting diploid nucleus proceeds through meiosis, the meiotic products undergo multiple rounds of mitosis, and the mitotic products are packaged into spores. The resulting spores bud to the surface of the basidium, producing four long spore chains that extend away from the basidium. Once dispersed spores encounter a hospitable environment, they undergo germination and resume vegetative growth as yeast, completing the life cycle of *C. neoformans*. Less is known regarding the molecular mechanisms controlling these later developmental processes relative to the network that regulates mate recognition and filamentation, but recent advances have revealed glimpses into how *C. neoformans* controls meiosis, spore formation, and germination.

Two of the targets of *ZNF3* have been shown to be required for wild type levels of sporulation and germination, and one of those targets (*SPO11*) is essential for proper meiosis (Feretzi and Heitman, 2013). *SPO11* is an endonuclease that makes double strand breaks that are required for the early stages of meiosis in a variety of organisms (Keeney and Neale, 2006). In *C. neoformans* crosses between *spo11* strains, the processes of meiosis, sporulation, and germination are severely affected (Feretzi and Heitman, 2013). Interestingly, the germination defect can be partially alleviated by subjecting mutant spores to x-ray irradiation, supporting a role for *SPO11* as a nuclease that makes breaks in DNA.

The other target of *ZNF3* known to be important for spore formation and germination is *UBC5*, a gene predicted to encode a ubiquitin-conjugating enzyme.

In addition to the requirements for proper meiosis and protein turnover, spore production is also affected by the amount of trehalose present in *C. neoformans* cells, a metabolic signal for glucose availability. Low trehalose levels (like those observed when both of the trehalases *Nth1* and *Nth2* are active) during development results in robust spore formation. However, when there are high amounts of trehalose present (such as during crosses of *nth1 nth2* cells), sporulation is dramatically decreased, and instead, chlamydospores (an alternative cell type) are produced (Botts *et al.*, 2014). For this shift in development to occur, both *NTH1* and *NTH2* must be deleted from the genome, suggesting that they play overlapping roles in trehalose degradation and sexual development signaling. While *NTH1* and *NTH2* have redundant roles in controlling developmental decisions, only *NTH2* appears to be related to virulence in a mouse model of disease, as *nth1* cells exhibit wild type levels of virulence both in mice and *C. elegans* models of infection, but *nth2* cells are hypervirulent (Petzold *et al.*, 2006; Botts *et al.*, 2014). Taken together, these results, along with the *Sxi2a/Sxi1a* data discussed above, further illustrate a connection between metabolism, development, and virulence.

Five other genes were recently identified in a proteomic study of *C. neoformans* spores as being involved in spore production to varying degrees (Huang *et al.*, 2015). Three (*DDI1*, *DST1*, and *TOP1*) were required for spore production and are known to have roles in processes as diverse as vesicle trafficking and ribosomal gene transcription in other organisms. The other two factors (*EMC3* and *GRE202*) are required for wild type levels of spore production, and mutant crosses produce markedly fewer spores. Interestingly, another protein (*Isp2*) is enriched in spores, and strains lacking this gene produce more spores than a wild type cross. How these spore production-related genes and proteins are regulated by transcription networks during development is an ongoing area of research and future work will shed light on how spore production is transcriptionally controlled.

Little is known regarding the molecular mechanisms that contribute to spore formation relative to mate recognition and filamentation, but even less is understood about spore germination and the resumption of vegetative growth after sexual development. Recently, the first bone fide germination factor was discovered in *C. neoformans* (*Isp2*), but it has no conserved domains or sequence homologs in any other organisms (Huang *et al.*, 2015). In *isp2* spores, the morphological changes associated with germination that occur in wild type spores appear, but the initiation of vegetative growth is delayed. Interestingly, this phenotype is not observed in spores produced from a cross containing a wild type mating partner (WT x *isp2*), indicating that *Isp2* is a basidium-derived protein (akin to the “maternally deposited” products of higher eukaryotes) and raises the possibility that other products found in basidia can be packaged into nascent spores as well.

Sexual development has facilitated the evolution of pathogenesis

As we have discussed here, many regulators and effectors of development also have links to pathogenesis (Figure 1). Sexual structures such as filaments, however, are rarely found in

patients with cryptococcosis, suggesting an indirect connection between virulence and sex. The influence sex has over pathogenesis is likely multi-faceted, and *C. neoformans* has proven to be a valuable tool for discerning how development can affect the evolution of pathogenesis.

Sex is known to be a creator of diversity in eukaryotic organisms and a producer of new phenotypes (Ni *et al.*, 2013). In addition, sex can transfer existing traits from a small group of individuals to a population, as has been discussed previously in *A. fumigatus* (Camps *et al.*, 2012). This has been observed in *C. gattii* as well where hypervirulence traits were transferred within a population via sexual reproduction, possibly facilitating an outbreak of cryptococcosis (Voelz *et al.*, 2013). It has also been proposed that sexual development played a major role in the evolution of the outbreak strains of *C. gattii* (Fraser *et al.*, 2005; Billmyre *et al.*, 2014).

In addition to its active role in mutation and evolution via meiosis, sexual development can also create genetic diversity indirectly through intermediates like dikaryons and diploid cells that may act as incubators for genetic changes that are advantageous for surviving in new environments but detrimental to immediate fitness. Dikaryons of the basidiomycete *Schizophyllum commune* adapt better than their monokaryotic counterparts to new selection pressures, and there is evidence of genetic exchange between the two nuclei (Clark and Anderson, 2004). Clark and Anderson went on to show that this genetic exchange occurs specifically during filamentation and prior to meiosis. Future studies are needed to determine if a similar adaptation mechanism exists in *C. neoformans* to respond to challenging environments. If so, the mutations and variation acquired during filamentous growth in a challenging environment could be implemented for survival in a susceptible host, eventually resulting in disease.

Diploidization allows for the evolution of both transcription factor networks and new traits such as the response to specific environmental stresses and virulence more broadly. The presence of two copies of each gene allows for individual copies to drift and acquire mutations that may be beneficial. In *Aspergillus nidulans*, transiently produced diploid nuclei in filaments are able to confer fitness advantages that promote increases in growth, and in *C. neoformans*, diploidization is proposed to be one of the mechanisms by which sex facilitates mutation and diversity (Schoustra *et al.*, 2007; Ni *et al.*, 2013).

Summary

Fungi have proven to be extremely useful models for the study of sex and the evolution of novel attributes such as pathogenesis. The mechanisms fungi use to control development (including complex transcription factor networks) are also implemented in other organisms and exhibit dramatic plasticity to accommodate new environments. In *C. neoformans* it appears this plasticity has facilitated the co-option of pathways originally used to survive challenging environments for use during survival in the human lung. Future work is needed to continue building the complex network controlling development in order to fully understand the mechanisms and evolutionary history of pathogenesis in this human fungal pathogen.

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Figure 1. A complex transcriptional control network regulates sexual development in *C. neoformans*

Genes involved in sexual development are represented with different-colored ovals. Colors are based on the stage at which null mutants exhibit phenotypes in development: blue – filamentation, red – spore production, green – spore germination, white – no known sexual development phenotype. Genes labeled with a white font are involved in virulence. White arrows - direct regulation via interactions between transcription factors and identified binding sites in the promoters of their targets. Black arrows - genetic interactions that may or may not occur via direct binding to target genes. Direct Sxi2a/Sxi1α targets of interest are boxed in gray.