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Cryptococcus neoformans: sex, morphogenesis, and virulence

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Abstract

Cryptococcus neoformans is a dimorphic fungus that causes lethal meningoencephalitis mainly in immunocompromised individuals. Different morphotypes enable this environmental fungus and opportunistic pathogen to adapt to different natural niches and exhibit different levels of pathogenicity in various hosts. It is well-recognized that *C. neoformans* undergoes bisexual or unisexual reproduction in vitro to generate genotypic, morphotypic, and phenotypic diversity, which augments its ability for adaptation. However, if and how sexual reproduction and the meiotic machinery exert any direct impact on the infection process is unclear. This review summarizes recent discoveries on the regulation of cryptococcal life cycle and morphogenesis, and how they impact cryptococcal pathogenicity. The potential role of the meiotic machinery on ploidy regulation during cryptococcal infection is also discussed. This review aims to stimulate further investigation on links between fungal morphogenesis, sexual reproduction, and virulence.

Keywords

Cryptococcus neoformans; Sexual cycles; Ploidy; Meiosis; DNA damage response; Morphogenesis; Dimorphism; Pathogenesis; Host-pathogen interactions; Vaccination

1. Introduction

Cryptococcus neoformans has been recognized as an environmental fungus and an opportunistic pathogen since its description in 1894 and 1895 when *C. neoformans* was isolated from a bone infection and fermented fruit juice (Otto, 1894; Sanfelice, 1895). This basidiomycete is ubiquitous in the environment and is commonly isolated from avian excreta, soil, and trees. Consequently, asymptomatic exposure through inhalation of spores or desiccated yeast cells is common in the general population, but it could lead to pulmonary and systemic cryptococcosis in individuals with compromised immune systems. Systemic cryptococcosis, with the most common clinical manifestation as cryptococcal meningoencephalitis, causes 15% of AIDS-related deaths globally (Dromer et al., 2011;

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Perfect, 2015). In the *C. neoformans* pathogenic species complex, *C. neoformans* generally causes systemic cryptococcosis in immunocompromised patients. A sibling species in this complex, *Cryptococcus gattii*, affects mostly immunocompetent individuals, although it also causes infections in immunocompromised patients (Dromer et al., 2011; Perfect, 2015). Compared to *C. neoformans*, *C. gattii* is more commonly isolated from tropical and subtropical regions, and it is responsible for the ongoing cryptococcosis outbreak in otherwise healthy individuals in North America and Canada (Kwon-Chung et al., 2002).

C. neoformans usually propagates mitotically in the unicellular yeast form through budding. Under conditions that induce sexual reproduction, such as dehydration or nitrogen starvation, the fungus undergoes morphological transition from yeast to hyphae. Hyphal formation enables nutrient scavenging from surroundings and the production of stress tolerant infectious spores. Because *Cryptococcus* yeast-to-hypha transition is uniquely and tightly associated with its bisexual and unisexual reproduction, it has not been considered a classic dimorphic pathogen, which typically encompasses thermally dimorphic species like *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Talaromyces marneffei* (Sil and Andrianopoulos, 2015). In this review, we will focus on recent discoveries on *Cryptococcus* sexual cycles, the regulation of morphogenesis, and their impact on cryptococcal pathogenicity. We will also discuss the potential impact of its meiotic machinery on ploidy regulation during cryptococcal infection.

2. *Cryptococcus* sexual cycles

The bipolar heterothallic sexual cycle of *C. neoformans* was discovered in the 1970s (Kwon-Chung, 1975). Under mating-inducing conditions (e.g. low nitrogen and dehydration), cells of the opposite mating type (α and **a**) conjugate in response to pheromone and fuse to form a dikaryon in which two parental nuclei congress but do not fuse (Figure 1). The resulting zygote then extends filamentous growth through the **a** parental cell in the form of dikaryotic hyphae. Clamp connections, a characteristic of heterothallic mating in basidiomycetes, ensure the inheritance of two parental nuclei in each newly generated hyphal compartment. The tips of aerial hyphae develop into club-shaped basidium heads where karyogamy and meiosis occur. The following repeated mitotic divisions in parallel with sporogenesis give rise to four chains of sexual spores (Figure 1). In addition to recombinant meiotic basidiospores formed on the fruiting structure of basidial heads, mitotic blastospores (yeast-like cells) bud off from hyphae or occasionally from the clamp connections. The mitotic blastospores inherit one of the parental nuclear genotypes. Although the dikaryotic hyphae, the blastospores, and the meiotic basidiospores inherit different nuclear genomes, all predominantly inherit mitochondrial DNA from the **a** parental strain (Matha and Lin, 2020). Thus, there is an amazing variety of cell types and genotypes generated during the sexual reproduction process.

C. neoformans produces equal numbers of **a** and α meiotic progeny from heterothallic sexual reproduction. However, natural cryptococcal populations are sharply distorted favoring the α mating type (99.9% α for serotype A, 95% α for serotype B, C, and D) (Cogliati, 2013; Kwon-Chung and Bennett, 1978; Yan et al., 2002), which spurs the view that the **a**-α bisexual reproduction may be rare or even absent in nature except in regions

where *MAT***a** isolates are more commonly present (e.g. *C. neoformans* serotype A VNB isolates in Africa or *C. gattii* isolates in Brazil) (Chen et al., 2015; Hagen et al., 2013). The discovery of unisexual reproduction in *Cryptococcus* provides a plausible explanation for the dominance of the α mating type. Monokaryotic fruiting or haploid fruiting, a process that resembles the **a**-α bisexual cycle with yeast-to-hypha transition and basidiospore production, involves cells of only a single mating type (Wickes et al., 1996). Since the opposite mating partner is absent, monokaryotic fruiting was previously considered asexual and mitotic. In 2005, Lin et al. demonstrated that meiosis and high levels of recombination occur during monokaryotic fruiting, indicating its sexual nature (Lin et al., 2005). Haploid cells of a single mating type can diploidize either through nuclear fusion or through endoreplication (Fu and Heitman, 2017; Lin et al., 2005; Lin et al., 2009b). Diploidization could take place either prior to or after the yeast-to-hypha transition. Meiosis and sporulation happen in the fruiting body basidial heads as observed in bisexual reproduction. Quantitative analyses of recombination during cryptococcal sexual reproduction (Roth et al., 2018; Sun et al., 2014) provide further evidence that monokaryotic fruiting is a meiotic process, the hallmark of sexual reproduction. The identification of natural homozygous and heterozygous α-α diploids, including αAAα, αADα and αBDα hybrids, further supports the occurrence of same-sex mating in nature (Lin et al., 2007; Lin et al., 2009b; Rhodes et al., 2017). Yadav et al. recently reported the occurrence of uniparental reproduction in *Cryptococcus*, known as hybridogenesis where both parents are physically required but only one parent contributes its genetic material to the hemi-clonal progeny (Yadav et al., 2020). Hybridogenesis might also contribute to the markedly skewed mating-type distribution in *C. neoformans*.

3. *Cryptococcus* yeast-to-hypha transition

3.1 *Cryptococcus* yeast-to-hypha transition and pheromone signaling

Yeast-to-hypha transition is tightly associated with sexual cycles in *C. neoformans* (Wang et al., 2014). Pheromone signaling triggers non-self-recognition and cell fusion during **a**-α bisexual mating, and the resulting dikaryotic zygote initiates hyphal growth (Figure 1). Therefore, yeast-to-hypha transition in *Cryptococcus* has historically been considered a pheromone-dependent process. Secreted pheromones are recognized by the G protein-coupled receptors (GPCRs), which reside on the plasma membrane of the recipient cells (Xue et al., 2008). Pheromone activation of the GPCR initiates a signal transduction cascade that includes a mitogen-activated protein kinase (MAPK) pathway (Figure 2) (Davidson et al., 2003). The central elements of the MAPK signaling are conserved among diverged fungal species, yet the downstream transcription factors that regulate mating usually differ between species (Figure 2) (Jones and Bennett, 2011). In *S. cerevisiae*, Ste12 is the transcription factor required for mating gene expression activated by the pheromone signaling (Roberts and Fink, 1994). A *STE12* homolog resides in the mating type locus in *C. neoformans*, but it is dispensable for mating (Wickes et al., 1997): deletion of *STE12* in *C. neoformans* reduces but does not abolish bilateral mating (Chang et al., 2000). In *Ustilago maydis*, a dimorphic basidiomycete that infects plants, it is the high mobility group (HMG)-box transcription factor Prf1 that recognizes the pheromone response elements (PREs) and activates mating genes (Hartmann et al., 1999). *C. neoformans* is evolutionarily close to *U.*

maydis, yet deletion of the *PRF1* homolog does not affect cryptococcal mating (Lin et al., 2010). The transcription factor Mat2 in *C. neoformans* was eventually identified through a forward genetics screening for mutants unable to initiate filamentation under mating-inducing conditions (Lin et al., 2010). Mat2, as an HMG-box transcription factor, functions downstream of the Cpk1 MAPK pathway, recognizes PREs, and is essential for pheromone sensing and response (Kruzel et al., 2012; Lin et al., 2010). Overexpression of *MAT2* is sufficient to increase the transcript levels of pheromone genes, evoke shmoo cell formation, and promote cell fusion (Wang et al., 2012), as expected for roles of the transcription factor in the pheromone sensing and response pathway.

For sexual reproduction, diploidization is necessary for meiosis, a reductive division. Diploidization is achieved through **a**-α nuclear fusion during bisexual reproduction, while it is more likely attained through endoreplication during unisexual reproduction as unisexual reproduction is largely independent from cell-cell fusion (Figure 1) (Fu and Heitman, 2017). Thus, the pheromone signaling pathway, which is required for non-self-recognition between **a** and α cells, should not be essential for unisexual development. Indeed, deletion of some major components of the pheromone pathway, including the pheromone receptor gene *CPR*α (*STE3*α), the pheromone transporter gene *STE6*, the G-protein α subunit genes *GPA2* and *GPA3*, or the pheromone *MF* genes abolishes bisexual mating but not self-filamentation (Chang et al., 2003; Hsueh and Shen, 2005; Hsueh et al., 2007; Lin et al., 2005). In addition, despite the essentiality of the cell identity homeodomain Sxi1α-Sxi2**a** complex for bisexual development, it is dispensable for unisexual development. However, deletion of the pheromone-responsive transcription factor gene *MAT2* abolished self-filamentation under all laboratory conditions that had been previously tested (Lin et al., 2010). The observation that Mat2 was necessary for both bisexual and unisexual development was contradictory to the notion that the pheromone pathway should not be essential for unisexual development. This conundrum was resolved recently when *mat2*Δ mutants were found to undergo robust self-filamentation in the presence of excessive copper, after heat-shock and exposure to high concentrations of calcium, or in the presence of glucosamine as the only carbon source (Gyawali et al., 2017; Xu et al., 2017). These discoveries strongly suggest that cryptococcal filamentation is regulated through complex pathways including the pheromone signaling pathway and other unidentified pathways. The natural or laboratory conditions and the unique signaling pathways that promote bisexual versus unisexual reproduction remain to be defined.

3.2 The regulation of *Cryptococcus* yeast-to-hypha transition

In many heterothallic fungi, the mating-type-specific homeodomain transcription factors form a heterodimer after cell-cell fusion, which regulates the subsequent sexual development. In *U. maydis*, the bE-bW heterodimer initiates the switch from budding yeast growth to polarized filamentous growth by targeting *RBF1*, which is necessary and sufficient for *b*-dependent filamentation (Heimel et al., 2010). In *C. neoformans*, Sxi1α and Sxi2**a** form a heterodimer that regulates post-zygotic bisexual development without any apparent pre-zygotic role in pheromone sensing or cell fusion (Hull et al., 2005; Hull et al., 2002). However, no homolog of *Ustilago* Rbf1 exists in *Cryptococcus* based on the analysis of the

Sxi1α-Sxi2**a** binding sites (Mead et al., 2015b). Thus, a Sxi heterodimer-independent transcription factor must govern the yeast-to-hypha transition in *C. neoformans*.

Transcriptome profiling of non-filamentous versus filamentous strains coupled with genetic studies revealed that Znf2 functions as the master regulator of yeast-to-hypha transition in *C. neoformans* (Lin et al., 2010). Deletion of this zinc finger transcriptional factor gene does not abolish cell fusion or pheromone response, similar to deletion of *SXI1*α or *SXI2***a**. However, Znf2 is required for cryptococcal hyphal growth during both bisexual and unisexual reproduction (Lin et al., 2010; Wang et al., 2012), in contrast to the Sxi1α-Sxi2**a** complex. Deletion of *ZNF2* locks *Cryptococcus* cells in the yeast form, and overexpression of *ZNF2* drives robust hyphal growth regardless of growth conditions (Wang et al., 2012). It is not surprising that *ZNF2* is not regulated by the Sxi1α/Sxi2**a** heterodimer (Mead et al., 2015a). By contrast, the pheromone transcription factor Mat2 is one of the upstream regulators of Znf2 (Figure 3) (Lin et al., 2010). A forward genetics approach to identify mutations that recapitulate the deletion of *ZNF2* revealed a long non-coding RNA (lncRNA) *RZE1* that regulates the total number of the *ZNF2* transcripts and the export of *ZNF2* transcripts from the nucleus to the cytosol for translation (Figure 3) (Chacko et al., 2015; Fu et al., 2018; Xu et al., 2017). Other upstream factors of Znf2 are yet to be identified (Figure 3).

In addition to the genetic factors, the role of epigenetic factors in regulating cryptococcal yeast-to-hypha transition has also been investigated. Feretzaki et al. found that RNAi is hyperactive during sexual reproduction. Znf3, a zinc finger protein that regulates pheromone production and cell fusion, is required for this sex-induced silencing (Feretzaki et al., 2016; Feretzaki and Heitman, 2013). Factors that modify chromatin structures, including histone modifications and chromatin remodeling complexes, also regulate cryptococcal filamentation. For instance, the histone deacetylase and acetyltransferase regulate mating and/or virulence (Brandao et al., 2018; O'Meara et al., 2010). Five out of 15 examined plant homeodomain (PHD) finger proteins, which are potential readers of histone modifications, regulate hyphal growth either as suppressors or as activators in *C. neoformans* (Meng et al., 2018). A PAS (Per-Arnt-Sim) domain-containing protein Pas3 regulates *Cryptococcus* hyphal growth through interacting with the E3 ubiquitin ligase Bre1, which mediates the ubiquitination of histone H2B (Zhao et al., 2018). Given the role of H2B mono-ubiquitination in regulating transcription activation (Deng et al., 2020), it is reasoned that the regulation of cryptococcal filamentation is a combination of genetic and epigenetic regulation. The SWI/SNF chromatin remodeling complex that slides or evicts nucleosomes is essential for cryptococcal filamentation (Lin et al., 2019; Walton et al., 2005). The SWI/SNF complex facilitates effective binding of Znf2 to the promoters of its many downstream targets and is required to open the chromatin for the transcription of these genes (Lin et al., 2019). Collectively, these studies suggest that epigenetic regulation intertwines with genetic regulation to control sexual development in *C. neoformans* (Figure 3).

4. *Cryptococcus* morphogenesis and virulence

Canonical *Cryptococcus* virulence traits, such as the production of capsule and melanin, and tolerance to the host temperature and CO_2 levels, have been previously reviewed

(Nosanchuk and Casadevall, 2006; Perfect, 2006; Zaragoza et al., 2009b) or recently published (Krysan et al., 2019). Here, we focus on recently emerging concepts in the relationship between *Cryptococcus* morphogenesis and virulence.

4.1 The effect of morphotypes on host-*Cryptococcus* interactions

Heterogeneity in morphotype provides a hedge-betting strategy for *C. neoformans* to adapt to different niches or stresses. Basidiospores produced during sexual reproduction are resistant to various environmental stresses tested, including high temperature, desiccation, and oxidative stress (Botts et al., 2009). Their small size (~1–2 μm) allows basidiospores to get deep into lung alveoli and prevents effective clearance by the airway ciliary movement (Botts and Hull, 2010). Previous studies showed that spores of serotype D are more infectious than yeast cells in a murine inhalation model (Sukroongreung et al., 1998), while disease progression by serotype A spores is modestly delayed compared to that of yeast cells (Velagapudi et al., 2009). These findings suggest that the difference in virulence between spores and yeast cells may be serotype-dependent, and the molecular bases for such difference are unclear. A recent study showed that parental yeasts that are not virulent by themselves produce meiotic basidiospores that cause fatal meningitis in mice (Walsh et al., 2019). This difference could be attributed to the association of spores, but not yeast cells, to the lung-draining lymph nodes. In addition, mice infected intranasally with a mixture of JEC20 and JEC21 yeasts (1:1) showed a lower fungal burden in lungs than those infected with equal inoculum of spores derived from a JEC20 × JEC21 cross (Walsh et al., 2019), consistent with the previous conclusion that spores of serotype D are more infectious than yeast cells (Sukroongreung et al., 1998).

Filamentation is critical for *Cryptococcus* to produce spores or defend itself against predation by soil amoeba in the environment (Casadevall, 2012; Lin et al., 2015; Steenbergen et al., 2001). However, during infection within a mammalian host, *C. neoformans* cells grow almost exclusively in the yeast form. This is opposite from the basidiomycete plant pathogen *U. maydis* or the ascomycete human pathogen *C. albicans*, of which the filamentous form is the characteristic morphotype associated with host invasion (Madhani and Fink, 1998). Intravenous or intracranial infection with purified filaments from a self-filamentous *C. neoformans* strain causes cryptococcosis at lower rates compared to yeast cells of the same strain (Shadomy and Utz, 1966; Zimmer et al., 1983). Furthermore, the RAM mutants that propagate as pseudohyphae are drastically attenuated in virulence in a murine model of cryptococcosis (Magditch et al., 2012).

The molecular link between morphogenesis and virulence in *C. neoformans* was further strengthened with the characterization of Znf2 (Lin et al., 2010; Wang et al., 2012). Overexpression of *ZNF2* drives cryptococcal hyphal formation and greatly attenuates virulence. Collectively, these studies using natural isolates and isogenic morphological mutants support an inverse relationship between *Cryptococcus* filamentation and virulence in a mammalian host. The difference in virulence of these morphotypes (yeast and filament) in mammalian hosts could be caused by the apparent physiological differences, chemical differences in molecular patterns at the cell surface, or the combination of all these factors. For instance, the surface of yeast and hyphal cells display different sets of molecules that

elicit different host immune responses. Previous studies showed that wild-type yeast cells induce Th2 response, while hyphae induce Th1 response (Zhai et al., 2015). In addition, the polysaccharide capsule, which masks pathogen-associated molecular patterns (PAMPs) and is antiphagocytic, is thinner in hyphae comparing to that in yeast cells (Zhao et al., 2019). The polysaccharide capsule helps *Cryptococcus* avoid and survive the attack from phagocytic cells such as macrophages, dendritic cells, and neutrophils (Zaragoza et al., 2009a). The ability of capsulated yeast cells to proliferate both intracellularly and extracellularly in host cells facilitates cryptococcal dissemination to other organs including into the central nervous system (Alvarez and Casadevall, 2006; Charlier et al., 2009; Ma et al., 2006; Tucker and Casadevall, 2002). Therefore, the yeast form of *Cryptococcus* is advantageous to establish infection, evade host immune response, disseminate, and to cause fatal disease.

4.2 Protective immunity against cryptococcal infection

Most human fungal pathogens reside in the environment and are opportunistic pathogens. Hosts recognize their presence by detecting fungal antigens or conserved PAMPs through pattern recognition receptors (PRRs) expressed on host immune cells. Early recognition and inflammation could clear fungal pathogens and/or stimulate the adaptive antifungal immunity, supporting the benefit of vaccination against fungal infections. In *B. dermatitidis*, the adhesin Bad1 is specifically expressed in the virulent yeast-form and it blocks T cell activation (Finkel-Jimenez et al., 2002). The *bad1*Δ mutant evokes a protective immune response and serves as a live-attenuated vaccine to protect the host from a subsequent lethal infection by a wild-type strain (McBride et al., 2018). The *C. albicans* hypha-specific surface adhesin Als3 mediates fungal attachment and invasion into host cells (Liu and Filler, 2011). An anti-*Candida* vaccine (*NDV-3A*) designed based on Als3 is now in clinical trials (Edwards et al., 2018). This is the first vaccine composed of a recombinant fungal protein antigen tested in humans. Those pioneering studies affirm that vaccination can be an effective strategy to prevent and/or treat fungal infections.

Animals inoculated intraperitoneally with live cryptococcal cells in the pseudohyphal form develop immunity against cryptococcosis. These animals became resistant to a subsequent challenge by virulent *C. neoformans* yeast cells (Fromtling et al., 1979). Interestingly, immunocompromised mice immunized with a live *C. neoformans* strain expressing interferon-γ are completely protected from a challenge by the wild-type *C. neoformans* H99 strain (Wormley et al., 2007). This suggests that cryptococcal vaccination could work even in immunocompromised hosts (Caballero Van Dyke and Wormley, 2018). We showed that either live or heat-killed *ZNF2*^{oe} filamentous cells can offer complete protection to mice from the subsequent infection with an otherwise highly aggressive isolate (Zhai et al., 2015). Recent discoveries of immune-protection from different cryptococcal mutants, including the live attenuated sterylglucosidase mutant (*sgl1*Δ) (Rella et al., 2015), the inactivated chitosan mutant (*cda1–3*Δ) (Lam et al., 2019), and the inactivated *fbp1*Δ mutant (Wang et al., 2019) further support the potential of inactivated whole cell vaccines or protein subunit vaccines to prevent the deadly cryptococcosis. Given that Znf2's regulon is enriched with extracellular proteins, the dimorphism-associated factors likely profoundly shape host-pathogen interactions, providing a fertile ground to identify vaccine candidates and guide the

development of immunotherapies. As most research is focused on molecules specific to the virulent morphological form, research on the virulence-attenuated morphological form is underexplored in *Cryptococcus* and other environmental dimorphic fungi.

4.3 Meiotic machinery-involved ploidy variation during infection

As an opportunistic fungal pathogen, *Cryptococcus* cells can stay dormant without causing any symptoms for decades in the lungs of immunocompetent individuals. Reactivation of latent pulmonary infections occur primarily in immunocompromised individuals. It is of great significance to unveil what promotes cryptococcal latency and how this fungus reactivates from dormancy.

Cryptococcus typically exists in a haploid state in the yeast form of 3–10 μm in diameter *in vitro*. However, heterogeneity in cryptococcal cell size in animal/human lungs has been documented for many decades. In a murine model of cryptococcosis, about 10 to 20 percent of cryptococcal H99 cells in the lungs form titan cells of 10–100 μm in diameter with polyploid DNA content (Okagaki et al., 2010; Zaragoza et al., 2010). The capsule layer of titan cells are highly cross-linked and tightly attached to a thicker cell wall compared to normal haploid cells (Zaragoza et al., 2010). These cells are resistant to phagocytosis, oxidative and nitrosative stresses, and antifungals (Crabtree et al., 2012; Gerstein et al., 2015; Okagaki and Nielsen, 2012). Interestingly, polyploid titan cells generate offspring with reduced-ploidy, primarily haploid daughter cells (Gerstein et al., 2015). Under stressful conditions (e.g. in the presence of antifungals), titan cells can produce diploid or aneuploid progeny with better adaptation to the stresses compared to progeny produced by normal yeast cells (Gerstein et al., 2015). It is proposed that titan cell formation may contribute to cryptococcal dormancy and regeneration of haploid cells may contribute to cryptococcal re-activation. What triggers polyploidization and what are the routes of depolyploidization remain unsolved.

During sexual reproduction, ploidy increase and reduction occur naturally. Meiosis is a form of reductive division that occurs following an increase in nuclear DNA resulting from karyogamy or endoduplication and pre-meiotic replication. In *C. neoformans*, meiosis occurs in the club-shaped basidial head developed at the tip of hyphae. Blocking meiosis does not affect hyphal growth or the development of basidial heads *in vitro*, but it does abolish or severely impair sporulation (Figure 1) (Feretzaki and Heitman, 2013; Liu et al., 2018). Given that yeast-to-hypha morphological differentiation associated with sexual reproduction *in vitro* is typically absent *in vivo*, and that disruption of the pheromone pathway specifically has no or minimal impact on cryptococcal virulence, meiosis had not been considered for any direct role during infection (Lin et al., 2009a). Meiosis is initiated intrinsically with programmed DNA double stranded breaks and might have been evolved prior to the emergence of the non-self-recognition system. Non-self-recognition chiefly primes cryptococcal cells for cell-cell fusion designed for bisexual reproduction (Gyawali et al., 2017). As diploidization during unisexual reproduction can be achieved through endoreplication, it is possible that meiosis can be uncoupled from non-self-recognition or morphogenesis under certain settings where bisexual reproduction is inhibited. We recently found that extreme genotoxic stresses, such as gamma radiation that causes DNA double

stranded breaks, can efficiently trigger cryptococcal cell enlargement and polyploidization *in vitro* (Zhao et al., 2020). *Cryptococcus* likely experiences similar stress in the lungs of immunocompetent hosts. When these polyploid cells are released to a stress-free condition, some cells form multiple smaller nuclei, mimicking the meiotic features. Interestingly, blocking meiosis increases the proportion of titan cells in the lungs, likely by impairing ploidy reduction rather than polyploidization (Zhao et al., 2020). Consistently, cryptococcal meiosis-specific genes are activated in a subset of the population during infection. Surprisingly, cryptococcal isolates with meiosis-specific genes activated *in vivo* displayed phenotypic diversity in terms of resistance to specific genotoxic stress that is not observed in sibling isolates recovered from the same tissue in the same host (Zhao et al., 2020). The findings strongly suggest that *Cryptococcus* uses the sexual program (gametogenesis) for direct adaptation to promote its individual survival in the host. So far, meiotic events in fungi occurring during invasive infection in a mammalian host have only been documented in the obligate pathogen *Pneumocystis* (Aliouat el et al., 1999; Almeida et al., 2015; Cushion et al., 2007; Kutty et al., 2010; Peters et al., 2001). That said, multiple eukaryotic pathogens, particularly parasites, undergo sexual differentiation during infection. Cancer cells, which in essence act like eukaryotic pathogens, are known to use the sexual program to promote their own cellular propagation at the cost of the whole organism (Erenpreisa et al., 2015). Thus, understanding sexual reproduction, even in a relatively 'simple' eukaryotic microbe like *C. neoformans*, will yield important novel insights into a conserved mechanism for adaptation and rejuvenation in diverse eukaryotic species.

5. Summary of key points

1. Cryptococcal sexual reproduction, the pheromone pathway for non-self-recognition, and morphogenesis are intimately associated. During unisexual reproduction, however, the sexual program could be uncoupled from the latter processes under certain conditions.
2. Znf2 is the master regulator that governs the ultimate yeast-to-hypha transition in *C. neoformans*. Multiple signaling pathways converge at Znf2 to promote or inhibit cryptococcal filamentation. The internal and external stimuli and their cognate receiving pathways that convey the information to Znf2 remain largely unknown.
3. Cell surface antigens likely elicit the immune-protection observed in filamentous strains (e.g. $ZNF2^{OE}$). Identifying the key immune-protective antigens would be the key to guide the development of subunit vaccines or to monitor the quality of inactivated whole cell vaccines.
4. The *Cryptococcus* sexual machinery likely contributes to its disease progression. Future challenges include identifying the host factors that activate sexual machinery and dissecting the molecular connection between sexual development and cryptococcal latency and reactivation during infection.

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Highlights

- 1) Cryptococcal sexual reproduction, the pheromone pathway for non-self-recognition, and morphogenesis are intimately associated.
- 2) Znf2 is the master regulator that governs the ultimate yeast-to-hypha transition in *C. neoformans.*
- 3) Cell surface antigens likely elicit the immune-protection observed in filamentous strains (e.g. *ZNF2*^{oe}).
- 4) The *Cryptococcus* sexual machinery likely contributes to its disease progression.

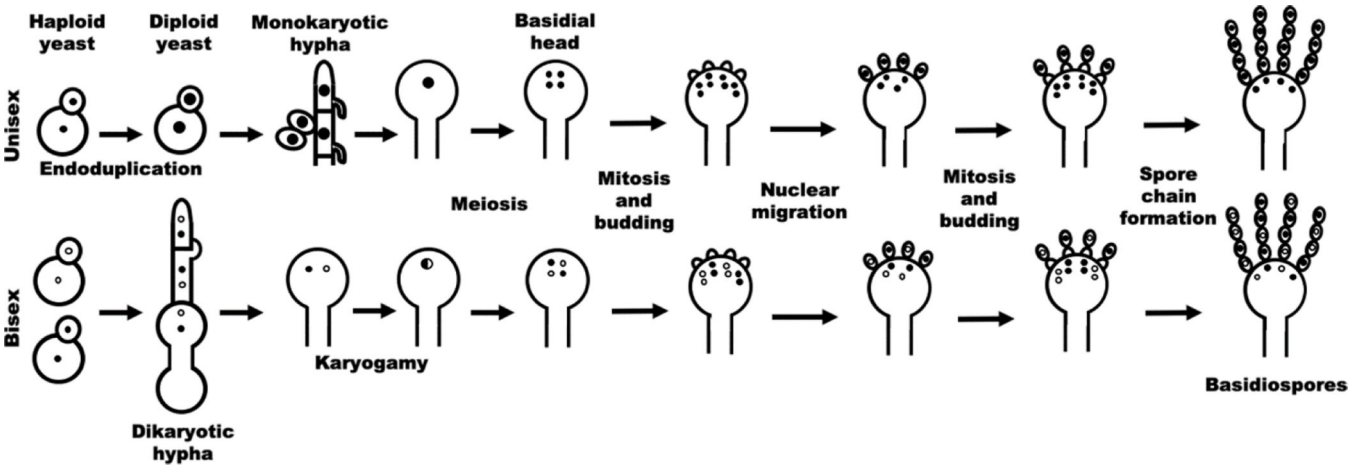

Figure 1. ***Cryptococcus*** **sexual cycles.**

Cryptococcal cells of a single mating type can undergo unisexual reproduction mainly through endoduplication to generate monokaryotic hyphae. The clamp connections formed during unisexual reproduction are unfused. Sporogenesis occurs in basidia through repeated mitosis and budding following one round meiosis. During bisexual reproduction, **a** and α cells fuse, undergo morphological switch to dikaryotic hyphae, generate fruiting bodies, and produce basidiospores.

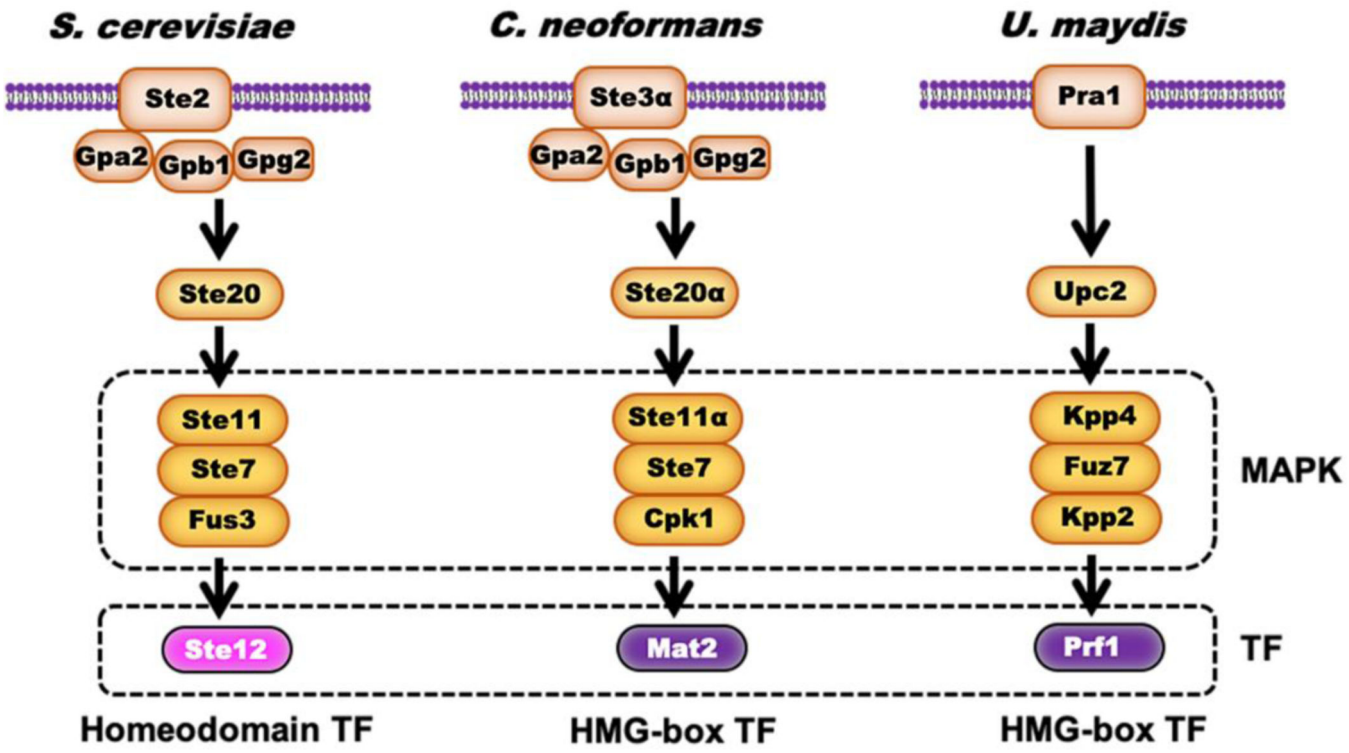

Figure 2. Pheromone signaling.

Pheromone binds to a cell surface receptor that in turn activates a downstream signaling cascade through the protein kinase Ste20 homologs in *S. cerevisiae* and *C. neoformans*, or Upc2 in *U. maydis*. The signal is relayed through a highly conserved MAPK cascade, in which the MAP kinase phosphorylates and activates the downstream transcription factor, resulting in gene transcription. The transcription factors diverge in different fungi. Ste12 in *S. cerevisiae* is a homeodomain transcription factor, while the Prf1 in *U. maydis* is an HMG-box transcription factor. In *C. neoformans*, the Prf1 homolog is dispensable for mating, but another HMG-box transcription factor Mat2 regulates pheromone signaling. Some components of this pathway are omitted for clarity.

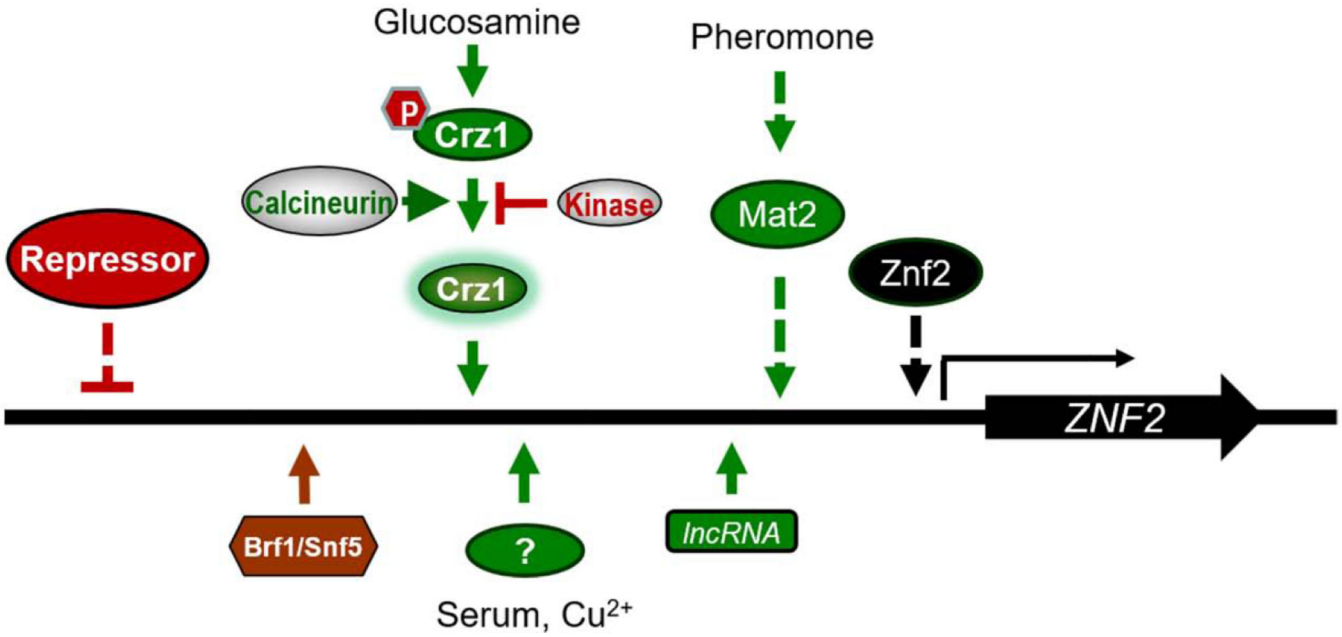

Figure 3.
The established and predicted regulatory pathways upstream of the master regulator of filamentation Znf2.