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Profiling a killer, the development of *Cryptococcus neoformans*

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Abstract

The ability of fungi to transition between unicellular and multicellular growth has a profound impact on our health and the economy. Many important fungal pathogens of humans, animals, and plants are dimorphic, and the ability to switch between morphological states has been associated with their virulence. *Cryptococcus neoformans* is a human fungal pathogen that causes life-threatening meningoencephalitis in immunocompromised and, in some cases, immunocompetent hosts. *Cryptococcus neoformans* grows vegetatively as a budding yeast and switches to hyphal growth during the sexual cycle, which is important in the study of cryptococcal pathogenicity because spores resulting from sexual development are infectious propagules and can colonize the lungs of a host. In addition, sexual reproduction contributes to the genotypic variability of *Cryptococcus* species, which may lead to increased fitness and virulence. Despite significant advances in our understanding of the mechanisms behind the development of *C. neoformans*, our knowledge is still incomplete. Recent studies have led to the emergence of many intriguing questions and hypotheses. In this review, we describe and discuss the most interesting aspects of *C. neoformans* development and address their impact on pathogenicity.

Keywords

morphogenesis; mating; sexual development; fungi; MAPK pathway; dimorphic fungi

Introduction

Some fungal species can shift between morphological forms during their life cycles. While mushrooms with elaborate and impressive fruiting bodies are considered monomorphic because their only morphological form is hyphae that develop directly from spores after germination, dimorphic fungi proliferate as unicellular organisms and can switch to multicellular growth under specific conditions. Dimorphic fungi are attractive models to study development due to their relatively simple morphological transition; for example a paradigm for morphological differentiation is the ascomycete *Saccharomyces cerevisiae*, whose yeast to pseudohyphal growth change has been studied extensively (Rua *et al*., 2001).

The ability of fungi to switch between unicellular and multicellular hyphal growth not only constitutes an important biological phenomenon, but also has a profound impact on our health and the economy. Many important fungal pathogens of humans, animals, and plants are dimorphic, and the ability to switch between morphological states has been associated with their virulence. Prominent examples of species with virulence-linked morphological

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transitions include the human opportunistic pathogen *Candida albicans* (Sudbery *et al*., 2004; Berman, 2006) and the maize pathogen *Ustilago maydis* (Nadal *et al*., 2008; Steinberg & Perez-Martin, 2008; Brefort *et al*., 2009). Several known systemic dimorphic fungi belonging to the ascomycete lineage grow as a spore-producing mold in the environment at ambient temperature and switch to yeast growth after being inhaled into the lungs of a human host as spores (Klein & Tebbets, 2007). Therefore, examining the mechanistic basis for morphological transitions in fungi may lead to the development of more effective antifungal therapies and strategies to prevent devastating crop diseases.

Cryptococcus neoformans is a human fungal pathogen that causes life-threatening meningoencephalitis in immunocompromised and, in some cases, immunocompetent hosts (Idnurm *et al*., 2005). Two *C. neoformans* serotypes are currently recognized: the most common causative agent of cryptococcosis, serotype A (*C. neoformans* var. *grubii*), and the relatively less virulent serotype D (*C. neoformans* var. *neoformans*) (Kwon-Chung & Varma, 2006; Lin & Heitman, 2006). *Cryptococcus gattii* has been classified into two additional serotypes, B and C, but these are currently considered to be sibling species (Kwon-Chung & Varma, 2006; Lin & Heitman, 2006).

Cryptococcus neoformans grows vegetatively as budding yeast and can be frequently found in tree hollows and pigeon guano. During the sexual cycle, *Cryptococcus* switches from yeast growth to hyphal growth. Despite this dramatic morphological transition, *Cryptococcus* is not considered by some to be a dimorphic fungus because yeast cells are the predominant form in the environment and in the human host, and it is likely that the morphological transition is not involved in infection. However, there are at least three important reasons why the development of *Cryptococcus* is relevant to its pathogenicity. First, spores that result from hyphal development during mating are infectious propagules. Upon inhalation, spores (in addition to desiccated yeast) can colonize the lungs of a host. *Cryptococcus neoformans* propagates to the bloodstream and crosses the blood–-brain barrier, ultimately colonizing brain tissue and leading to fatal consequences if not treated. Second, sexual reproduction contributes to the genotypic variability of *Cryptococcus* species, which may lead to increased fitness and virulence. Third, some genes located within the *MAT* locus are important during mating and during infection. Therefore, the development of *Cryptococcus* is not only an interesting paradigm for biologists, but is also important in the study of cryptococcal pathogenicity.

Despite significant advances in our understanding of the mechanisms behind the *Cryptococcus* development, our knowledge is still limited. Recent studies have led to the emergence of intriguing questions and hypotheses, many of which are discussed below. Several excellent reviews describe the sexual reproduction of *Cryptococcus*, and the species' mating-type locus structure and the signal transduction involved in mating (Wang & Heitman, 1999; Idnurm *et al*., 2005; Nielsen & Heitman, 2007; Hsueh & Heitman, 2008; Lin, 2009; Morrow & Fraser, 2009; Kruzel & Hull, 2010; Raudaskoski & Kothe, 2010; Wang, 2011), or discuss the unique properties of the *Cryptococcus* mating types (McClelland *et al*., 2004). In this review, we describe development in a broad sense as the events leading to all morphological transitions that contribute to the proliferation and increased fitness of *C. neoformans* both in the environment and within a human host. While we discuss the most salient aspects and questions of cryptococcal development, we refer the reader to a recently published work describing *Cryptococcus* biology for more detailed information (Heitman *et al*., 2011).

Morphological forms of *C. neoformans*

The *Cryptococcus* life cycle consists of vegetative and sexual growth phases (Fig. 1). *Cryptococcus neoformans* exists in at least two morphological states during vegetative growth. The most prevalent form in its natural habitat and in clinical samples is unicellular budding yeast, which reproduce by mitotic division. Alternative vegetative forms are pseudohyphae. Pseudohyphae are linked yeast cells that do not completely separate after mitotic divisions and serve as an intermediate form between yeast and true hyphae. *Cryptococcus neoformans* pseudohyphae have only occasionally been reported in clinical samples (Shadomy & Utz, 1966; Freed *et al*., 1971; Lurie & Shadomy, 1971; Williamson *et al*., 1996; Gazzoni *et al*., 2009) and in the environment and may represent a strategy to avoid natural predators (Neilson *et al*., 1978). Similar to *S. cerevisiae*, *C. neoformans* utilizes the RAM pathway to control the switch from yeast to pseudohyphae, but mechanistic details of this transition are lacking at present (Walton *et al*., 2006; Verma-Gaur *et al*., 2008).

Mating of *C. neoformans* results in a third growth form: hyphal growth. During mating, two compatible yeast cells fuse, but the two parental nuclei remain separate, leading to the formation of dikaryotic hyphae, a hallmark feature of basidiomycetes (Fig. 1). Another basidiomycete-specific characteristic is the specialized clamp cells that form between each cellular compartment in the hyphae to maintain the dikaryotic state. The ultimate developmental stage in the sexual cycle of *C. neoformans* occurs in a terminal, specialized cell called the basidium, in which the fusion of the parental nuclei and meiosis take place. Nuclear meiotic products undergo rounds of mitotic division, and the mitotic nuclei are packaged into spores that bud from the apical surface of the basidium to form four spore chains, a feature that distinguishes the genus *Filobasidiella* from other members of the *Basidiomycota*. Upon germination, spores develop into yeast, which concludes the sexual cycle. Opposite- sex mating can also produce diploids that grow as yeast at 37 °C and filament under inducing conditions, resulting in monokaryotic hyphae, which are decorated with unfused clamp cells and capable of producing basidia and spores (Sia *et al*., 2000).

In addition to classical opposite-sex mating, *C. neoformans* can also undergo same-sex mating, which is referred to as monokaryotic or haploid fruiting (Wickes *et al*., 1996; Lin *et al*., 2005) and was originally reported by Erke (1976) as homothallism. Similar to opposite-sex mating, monokaryotic fruiting also results in the formation of hyphae and leads to the formation of basidia and spores. However, the filaments are monokaryotic and the clamp cells are not fused, similar to hyphae produced by **a**/α diploids. Although this unusual form of sexual reproduction is not α-mating type specific (Tscharke *et al*., 2003), it is strongly associated with the α-mating type (Wickes *et al*., 1996; Lin *et al*., 2006).

Mating is a possible source of hybrid *C. neoformans* species. Hybrids are most frequently formed between the A and the D serotypes of either the same or opposite mating types (αAD**a**, **a**ADα, αADα) and are of significant clinical importance (Litvintseva *et al*., 2005a; Lin *et al*., 2007). Diploids derived from genetically distinct strains of the same serotype (αAAα) have also been reported (Lin *et al*., 2009). Interestingly, even hybrids between *C. neoformans* and *C. gattii* have been described from the environment (Bovers *et al*., 2006) and in clinical samples (Bovers *et al*., 2008).

Two groups recently described a peculiar morphological form of *C. neoformans* that occurs during infection (Okagaki *et al*., 2010; Zaragoza *et al*., 2010). These studies found unusually large yeast-like *Cryptococcus* cells in clinical samples, now known as giant cells, and it remains to be elucidated how this morphological change is triggered.

Cryptococcus neoformans mating

Cryptococcus neoformans is a heterothallic fungus with a bipolar mating system in which a single mating locus defines each mating type (**a** and α) (Kwon-Chung, 1976b). The mating-type locus of *C. neoformans* is significantly larger than the *MAT* loci of most other fungi, spanning over 100 kb with > 20 genes. Several *MAT* locus genes function in mating, including genes encoding the Ste20 p21-activated kinase (PAK), the MAPKKK Ste11, and the transcription factor Ste12 (Lengeler *et al*., 2002; Fraser *et al*., 2004). The bipolar *MAT* locus of *Cryptococcus* may have evolved from an ancestral tetrapolar *MAT* system through a series of genomic rearrangements (Fraser *et al*., 2004; Hsueh *et al*., 2008). Similar to other fungi, the cell-type identity of *Cryptococcus* is specified by pheromone and receptor genes and homeodomain transcription factor genes within the *MAT* locus that serve as master regulators of sexual reproduction (Lengeler *et al*., 2002).

Cryptococcus species and serotypes vary in their ability to undergo the sexual cycle. Most serotype D strains mate, while the mating ability of serotype A and *C. gattii* is strain specific (Fraser *et al*., 2003; Halliday & Carter, 2003; Nielsen *et al*., 2003). However, the genomes of all strains suggest that the ability to undergo sexual reproduction is conserved.

Mating of *C. neoformans* has never been directly observed in nature or within the host, and only specific conditions in the laboratory can trigger sexual reproduction between compatible yeast cells (Kwon-Chung, 1976a; Heitman, 2006). However, both mating and sporulation have been observed on media containing pigeon guano and on live plants under laboratory conditions, suggesting that the sexual cycle occurs occasionally in its natural environment (Nielsen *et al*., 2007; Xue *et al*., 2007). This is further supported by recent population genetics studies that provide compelling evidence for sexual reproduction in both *C. neoformans* and *C. gattii* in nature (Campbell *et al*., 2005; Bui *et al*., 2008; Hiremath *et al*., 2008). Currently, the filamentous form (teleomorph) of *C. neoformans* is classified under the genus *Filobasidiella*, which also contains the closely related species *Filobasidiella depauperata* (Heitman *et al*., 2011). Interestingly, *F. depauperata* grows only as hyphae and has no yeast state (Rodriguez-Carres *et al*., 2010). It will be interesting to uncover the underlying genetic and/or epigenetic factors that govern yeast and hyphal growth in these sister species.

One of the interesting, but unexplained aspects of *C. neoformans* biology is the overwhelming predominance of the α-mating type in the environment and clinical isolates. This phenomenon could explain why mating in nature is rare (Idnurm *et al*., 2005). One exception is the recently documented population of *Cryptococcus* in sub-Saharan Africa, which contains an equal ratio of **a**- and α-mating-type cells and has a relatively high recombination rate (Litvintseva *et al*., 2003, 2005b). In serotype D, the α-mating type is more virulent than the **a**-mating type (Kwon-Chung *et al*., 1992), which led to the hypothesis that α cells have a higher fitness than the **a** cells (McClelland *et al*., 2004). However, in serotype A, both mating types have similar virulence (Nielsen *et al*., 2003). Therefore, the mechanism underlying the skewed mating-type proportion in natural populations is still unknown.

What signals trigger mating in *C. neoformans*?

In contrast to *S. cerevisiae*, which prefers the diploid state in nature and for which pheromone stimulation is sufficient to initiate mating, *C. neoformans* proliferates as a haploid yeast and specific environmental conditions in addition to pheromone signals are necessary to initiate the sexual cycle. Several external cues can promote or inhibit mating in *C. neoformans*. Nutritional signals that promote mating have been identified by generating defined media and analyzing media of unknown compositions. Among these factors, *myo-*

inositol, copper ions, and nitrogen starvation were most significant (Xue *et al*., 2007; Kent *et al*., 2008). These results were also supported by genetic studies (Torres-Guererro & Edman, 1994; Walton *et al*., 2005; Rutherford *et al*., 2008). Xue *et al*. (2007) established that mating can occur on plant surfaces using *Arabidopsis thaliana* and *Eucalyptus camaldulensis* under laboratory conditions. Furthermore, *myo*-inositol and the plant hormone indole acetic acid were identified as two major plant-derived stimulators of mating (Xue *et al*., 2007). *Cryptococcus neoformans* var. *grubii* and var. *neoformans*, but not *C. gattii* grow and mate robustly on media prepared with pigeon guano (Staib, 1981; Staib & Blisse, 1982), indicating that the nutritional composition of pigeon guano provides a highly favorable environment for *C. neoformans* growth and mating. The actual components in pigeon guano that stimulate mating are unknown, but these studies provide an insight into why avian excreta are a common ecological niche for *C. neoformans*.

Three environmental factors found in the human host inhibit the mating of *C. neoformans*: a temperature of 37 °C, high humidity, and 5% carbon dioxide (CO_2). This is consistent with the hypothesis that mating does not occur during infection. Similar to some thermodimorphic fungal pathogens such as *Histoplasma capsulatum*, a temperature of 37 °C promotes yeast growth of *C. neoformans* and blocks hyphal growth (Sia *et al*., 2000; Klein & Tebbets, 2007; Nguyen & Sil, 2008). Mechanisms of temperature-controlled morphogenesis in *Cryptococcus* are unknown, but the regulatory pathways may involve calcineurin and Ras1, as these components are essential for mating and growth at a high temperature (Odom *et al*., 1997; Alspaugh *et al*., 2000; Nichols *et al*., 2007; Kozubowski *et al*., 2009). Mating of *C. neoformans* is more efficient under dry compared with humid conditions and has never been observed in liquid, but the mechanism of this regulation is unknown.

Cell fusion during mating is inhibited by 5% CO_2, likely because of decreased pheromone production (Bahn *et al*., 2005). Bahn *et al*. (2005) proposed that CO_2-mediated mating inhibition results from increased intracellular levels of HCO_3^- produced by the carbonic anhydrase Can2 at high CO_2 levels. In the *can2*Δ mutant, the small amount of bicarbonate generated through nonenzymatic spontaneous hydration of CO_2 is sufficient to support vegetative growth, but not to inhibit mating. However, proper development of basidia and sporulation requires *CAN2*; while sporulation is dependent on the correct nuclear distribution within hyphae, *CAN2* was not associated with nuclear migration (Bahn *et al*., 2005).

Mating of *C. neoformans* on a medium supplemented with V8 juice is inhibited by continuous exposure to white light, whereas mating on plants is not significantly inhibited by light (Xue *et al*., 2007). Two conserved photoreceptor genes, *BWC1* and *BWC2*, mediate mating inhibition by light (Idnurm & Heitman, 2005; Lu *et al*., 2005; Yeh *et al*., 2009). Idnurm & Heitman (2005) demonstrated that Bwc1 and Bwc2 interact physically in a two-hybrid assay and function either directly or indirectly to repress the transcription of *MFα1* and *SXI1α*, two key genes that regulate mating and completion of the sexual cycle. Accordingly, *BWC1* and *BWC2* were required for efficient light-driven inhibition of cell fusion (dependent on *MFα1*) and subsequent filamentation (dependent on *SXI1α*). The inhibition of mating by light, specifically UV irradiation, which induces DNA damage, may have evolved to avoid the DNA damage-sensitive process of meiosis (Idnurm & Heitman, 2005; Heitman *et al*., 2011). Interestingly, *bwc1*Δ and *bwc2*Δ mutants were significantly less virulent in a murine model of infection, suggesting an intriguing connection between the ability to sense light and pathogenicity (Idnurm & Heitman, 2005).

Cell fusion, the initial step during sexual development

For cell fusion to occur, at least one mating cell usually produces a conjugation tube directed towards the mating partner. This early mating response is orchestrated by a dedicated mating signaling pathway. Pheromone stimulation in *C. neoformans* is an essential, although not sufficient, mating signal necessary for fusion to occur between opposite mating-type cells. The mating-type locus of each mating type encodes specific pheromones and receptors that determine the compatibility of mating partners (Stanton *et al*., 2010). Similar to *S. cerevisiae*, in *C. neoformans*, pheromone stimulation of the pheromone receptor transduces the signal through heterotrimeric G proteins and triggers signaling via the mitogen-activated protein kinase (MAPK) signaling pathway (Fig. 2) (Alspaugh *et al*., 1998; Wang & Heitman, 1999).

Two G protein-dependent signaling pathways that influence mating in *C. neoformans* have been characterized. One pathway responds to pheromone while the other is stimulated by nutritional signals (Fig. 2) (Alspaugh *et al*., 1998; Wang & Heitman, 1999). Similar to *S. cerevisiae*, in *C. neoformans*, GTP binding activates the G protein α subunit when pheromone binds to the pheromone receptor, releasing the Gβγ complex to activate downstream effectors. Despite similarities, G protein signaling in *C. neoformans* is more complex than in *S. cerevisiae* due to the more complex nature of its development. For example, among the three G protein α subunits expressed in *C. neoformans* (Gpa1, Gpa2, and Gpa3), Gpa1 functions upstream of adenylyl cyclase and protein kinase A (PKA) and is required for the pathogenesis and development of *C. neoformans*, while Gpa2 and Gpa3 are involved in mating (Alspaugh *et al*., 1997, 2002; D'Souza *et al*., 2001). Similarly, *GIP2* and *GPB1* encode β-subunits that function in nutrient sensing and mating, respectively (Wang *et al*., 2000; Hsueh *et al*., 2007; Li *et al*., 2007), and γ- subunits are encoded by *GPG1* and *GPG2* (Li *et al*., 2007). Both the Gpa2 and the Gpa3 α subunits interact physically with the pheromone receptor Ste3. However, only Gpa2 was shown to interact with the β-subunit Gpb1 (Li *et al*., 2007). Moreover, while the expression of *GPA2* is induced during mating, *GPA3* is induced by nutrient limitation (Hsueh *et al*., 2007) and the two genes seem to have both overlapping and opposing functions in pheromone sensing (Hsueh *et al*., 2007; Li *et al*., 2007). The α subunits are negatively regulated by the RGS domain proteins Crg1 and Crg2, which stimulate GTP hydrolysis. Similar to what was described in *S. cerevisiae*, Crg1 is in a complex with the pheromone receptor Ste3, and Crg2, which is membrane bound, also interacts with the pheromone receptor, possibly with a lower affinity (Hsueh *et al*., 2007). While Crg1 is induced by pheromone and acts exclusively in pheromone sensing, Crg2 is constitutively expressed and can inhibit both the mating and the Gpa1-cAMP pathways (Hsueh *et al*., 2007; Shen *et al*., 2008; Xue *et al*., 2008). The cAMP/PKA pathway regulates responses to several nutritional starvation signals, including nitrogen, iron, and glucose, and may be responsible for the nutritional regulation of the *MFα1* gene (Alspaugh *et al*., 1997). In addition, mutants lacking the α subunit Gpa1 exhibit mating defects, further suggesting cross-talk between the pheromone signaling and the cAMP/PKA pathway.

Similar to other fungi, the MAPK cascade transduces the signal triggered by pheromone in *C. neoformans* (Fig. 2) (Davidson *et al*., 2003). Upon pheromone stimulation, the Gβγ subunit heterodimer activates a member of the PAK family. *Cryptococcus neoformans* genome encodes two PAK homologues, Ste20 and Pak1, which together are essential for cell viability (Wang *et al*., 2002) and involved in mating (Wang *et al*., 2002; Nichols *et al*., 2004). PAK transduces the signal to the MAPK pathway components Ste11 (MAPKKK), Ste7 (MAPKK), and Cpk1 (MAPK), resulting in the activation of the mating response. Another conserved component of the mating pathway was described recently: the adaptor protein Ste50 (Jung *et al*., 2011). Unlike in *S. cerevisiae*, where Ste50 acts in multiple MAPK pathways, *C. neoformans* Ste50 appears to be mating specific. Further, it does not

appear to be important for virulence, in contrast to the *U. maydis* homologue. Fu *et al*. (2011) have shown that the *STE50* gene is required for response to pheromone, cell fusion, and the production of dikaryotic hyphae. Interestingly, Ste50 is also required for the production of monokaryotic hyphae during monokaryotic fruiting (Fu *et al*., 2011). The main role of Ste50 appears to be similar to the *S. cerevisiae* homologue in that it serves as an adapter protein for bringing Ste20 and Ste11 together to activate Ste11 (Fu *et al*., 2011).

Another unique characteristic of the *C. neoformans* MAPK pathway is that *STE20* and *STE11* are represented by mating-type-specific alleles (Clarke *et al*., 2001; Davidson *et al*., 2003). Initial cell fusion is blocked in *ste11***a**/α, *ste7*, and *cpk1* mutants (Davidson *et al*., 2003), but cell fusion is not dependent on Ste20 (Nichols *et al*., 2004), indicating that Pak1 may share this role.

A homologue of the *S. cerevisiae* pheromone-responsive transcription factor Ste12 has been cloned from *C. neoformans* (Wickes *et al*., 1997). *STE12*α plays only a minor role in mating, but may be one of several transcription factors regulating the response to pheromone (Yue *et al*., 1999; Davidson *et al*., 2003). Overexpression of *STE12*α restores mating and fruiting in MAPK mutants (Davidson *et al*., 2003) and dramatically induces the expression of the pheromone *MFα1* gene, but the deletion of *STE12*α has no effect on *MFα1* expression, suggesting that Ste12α does not function downstream of the MAPK cascade in a simple linear pathway (Davidson *et al*., 2003).

In *S. cerevisiae*, the response to pheromone involves two major events: cell cycle arrest and conjugation between two cells of opposite mating type. Cell cycle arrest is mediated by Fus3 (a member of the MAPK family) and its substrate Far1 by inhibiting G1 cyclins (Peter *et al*., 1993). It is unclear whether cell cycle arrest occurs in response to a pheromone signal in *C. neoformans*, and if it does, the stage of the cell cycle at which the arrest takes place (Kruzel & Hull, 2010).

Unlike in *S. cerevisiae*, where both mating partners extend a projection during mating, only one of the cells typically generates a projection in *Cryptococcus*. Some reports indicate that only cells of the α-mating type generate conjugation tubes, and cells of the **a**-mating type swell (Davidson *et al*., 2000; Wang *et al*., 2000; McClelland *et al*., 2004). This divergent response of cells of opposite mating type may be characteristic only of serotype D, because in serotype A, the formation of conjugation tubes by either mating partner has been observed and swelling of **a** cells does not seem to occur (Nichols *et al*., 2004; L. Kozubowski & J. Heitman, unpublished data). Future studies should determine whether conjugation tube formation is serotype specific.

Mating projections are visible by ~4 h, which coincides with an increase of pheromone transcript levels (Shen *et al*., 2002; Chang *et al*., 2003), suggesting that these phenomena are related. It is expected that polarisome components analogous to those in *S. cerevisiae* govern the formation of conjugation tubes in *C. neoformans*, but these mechanisms have not yet been explored in detail. A PAK homologue, Pak1, is necessary for the establishment of polarized protrusions during mating (Nichols *et al*., 2004). Surprisingly, Pak1 is not involved in pheromone induction during mating, in contrast to the role of its homologue in *S. cerevisiae*, Ste20 (Nichols *et al*., 2004).

After one or both cells of opposite mating type produce a conjugation tube, fusion between the two cells occurs. Under laboratory conditions, only a relatively small percentage of the cell population exhibits a mating response and undergoes fusion events. Less than four fusion events per 50 cells were reported in serotype A after 24 h (Nichols *et al*., 2004). The exact mechanism of cell fusion has not been studied in *Cryptococcus*. In *S. cerevisiae*, the initial cell wall attachment between two cells is mediated by GPI-anchored agglutinin

proteins (Chen *et al*., 2007). This is followed by cell wall remodeling and the formation of a pore through which fusion of the plasma membrane takes place (White & Rose, 2001). Only two proteins are known to participate in cell fusion during mating in *S. cerevisiae*: Prm1 and Fig1. Only a Prm1 homologue is encoded in the *C. neoformans* genome, but its role has not been investigated (Heiman & Walter, 2000).

Development of postmating hyphae

A common feature of fungi belonging to the basidiomycete phylum is the formation of dikaryotic hyphae, which ultimately give rise to spore-producing structures. For example, *U. maydis* develops dikaryotic filaments upon mating that penetrate host tissue during plant infection (Banuett, 1992). The initial step in the regulation of the dikaryotic growth phase is conserved and involves homeodomain mating-type-specific transcription factors that heterodimerize (Kues & Casselton, 1992). Cell fusion in *C. neoformans* is followed by the development of hyphae that grow as a dikaryon until sporulation occurs (Figs 1 and 3). Then, the two homeodomain transcription factors Sxi1α and Sxi2**a**, which are derived from opposite mating partners, dimerize and trigger hyphal transition and development (Hull *et al*., 2002, 2005). The Sxi1α/Sxi2**a** heterodimer binds to DNA sequences that differ from sequences described in other fungi (Stanton *et al*., 2009). In the absence of one or the other homeodomain protein, cells can fuse, but subsequent hyphal development fails to progress. Hence, the Sxi1α/Sxi2**a** heterodimer plays a central role in establishing the zygotic developmental fate that follows cell fusion during mating. *SXI1*α and *SXI2***a** expression is elevated upon cell fusion and does not require the presence of the Sxi1α/Sxi2**a**-regulatory heterodimer or pheromone, indicating that other mating-specific factors are responsible for this gene expression upregulation. However, the expression of *SXI1*α and *SXI2***a** results in the repression of pheromone gene expression, underscoring the importance of such repression in sexual development following cell fusion. When Sxi1α was expressed in **a** cells or Sxi2**a** in α cells, complete sexual development with sporulation occurred, even in the absence of the opposite mating-type partner (Hull *et al*., 2002, 2005). In this artificially induced sexual cycle, the hyphae produced were monokaryotic, and the clamp cells did not fuse, reminiscent of same-sex mating or self-filamentation of a diploid (Sia *et al*., 2000).

After fusion has occurred, only one of the parental cells initiates hyphal growth. McClelland *et al*. (2004) demonstrated that the initial hypha always originates from the **a**-mating type. This phenomenon is one explanation for the uniparental mitochondrial inheritance observed during mating in *C. neoformans* (Yan & Xu, 2003). The exclusive inheritance of mitochondria from the **a**-mating partner is influenced by the migration of the α nucleus to the **a** cell after cell fusion, an event that could contribute toward limiting or excluding the mitochondria of the α cell from the progeny. Uniparental mitochondrial inheritance requires *SXI1*α and *SXI2***a**, suggesting that these transcription factors may control nuclear migration within the zygote (Yan *et al*., 2007).

While it is clear that *SXI1*α and *SXI2***a** are essential for hyphal development following cell fusion, the mechanism and targets of these transcription factors are relatively unknown (Kruzel & Hull, 2010). One gene target homologous to the *CLP1* gene first described in *Coprinopsis cinerea* is necessary for clamp cell formation (Inada *et al*., 2001; Ekena *et al*., 2008). In *C. neoformans*, *CLP1* transcription is upregulated during mating and directly controlled by *SXI1*α and *SXI2***a**. Following cell fusion, at least one copy of *CLP1* is required for hyphal development. *CLP1* is also essential for growth after cell fusion during the formation of diploids. This observation supports the hypothesis that Clp1 acts as a cell cycle regulator, enabling growth after cell fusion (Ekena *et al*., 2008). *CLP1*, but not *SXI1*α or *SXI2***a**, is necessary for filamentation during same-sex mating, suggesting that Clp1 responds to an alternative signaling pathway during same-sex mating (Ekena *et al*., 2008). Because

CLP1 deletion prevents hyphal formation similar to *SXI1*α and *SXI2***a** disruption, it is difficult to establish whether these genes also control developmental events during subsequent hyphal growth. Expression of *CLP1* from a regulatable promoter would address this question.

Similar to other basidiomycetes, *C. neoformans* clamp cells are formed during each hyphal cell division and facilitate proper nuclear distribution and maintenance of the dikaryotic state (Casselton & Olesnicky, 1998) (Fig. 1). The clamp cell develops at the most apical cell of the hypha as a bud, initially bending backwards to produce a hookshaped appendix that eventually fuses with the hyphal cell. Clamp cell development is coordinated with nuclear division of the two genetically distinct haploid nuclei. The nucleus that is closer to the apex of the hypha divides, with the two resulting nuclei moving to the hyphal cell and the clamp cell, whereas the subapical nucleus divides within the hyphal cell, maintaining the dikaryotic state. In basidiomycetes, clamp cell formation and coordinated nuclear divisions are controlled by homeodomain transcription factors, but clamp cell fusion is dependent on pheromone and pheromone receptor genes (Casselton & Olesnicky, 1998). *Coprinopsis cinerea* clampless 1 protein (Clp1) participates in clamp formation (Inada *et al*., 2001) and *U. maydis* Clp1 is required for the formation of clamp-like structures (Scherer *et al*., 2006); however, further investigations are required to establish whether *C. neoformans* Clp1 acts during clamp cell formation (Ekena *et al*., 2008).

Septins, which are filament-forming GTPases, localize to the base of the clamp cell near the point of fusion between the clamp cell and the hypha (Kozubowski & Heitman, 2010). Septin mutants exhibit defects in nuclear distribution and fail to fuse clamp cells in the postmating hyphae, indicating that septins are involved in clamp cell development and nuclear migration. A lack of septins leads to an aberrant nuclear distribution during monokaryotic fruiting, a growth mode characterized by unfused clamp cells. This suggests that septins play a role in nuclear distribution independent of clamp cell fusion. However, it is possible that septins also contribute directly to clamp cell fusion during opposite-sex mating (Kozubowski & Heitman, 2010).

Hsueh *et al*. (2009) described a constitutively active G protein-coupled receptor in *C. neoformans*, Cpr2, which protects clamp cells from pheromones generated by other cells and promotes clamp cell fusion. During mating between *cpr2*Δ mutants, unusual haustorial filaments are produced at sites where clamp cells are normally initiated. Consistent with a hyphae-specific role, Cpr2 expression is highly induced during mating after cell fusion. Although *cpr2*Δ cells exhibit a modest defect in mating cell fusion assays, this is likely due to the requirement of Cpr2 for cell survival after cell fusion has occurred (Hsueh *et al*., 2009).

A septum forms immediately adjacent to the clamp cell to divide individual hyphal compartments. *Cryptococcus neoformans* possesses a specialized channel in the center of the septum called the dolipore, which facilitates communication between individual compartments of the hyphae and whose precise structure is unknown (Kwon-Chung & Popkin, 1976; Rhodes *et al*., 1981). *Cryptococcus neoformans* septin proteins localize to the medial point in the septa, presumably a dolipore, unlike in ascomycete fungi, where septins localize to the entire septum (Westfall & Momany, 2002; Kozubowski & Heitman, 2010). Therefore, the structure and function of the dolipore may depend on septins.

Whereas most hyphae are thick and straight for several days following cell fusion, an outgrowth of a relatively narrow, wandering hypha has been observed in serotype A *C. neoformans* after the first clamp cell is formed (Kozubowski & Heitman, 2010). These ‘pioneer hyphae’ grow either from hyphal tips or from clamp cells (Kwon-Chung, 1976a).

The significance of pioneer hyphae is unknown, but it may allow the hyphal tip to forage for nutrients or contact neighboring hyphae to initiate hyphal connections. Pioneer hyphae are reminiscent of haustorial hyphae described in other fungi and frequently observed in the *C. neoformans cpr2*Δ mutant (Hsueh *et al*., 2009). Thus, the deletion of *CPR2* may result in an intensification of a physiological phenomenon.

Postmating hyphae of *C. neoformans* are occasionally connected (Kwon-Chung, 1976a; Kozubowski & Heitman, 2010). Whether such connections are formed based on pheromone-mediated signaling or through self-fusion mechanisms characteristic of other fungi is not known (Read *et al*., 2009).

Mating filaments in *C. neoformans* grow significantly slower than in other 'classic' filamentous fungi, consistent with the presumed lack of a Spitzenkörper, a vesicle supply center at the growing apex of hyphal cells (Steinberg, 2007). Interestingly, the filamentous sibling species *F. depauperata* grows at a very slow rate, suggesting that the mode of hyphal growth in this fungus is analogous to the postmating hyphae generated in *C. neoformans* (Rodriguez-Carres *et al*., 2010).

Cryptococcus neoformans utilizes polarity-associated proteins to maintain polar extensions. One such protein, Ste20 (a homologue of *S. cerevisiae* Cla4), maintains filament polarity (Nichols *et al*., 2004). A bilateral *ste20*Δ cross results in the formation of aberrant filaments characterized by excessive branching, abnormal tip splitting, and aberrant basidia that do not produce spores (Nichols *et al*., 2004). Ste20 physically interacts with and is likely responding to the Rho-like GTPases Rac1 and Cdc42 (Vallim *et al*., 2005). Similar to Ste20, Rac1 is not required for cell fusion during mating and strains lacking Rac1 display an aberrant hyphal morphology in bilateral crosses, including shorter and thicker hyphae and extensive branching (Vallim *et al*., 2005). However, unlike *ste20*Δ mutants, *rac1*Δ mutants have no defect in sporulation, suggesting that the Ste20 PAK kinase plays a more substantial role in mating hyphal morphology than Rac1. Overexpression of either *RAC1* or *STE20*α restores high-temperature growth to the *ras1* mutant, suggesting that Ras1 signals through Rac1 and Ste20 (Alspaugh *et al*., 2000). While Ras1 functions in the initial events of mating, including pheromone production, cell fusion, and the initiation of filamentous growth, Rac1 and its effector kinase Ste20 may act specifically during hyphal growth (Alspaugh *et al*., 2000; Waugh *et al*., 2003; Nichols *et al*., 2004; Vallim *et al*., 2005).

Staudt *et al*. demonstrated a conserved role of the microtubule- associated protein Bim1 in hyphal morphology. Crosses between *bim1*Δ mutants produced unusually short and curved filaments with missegregated nuclei (Staudt *et al*., 2010). Similarly, in *Schizophyllum commune*, nuclear migration was associated with microtubule tracks and microtubule-associated motors (Raudaskoski, 1998).

Two types of growth associated with *C. neoformans* hyphae produce yeast cells. Oval-shaped cells called blastospores can form directly from hyphae by mitotic budding from the edge of the hyphal cell (Lin *et al*., 2005). Another mode involves yeast cells budding from chlamydospores. Chlamydospores form as a result of the conversion of the hyphal compartment into a large, round structure. Although chlamydospores in *Cryptococcus* were first reported 40 years ago to be abundant in postmating hyphae, they have only been described recently in *C. neoformans* (Kurtzman, 1973; Lin & Heitman, 2005). Chlamydospores are enriched in glycogen and may serve as energy stores, but the molecular mechanisms controlling chlamydospore formation are unknown (Lin & Heitman, 2005).

The ultimate stage of sexual development is the production of sexual spores. Basidiomycetes differ from other fungi in that they produce spores outside of the basidium, a specialized, spore-producing cell. Typically, a basidium is a unicellular, bottle-shaped structure

originating from the terminal hyphal cell. In the terminal cell, two parental nuclei fuse (karyogamy) and undergo meiosis. The products from a single meiosis and subsequent mitotic divisions give rise to spores that bud from the surface of the basidium in chains (Idnurm, 2010).

Cryptococcus neoformans produces holobasidia from which four spore chains grow (Figs 1 and 3). The mechanism and signals responsible for initiating karyogamy and differentiation of the terminal cell into basidium are not known. Microscopic studies show four nuclei clustered together in some terminal hyphal cells, suggesting that nuclear fusion and meiosis can occur before the terminal cell transforms into the final, globose-like shape characteristic of the basidium (Kozubowski & Heitman, 2010) (Fig. 1). Ploidy-dependent cell growth is a well-established phenomenon (Kondorosi *et al*., 2000; Larkins *et al*., 2001); it is possible that nuclear fusion or the subsequent meiosis trigger a signal for the morphological transition. Alternatively, morphological changes may be necessary for nuclear fusion to occur. The deletion of genes encoding the meiosis-specific proteins Dmc1 and Spo11 severely affects sporulation, but basidia form, suggesting that a morphological transition to basidium is not dependent on meiosis (Lin *et al*., 2005). Whether nuclear fusion with meiosis and the morphological transition from hypha to basidium are dependent on one another will require further studies.

The formation of four spore chains on the basidium is an interesting biological phenomenon. It is unclear what governs the establishment of four areas of polarized growth on the surface of the basidia. This number correlates with four meiotic products, suggesting that the number of nuclei influence the initial protrusions. Even more intriguing is that subsequent mitotic divisions may occur simultaneously within the basidium. In some basidiomycetes, the basidium is divided into four compartments that could potentially provide spatial isolation for the budding of individual protrusions (Boekhout *et al*., 1991); however, *Cryptococcus* produces uncompartmentalized holobasidia. Moreover, when nuclei are visualized in the basidium, five or six nuclei are frequently observed in one single cell (Kozubowski & Heitman, 2010). This suggests that postmeiotic mitosis and the budding of subsequent spores are not tightly coordinated.

Several *C. neoformans* mutations have been described that result in defects in spore chain formation. For example, a lack of spore chains was reported in strains lacking *CAN2*, or *CRG2*, which encode β-carbonic anhydrase, and a regulator of G protein signaling, respectively (Bahn *et al*., 2005; Xue *et al*., 2008). In the presence of 5% CO_2, hyphae of the *can2*Δ mutant do not develop proper basidia and spores, indicating that the conversion of CO_2 to HCO_3^- by Can2 is necessary for sporulation (Bahn *et al*., 2005).

In contrast to the *can2*Δ mutant, postmating hyphae of the *crg2*Δ mutant form normal basidia, but sporulation efficiency was significantly reduced (Xue *et al*., 2008). Xue *et al*. (2008) speculated that hyperactive PKA signaling (via Crg2 or dominant active Gpa1) and inhibition of PKA signaling (via reduced cyclase activation in *can2*Δ mutants) impair the production of long spore chains. Spore chain production is also affected in mutants lacking Cdc42 homologues (Ballou *et al*., 2009); these defects may be associated with the inability to assemble septins at sites of polarized growth, given that *cdc42*Δ mutants exhibit defects in septin organization and septin mutants fail to sporulate properly (Ballou *et al*., 2009; Kozubowski & Heitman, 2010).

Same-sex mating

When exposed to environmental conditions conducive for mating, a haploid *C. neoformans* strain can produce hyphae decorated with basidia and spore chains, similar to hyphae

resulting from mating between opposite-sex partners (Fig. 1) (Shadomy & Utz, 1966; Erke & Schneidau, 1973). This type of haploid-derived hyphal growth was initially considered asexual haploid fruiting that only occurred in the α-mating type (Wickes *et al*., 1996), but the exclusive ability of α cells to fruit was subsequently challenged when others demonstrated that mating type **a** cells can also undergo haploid fruiting (Tscharke *et al*., 2003; Lin *et al*., 2006). The discovery of genetic recombination and ploidy changes during haploid fruiting revealed that this form of growth is an alternative mode of sexual reproduction involving only one mating type (Lin *et al*., 2005; Wang, 2011). Haploid fruiting is also referred to as monokaryotic fruiting, unisexual reproduction, or same-sex mating. The efficiency of same-sex mating is relatively low, and it is not a common phenomenon; in serotype D strains, fruiting of the **a**-mating type is rare, and neither of the serotype A mating types derived from H99 strain can self-filament under laboratory conditions. Similar to opposite-sex mating, same-sex mating has not been observed directly in the environment. The biological significance of same-sex mating is evident from population genetics studies on environmental and clinical isolates (Lin *et al*., 2007, 2009; Bui *et al*., 2008; Saul *et al*., 2008). These studies demonstrate that same-sex mating plays an important role in generating diversity in *C. neoformans* populations and contributes to the global spread of cryptococcosis. Given the predominance of the α-mating type in nature, same-sex mating may be one way in which *C. neoformans* can undergo genetic recombination in the environment (Lin *et al*., 2005).

It is striking that both same-sex and opposite-sex mating are triggered and regulated by similar environmental conditions and involve the pheromone signaling pathway. However, same-sex mating is a significantly more plastic process compared with opposite-sex mating and relies on alternative signaling pathways in addition to pheromone signaling (Wang, 2011). This is exemplified by several mutants defective in bisexual mating, but capable of unisexual mating (Hsueh & Shen, 2005; Hsueh *et al*., 2007; Ekena *et al*., 2008). During opposite-sex mating, a diploid nucleus is formed by nuclear fusion before sporulation, but diploidization in same-sex mating may occur via endoreplication (Lin *et al*., 2005). Under a low magnification, hyphae produced during same-sex mating resemble those resulting from opposite-sex mating, but appear more sporadically. However, unlike opposite-sex mating hyphae, hyphal cells produced by same-sex mating contain single nuclei (monokaryotic), clamp cells do not fuse, and spores are smaller and rounder (Wickes *et al*., 1996; Lin *et al*., 2005).

Sexual development and RNAi

RNAi is a sequence-specific gene silencing mechanism involving small (20–30 nucleotides) RNAs (Hannon, 2002). A novel repeat-silencing mechanism during *C. neoformans* mating was discovered recently: sex-induced silencing (SIS) (Wang *et al*., 2010). Wang *et al*. conducted a comparative transcriptome analysis and identified a group of retrotransposons located in the candidate centromeric regions that were highly induced in the RNAi-deficient strain specifically under mating conditions. Wang *et al*. demonstrated that an increase of transposition during mating is prevented through SIS, which involves the classic RNAi components Dicer, Argonaute, and an RNA-dependent RNA polymerase. Mating between serotype A strains lacking some RNAi components resulted in significant morphological abnormalities in postmating hyphae, frequently characterized by defects in spore chain formation. Wang *et al*. hypothesize that SIS plays a dual role as a defense mechanism against introduced repeats of foreign DNA and against sex-induced transposition. Silencing occurred before the onset of meiosis, and regulation was at the level of translation of the RNAi components. This phenomenon likely involves other mating- dependent mechanisms, because the translation of RNAi components was also induced under nutrient-limiting conditions without the mating partner (Wang *et al*., 2010). This also indicated that the

translational regulation of RNAi components is not specific to sexual reproduction and may also occur during stress, but the exact function of RNAi during stress remains to be defined.

Several fungal species in the ascomycete and basidiomycete lineages have lost the RNAi machinery, indicating that this form of gene silencing is not essential. *Cryptococcus neoformans* serotype A contains all known components of the RNAi machinery, including two Dicer homologues (Dcr1 and Dcr2), one Argonaute homologue (Ago1), and one RNA-dependent RNA polymerase (Rdp1). Whereas the *C. gattii* VGI strain WM276 genome contains two Argonaute homologues, *C. gattii* strain VGII R265 lacks an Argonaute homologue, suggesting that this cryptic species may have lost the ability to silence through RNAi. This is striking because the R265 strain is the cause of the recent outbreak of cryptococcosis in the Pacific Northwest. Therefore, increased transposition activity may be one way in which R265 acquired increased virulence (Wang *et al*., 2010). In contrast, the *C. neoformans* serotype D strain JEC21 contains two Argonaute homologues. Whether this would result in more robust silencing or more RNAi-driven pathways operating in this strain will require further investigation (Wang *et al*., 2010).

SIS mechanisms acting to suppress transposable elements have been described in other organisms (Bourc'his & Bestor, 2004; Kelly & Aramayo, 2007). For example, *Neurospora crassa* has developed a number of genome defense strategies operating at different stages of its life cycle, including sex-specific repeat-induced point mutation and meiotic silencing of unpaired DNA (Selker, 1997; Cogoni & Macino, 1999; Galagan & Selker, 2004; Kelly & Aramayo, 2007). The mating-induced silencing described by Wang *et al*. represents a novel mechanism that does not involve unpaired DNA and is regulated by the translational induction of RNAi components. It is plausible that similar mechanisms are conserved in other fungal species, especially in those containing all of the RNAi machinery, but missing a meiotic silencing pathway (Kelly & Aramayo, 2007).

Development and virulence

Spores can infect animals and humans through inhalation, underscoring the critical role of sexual development in the virulence of *C. neoformans* (Giles *et al*., 2009; Velagapudi *et al*., 2009; Botts & Hull, 2010). In addition to spores, small, desiccated yeast cells are also candidates for infectious propagules of *C. neoformans*. Yeast is the most common morphological form in host tissue, although filamentous *Cryptococcus* during infection has been reported occasionally (Freed *et al*., 1971; Anandi *et al*., 1991;Williamson *et al*., 1996; Bemis *et al*., 2000). As described above, the host environment inhibits sexual development and hyphal growth. Experimentally introducing *C. neoformans* strains growing as hyphae to a model host confirmed the inability of filaments to persist during infection (Shadomy & Utz, 1966; Shadomy & Lurie, 1971; Zimmer *et al*., 1983). Strains collected from the environment as pseudohyphae were avirulent in a murine model of infection, while yeast cells derived from the same strain were pathogenic (Neilson *et al*., 1978). Thus, it appears that the formation of hyphae in *C. neoformans* during infection is rare and not advantageous for pathogenicity.

Several genes involved in the sexual development of *Cryptococcus* contribute to virulence. Interestingly, the requirement for a given gene for virulence is often dependent on the *C. neoformans* serotype. For example, the Ste12 transcription factor is needed for full virulence in serotype D, but is dispensable in serotype A (Yue *et al*., 1999). Conversely, Ste20 is required for virulence in serotype A, but not in D. The latter may be explained by the serotypespecific requirement for Ste20 for growth at 37 °C (Wang *et al*., 2002). At least in the case of Ste20, this is not due to a difference in the actual protein function between the two serotypes, but is instead associated with differences in related pathways.

Cryptococcus neoformans strains obtained from infected individuals or animals are highly variable with respect to yeast cell size (Cruickshank *et al*., 1973; Love *et al*., 1985; Feldmesser *et al*., 2001). Significant proportions of cells have been described as unusually large, often up to 10 times larger than cells found in the environment or cultured under laboratory conditions. Okagaki and colleagues used a murine inhalation model of cryptococcosis to detect yeast cells ranging from 10 to 100 μm in diameter in the host. These unusually large cells, referred to as giant cells, were mostly present in the lungs and were less abundant in the spleen and the brain (Okagaki *et al*., 2010). Cells of the **a**-mating type increased in size when present during coinfection with α-mating-type cells more frequently than either of the mating types alone, and this was dependent on the pheromone receptor Ste3. The authors hypothesize that pheromone sensing by **a**-mating-type cells during coinfection is necessary for this morphological change. This is particularly intriguing given that **a**-mating-type cells of serotype D typically swell in response to pheromone, while α-matingtype cells generate conjugation tubes. However, Okagaki and colleagues used a serotype A strain that normally does not swell during mating. Moreover, it is interesting that conditions in the host do not induce conjugation tubes in α-mating-type cells during coinfection. Importantly, giant cells were resistant to phagocytosis and oxidative and nitrosative stress (Okagaki *et al*., 2010; Zaragoza *et al*., 2010), which led to the hypothesis that cell gigantism in *Cryptococcus* may help the fungus avoid the early immune response. Giant cells are polyploid, but did not arrest during the cell cycle and produced daughter cells at a significantly higher rate than regular-sized yeast cells (Okagaki *et al*., 2010; Zaragoza *et al*., 2010). Zaragoza and colleagues visualized giant cells with multiple vesicles, a very thick cell wall, and an unusually thick, extensively cross-linked capsule. Zaragoza and colleagues also demonstrated that the cell gigantism is dependent on the cAMP pathway, but not on the Ras1 pathway, although it is conceivable that Ras1 could still be involved, given that the *ras1*Δ mutant produces larger cells at 37 °C in standard growth media. Nuclear staining and viability assays of *ras1*Δ giant cells obtained from mice may provide answers. Nonetheless, these data suggest that the pathways involved in mating (the pheromone and cAMP pathways) participate in this morphological transformation and contribute to the survival of *C. neoformans* during the early stages of infection.

Several features of giant cells are reminiscent of hyphae-derived chlamydospores, including a large size, a thick cell wall, and multiple vacuoles. Both giant cells and chlamydospores may serve a common purpose: to survive harsh environmental conditions. The signaling pathways involved in chlamydospore formation may differ from those involved in giant cell formation, as the formation of chlamydospores does not depend on the cAMP pathway (Lin & Heitman, 2005).

Concluding remarks

The first detailed description of cryptococcosis was made by Otto Busse in 1894 (Busse, 1894). Nearly a century later, with the advent of potent genetic tools and molecular biology techniques, we have started to uncover the biology of *Cryptococcus* and have learned some of the principles governing its pathogenicity.

The prevalent morphological form of *C. neoformans* in the environment and the host is a budding yeast. However, sexual differentiation in *C. neoformans* is well established and spores produced during mating may contribute to pathogenicity. Furthermore, some factors operating during mating contribute to virulence and it is now evident that *C. neoformans* undergoes a morphological transition to giant cells during infection, which influences disease progression. Despite these important connections, current knowledge of the development of *C. neoformans* remains limited. So far, the basic principles have been

established, and a number of important questions have emerged that should stimulate progress in our understanding of the biology of *C. neoformans*.

It is apparent that *C. neoformans* is unique in many respects. While some paradigms from classic fungal model systems apply to its biology, many aspects are strikingly different. For example, mating is orchestrated by pheromone-activated G protein couple receptors and a MAPK pathway, but the detailed architecture of these signaling routes differs from the *S. cerevisiae* paradigm. Moreover, while we can apply certain principles established from other model yeasts, including *S. cerevisiae*, *C. albicans*, and *S. pombe*, studies on hyphal development must rely on the relatively less complete knowledge from other basidiomycetes.

There are several outstanding questions regarding the sexual development of *C. neoformans*. The transcriptional programs orchestrating sexual development, including the formation of basidia and sporulation, are largely unknown. Additional downstream targets of Sxi1 and Sxi2 and the mechanisms of how these homeodomain transcription factors govern hyphal growth are also still largely unknown. The mechanistic details of clamp cell formation and septation are lacking, and we do not know how nuclear distribution and dynamics are regulated in hyphae. Although the formation of blastospores and chlamydospores has been described, the exact mechanisms behind their development and the biological significance remain unclear. Despite useful genetic tools, precise evaluation of events leading to spore chain development has been challenging due to difficulties in conducting time-lapse microscopy of hyphae. Unlike yeast, which can be easily grown on a microscope slide, postmating hyphae develop basidia, which are mostly aerial and difficult to image. In addition, light inhibits mating and hyphal development. Overcoming these challenges would allow investigations to uncover the sequence of events that occur during spore chain formation.

Some other intriguing aspects of the biology of *C. neoformans* still remain unexplained. Most notably, the skewed population towards the α-mating type in the environment and clinical samples remains puzzling, despite evident genetic recombination. A better understanding of mechanisms orchestrating unisexual mating will help to resolve this conundrum. Recent findings on RNAi-dependent genome defense mechanisms during mating have opened a new area that needs further exploration. It will be important to establish other possible silencing mechanisms that operate in *C. neoformans* during mating and in response to cellular stress, including possible responses associated with infection of a human host. Future advances in deciphering the development of *C. neoformans* will not only help to understand basic biological phenomena, but will also contribute toward better treatments against this emerging pathogen.

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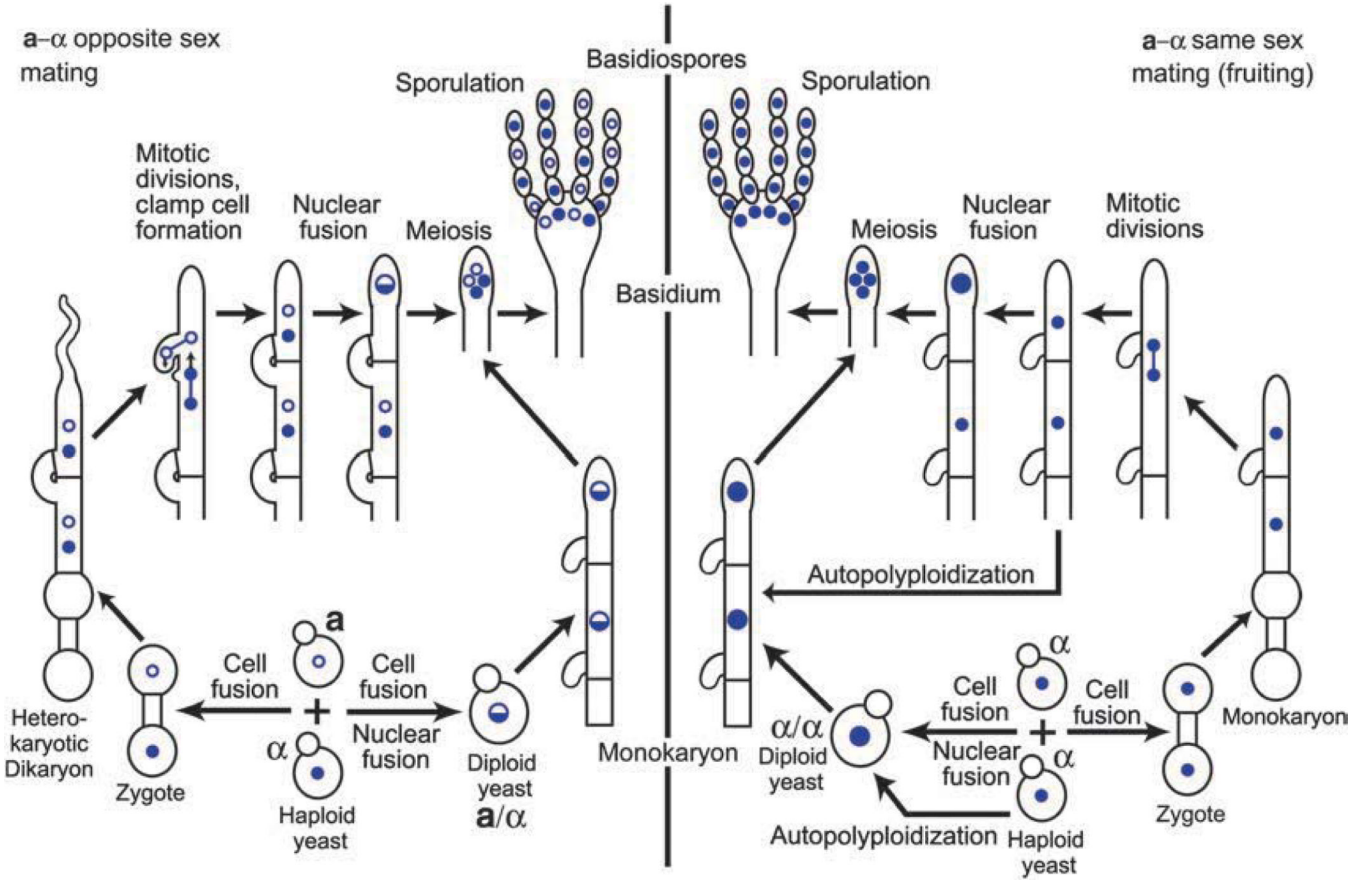

Fig. 1.

Sexual cycle of *Cryptococcus neoformans*. During the opposite-sex mating, **a** and α haploid yeast cells secrete peptide pheromones that stimulate cell–cell fusion. The resulting zygote develops first as hyphae, which are dikaryotic. After the first clamp cell is formed, a narrow 'pioneer' hypha is present at the apex. During hyphal growth, a basidium develops from the apical cell. Nuclear fusion and meiosis take place most likely concomitant with the formation of the basidium. Postmeiotic nuclei undergo rounds of mitotic divisions and four chains of spores are formed by subsequent budding from the surface of the basidium. Alternatively, **a** and α haploid yeast cells can form an **a**/α diploid, which grows as yeast at 37 °C in a rich medium and forms hyphae at 24 °C on mating-inducing medium. These diploid-derived hyphae are monokaryotic, have unfused clamp cells, and produce **a** and α spores. During same-sex mating, two yeast cells of the same mating type (α is depicted) undergo fusion and form monokaryotic hyphae with haploid nuclei. Concomitant with the formation of the basidium, nuclear fusion and meiosis occur. The resulting recombinant spores are of a single mating type. A single yeast cell can undergo autopolyploidization (i.e. endoreplication), resulting in a diploid, which develops into monokaryotic hyphae with diploid nuclei and unfused clamp cells. Autopolyploidization can also occur in the hyphae. Both opposite- and same-sex mating hyphae can develop chlamydospores and blastospores (not depicted) (see the text for a more detailed description).

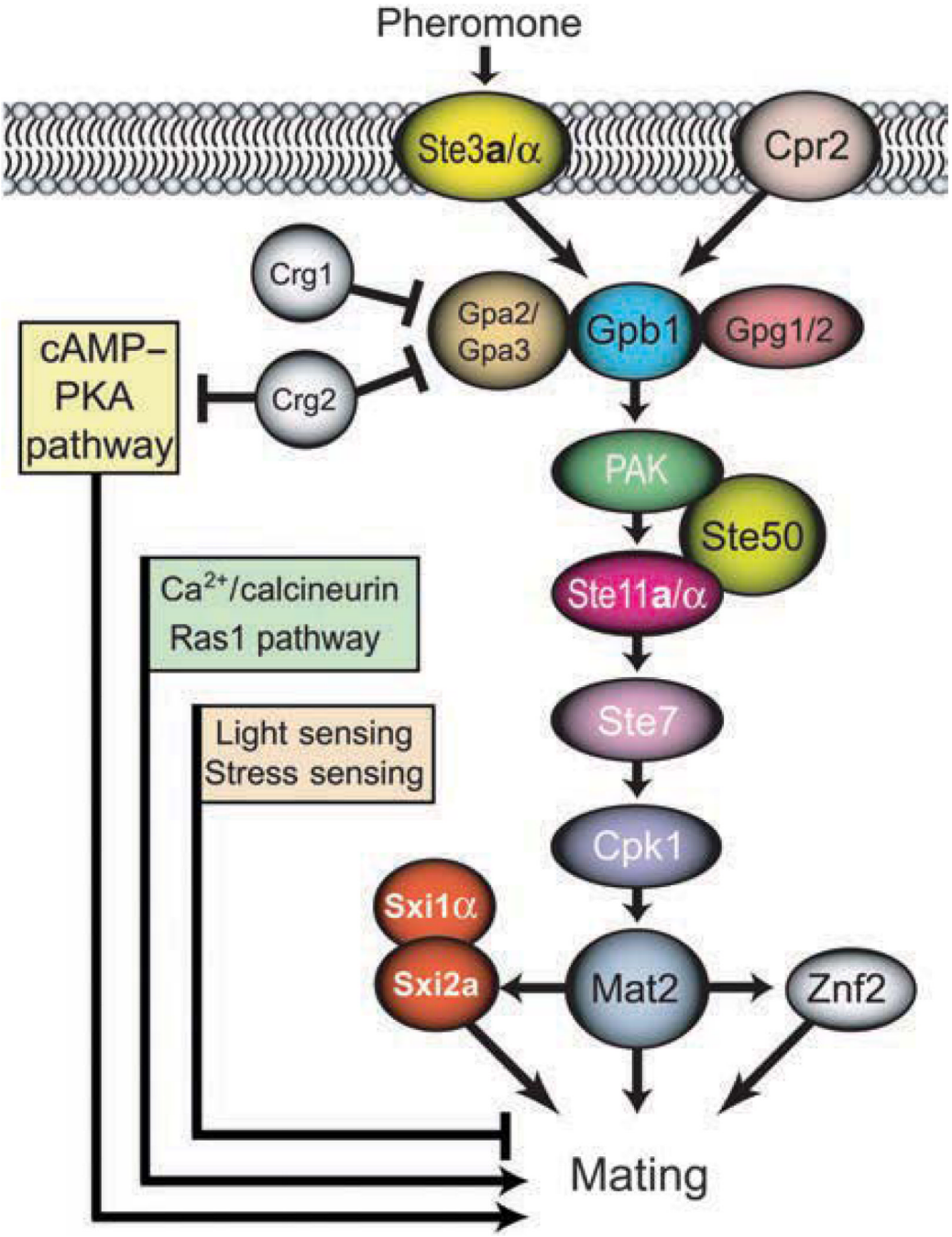

Fig. 2.

The pheromone response pathway in *Cryptococcus neoformans*. Other pathways that either positively regulate sexual development (cAMP-PKA pathway, Ca^{2+}-calcineurin pathway) or inhibit mating (light sensing, stress sensing) are also indicated (see the text for details).

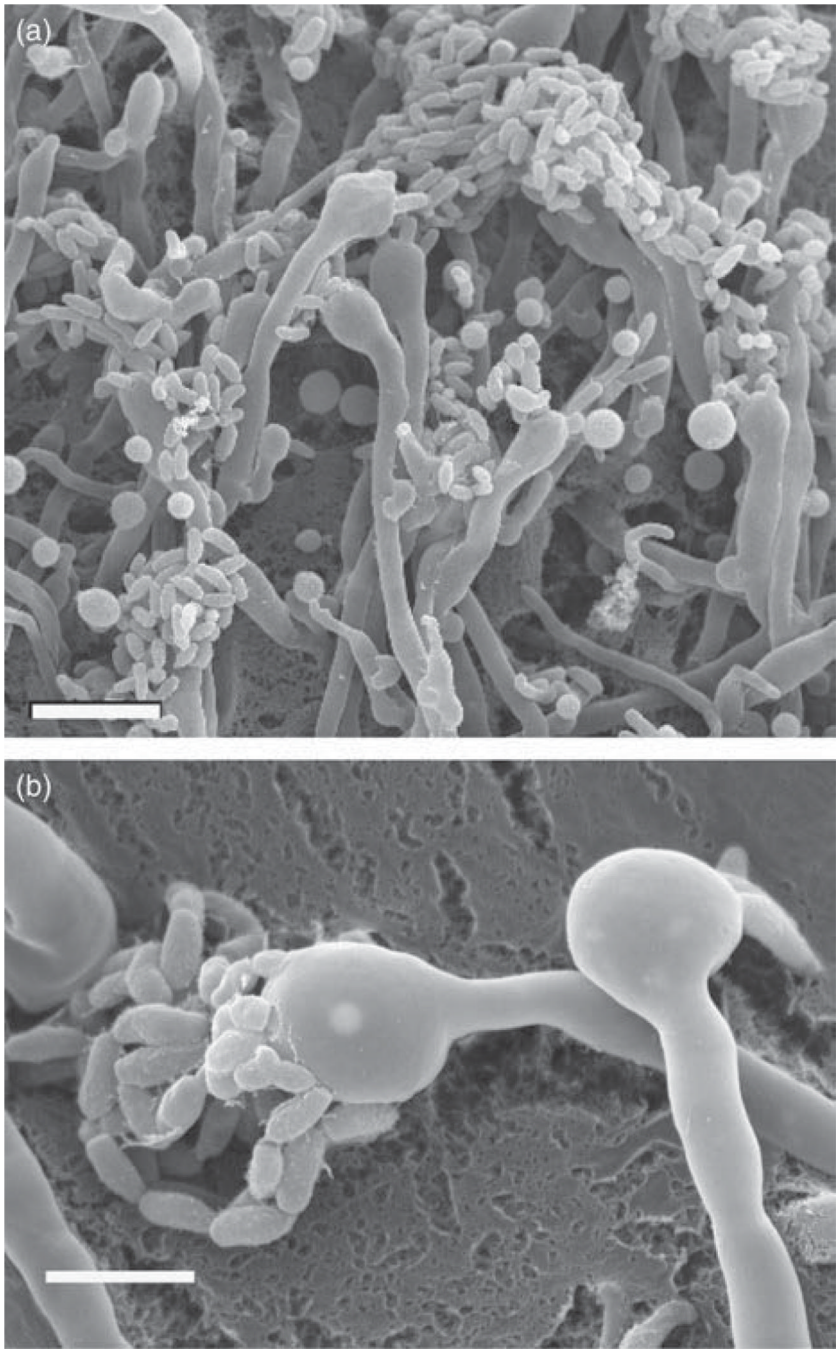

Fig. 3.
Postmating hyphae (a) of *Cryptococcus neoformans* serotype A and basidia (b) imaged by scanning electron microscopy. Images were taken by Lukasz Kozubowski (a) and Soo Chan Lee (b) and Valerie Knowlton (a, b).