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***Cryptococcus neoformans*: Historical curiosity to modern pathogen**

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I. Introduction

Two serious pathogens of mammals, *Cryptococcus neoformans* and *Cryptococcus gattii*, comprise the *C. neoformans* species complex in the fungal phylum Basidiomycota. These yeasts are facultative intracellular microbes: in the environment they grow either independently or possibly within soil amoeba (Steenbergen, et al., 2001), and in mammalian hosts they similarly occur either free in tissues or body fluids or within phagocytic cells. The importance of *C. neoformans* to human health has stimulated its development as an experimental model for both basic physiology and pathogenesis. Below we briefly review the history of this fascinating and versatile fungus, current tools available for its study, and some notable aspects of its biology that contribute to virulence. We hope this article will introduce a compelling and important organism to researchers in the other areas of mycology.

II. Discovery, nomenclature, and classification

In 1894 pathologist Otto Busse and surgeon Abraham Buschke first described this yeast as a human pathogen when they isolated a '*Saccharomyces-like*' organism from a bone infection in a young woman (Busse, 1894). Later that year Francesco Sanfelice reported the isolation from fermenting peach juice of a similar yeast, which he termed *Saccharomyces neoformans* because of its unique colony form (Sanfelice, 1894). Finally, in 1901, Jean-Paul Vuillemin renamed the organism *Cryptococcus neoformans* because it did not produce ascospores (Barnett, 2010), which is a defining characteristic of the genus *Saccharomyces*.

Observers of *C. neoformans* have consistently noted its thick cell walls and extensive capsule. During the mid 20th century, rabbit antisera were used to define four capsule serotypes (A through D (Evans, 1950; Wilson, et al., 1968)), a categorization that was later refined by analysis of DNA sequences, ecology, epidemiology, and pathobiology (Franzot, et al., 1999; Kwon-Chung and Varma, 2006). The current classification defines two species: *C. neoformans*, encompassing var. *grubii* (serotype A) and var. *neoformans* (serotype D), and *C. gattii* (serotypes B and C) (Kwon-Chung and Varma, 2006). The two species are also divided into eight major molecular types: VNI and VNII (var. *grubii*), VNIV (var. *neoformans*), VNIII (AD hybrids), and VGI – VGIV (*C. gattii*) (Igreja, et al., 2004; Kidd, et al., 2004; Litvintseva, et al., 2006; Meyer, et al., 2009). The latter system allows more precise genetic typing, which is particularly important in classifying inter- and intra-varietal diploid or aneuploid hybrids that have been recovered in the laboratory and from the environment.

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The genomes of *C. neoformans* and *C. gattii* diverged over 34 million years ago, yielding species with marked ecological and pathological differences (D'Souza, et al., 2011; Sharpton, et al., 2008). *C. neoformans* is found worldwide, is associated with avian excreta (particularly that of pigeons), and causes the vast majority of human infections. In contrast, *C. gattii* has historically been found in tropical and subtropical regions associated with various tree species, notably eucalyptus trees (Ellis and Pfeiffer, 1990; Sorrell, et al., 1996), and is responsible for less human illness.

III. Cryptococcal disease

a. Epidemiology

The spectrum of cryptococcal disease ranges from self-limiting cutaneous infections to fatal systemic ones (Mitchell and Perfect, 1995; Perfect, et al., 2010). Systemic disease is contracted by inhalation of the infectious particle (either desiccated yeast or spores (Botts and Hull, 2010; Giles, et al., 2009; Velagapudi, et al., 2009)), which leads to a primary pulmonary infection. This can remain latent for extended periods of time, but emerges and disseminates if the host becomes immunocompromised. Upon dissemination the organism shows particular tropism for the central nervous system (CNS), frequently causing fatal meningitis.

In the 1950s fewer than 300 cases of cryptococcosis were reported worldwide (Littman ML, 1956). This number rose dramatically in the ensuing years with the increase in numbers of patients with AIDS or other states of immune compromise. A recent study estimated over a million total cases of cryptococcal meningitis in 2006 (Park, et al., 2009). Most of these were *C. neoformans* infections in sub-Saharan Africa and other developing regions where treatment is limited by infrastructure and cost. Over half of these patients die from this disease, yielding fatalities in the range of those due to tuberculosis or diarrheal diseases in these regions (Figure 1).

C. neoformans generally affects immunocompromised individuals, although a few cases have been reported in individuals with no apparent underlying immunodeficiency (Chen, et al., 2008). In contrast *C. gattii* primarily affects immunocompetent individuals in endemic regions. Notably, its range has expanded in the last decade (Byrnes, et al., 2009; Fyfe, et al., 2008), starting with a 2001 outbreak on Vancouver Island (Byrnes, et al., 2009) and followed by associated outbreaks in nearby regions of the United States (Dixit, et al., 2009) and Canada. The reported incidence, several hundred human cases (Kidd, et al., 2004) and a smaller number of veterinary cases (MacDougall, et al., 2007), is low compared to that of *C. neoformans*. However, this documented spread of a pathogen from a previously limited geographic distribution may represent a new threat to public health. This review concentrates on *C. neoformans*, the better-studied species and more common pathogen.

b. Infection

C. neoformans proliferates within host phagocytic cells (Figure 2), which may confer advantages in terms of dissemination and immune protection. Initial interactions of the yeast with host cells may be mediated by an adhesin and may also involve host recognition of fungal capsule components (Del Poeta, 2004; Mansour and Levitz, 2002; Wang, et al., 2012). Once adherent cells are internalized, they traffic to the vacuole where they are able to survive and replicate, despite normal acidification of that compartment (Alvarez and Casadevall, 2006; Artavanis-Tsakonas, et al., 2006; Feldmesser, et al., 2001; Levitz, et al., 1999). Notably, the intracellular replication rate correlates with virulence (Alvarez, et al., 2009; Ma, et al., 2009; Mansour and Levitz, 2002). The outcome of internalization is likely influenced by the host immune status. The best outcome for the host is fungal death;

alternatively cryptococci may also continue to replicate within the host cell, or they may exit either by a lytic process lethal to the host cell or by a non-lytic process that leaves both cells intact (Alvarez and Casadevall, 2006; Alvarez and Casadevall, 2007; Johnston and May, 2013; Ma, et al., 2006; Ma, et al., 2007).

Entry to the CNS is a key step in cryptococcal pathogenesis. Various reports suggest that both internalized and free cryptococci traverse the blood-brain barrier to reach the brain, either by transcytosis through barrier epithelial cells or between them via breach of the tight junctions (Charlier, et al., 2005; Chen, et al., 2003; Jong, et al., 2012; Jong, et al., 2008a; Jong, et al., 2008b; Kim, 2008). A combination of these events may also occur.

C. neoformans may itself change during infection. The host environment induces metabolic alterations in the fungi, as well as changes in the size and structure of the capsule (Charlier, et al., 2005; Gates-Hollingsworth and Kozel, 2009; Zaragoza, 2011; Zaragoza, et al., 2009) that may help them evade the host immune response (Doering, 2009). A particularly dramatic process observed during primary pulmonary infection is the formation of 'titan cells' that may reach up to 100 μm in diameter (Okagaki and Nielsen, 2012; Okagaki, et al., 2010; Zaragoza, et al., 2010; Zaragoza and Nielsen, 2013). These cells cannot be readily phagocytosed but do produce normal sized progeny, leading to the suggestion that they act as dissemination points for *Cryptococcus* (Zaragoza and Nielsen, 2013). Formation of titan cells also leads to improved survival in conditions of stress (Zaragoza and Nielsen, 2013).

IV. Virulence factors

a. Capsule

The best-studied virulence factor of *C. neoformans* is its polysaccharide capsule (Doering, 2009; Gates, et al., 2004; Janbon and Doering, 2011; Kumar, et al., 2011; O'Meara and Alspaugh, 2012; Vecchiarelli, et al., 2013) (Figure 3). This dynamic structure, which can grow to several times the cell diameter in thickness, is unique among fungal pathogens. Cells lacking this structure are avirulent and the capsule, as well as shed polysaccharide, has been shown to inhibit phagocytosis of yeast and other host immune responses (Doering, 2009; Janbon and Doering, 2011). The capsule is associated with the yeast cell wall (Reese and Doering, 2003) and is composed primarily of two large polysaccharides, glucuronoxylomannan (GXM) and glucuronoxylomannogalactan (GXMGal) (Doering, 2009; Heiss, et al., 2013; Janbon and Doering, 2011); both of these have been implicated in virulence (Janbon and Doering, 2011).

The unique capsule polysaccharides are a rich area for investigation of carbohydrate structures and their biosynthetic pathways (Doering, 2009; Janbon and Doering, 2011). Numerous mutants with defects in capsule synthesis have been generated, many of them exhibiting virulence defects in animal models of infection (Perfect, 2005), and capsule regulation is an active area of research (Kumar, et al., 2011). Defining the capsule synthetic machinery may suggest new targets for chemotherapy of this serious disease.

b. Other virulence factors

C. neoformans expresses an arsenal of factors beyond the capsule that have been associated with virulence and therefore have attracted significant research interest (Heitman, 2011). To cause disease in humans, this yeast must not only survive at 37°C, but thrive in this environment, a characteristic that distinguishes it from nonpathogenic species of *Cryptococcus*. In the presence of appropriate precursors *C. neoformans* also produces melanin (Figure 4A), which provides protection from environmental stresses including antifungal compounds (Casadevall and Pirofski, 2001; Eisenman, et al., 2007; Nosanchuk

and Casadevall, 2006; Steenbergen and Casadevall, 2003); the enzyme that catalyzes melanin formation has been suggested as a therapeutic target (Zhu and Williamson, 2004).

Another factor that has been implicated in virulence is mannitol production, which is correlated with increased resistance to osmotic, heat, and oxidative stress (Wong, et al., 1990). Superoxide dismutase, proteases, and phospholipases have also been suggested as cryptococcal virulence factors (Brown, et al., 2007; Chen, et al., 1997; Jacobson, et al., 1994; Missall, et al., 2004).

V. Molecular and research tools

a. *C. neoformans* as a model organism

Primarily because of its role in human disease, *C. neoformans* has been actively developed as a tractable model system. The cells are round, typically 5–6 μm in diameter, and reproduce by budding (Figure 4B, C). They grow readily in liquid culture and form smooth, mucoid colonies on solid media. These cells also can undergo a sexual cycle leading to spore production (Figure 4D).

Early studies of *C. neoformans* were hampered by a lack of molecular tools and the challenges presented by cells with thick cell walls, an extensive capsule, and a propensity for non-homologous recombination. These obstacles have been largely overcome by the concerted efforts of multiple research groups, including community-driven genome sequencing (Heitman, et al., 1999b). Today, *C. neoformans* is being used to discover novel biology in areas ranging from sexual reproduction and signal transduction to polysaccharide synthesis, contributing to our knowledge of basic physiology and fungal pathogenesis. We next discuss selected research tools that have allowed this transformation.

b. Genome and genetics

Two *C. neoformans* genome sequences have been published (Loftus, et al., 2005), with initial studies aided by high-resolution linkage maps (Forche, et al., 2000; Schein, et al., 2002). The two sequences, of related var. *neoformans* strains, show a high degree of synteny despite a large segmental duplication in one of them (Fraser, et al., 2005). Genome sequences are also available for a var. *grubii* strain (http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/MultiHome.html), important because this variety includes almost 99% of the isolates recovered from AIDS patients, and for two strains of *C. gattii* (serotype B, VGI and VGII; <http://www.bcgsc.ca/project/cryptococcus>).

The 19 Mb *C. neoformans* genome generally consists of 14 chromosomes, although significant heterogeneity in chromosome number and size has been reported (Fries, et al., 1996; Polacheck and Lebens, 1989) that does not correlate with taxonomic variety. The genome encodes an estimated 6,500 genes, each with an average of 5.3 introns of about 50–70 nucleotides (Loftus, et al., 2005). Most splice sites are of the form 5'GU. . .CURAY. . .AG3', where A is the invariable branch point, R represents purines, and Y represents pyrimidines (Kupfer, et al., 2004); these sequences resemble those of higher eukaryotes (Kupfer, et al., 2004). The G + C content of the cryptococcal genome is relatively high (49% vs. 38% in *S. cerevisiae* or 33% in *C. albicans*) and varies considerably between coding regions (51%) and introns (43%).

A turning point in *C. neoformans* genetics was the description of a hyphae-forming strain in 1966 ((Shadomy and Utz, 1966); Figure 4D). This report paved the way for June Kwon-Chung's discovery of sexual reproduction, which in turn revolutionized the study of *C. neoformans* by enabling genetic manipulation and contributing to our understanding of the species complex (Kwon-Chung, 1975; Kwon-Chung, 1976). Genetic crossing has also been

used to establish congenic strains for research (Heitman, et al., 1999a; Nielsen, et al., 2003; Zhai, et al., 2013).

c. Mating

June Kwon-Chung showed that *C. neoformans* is a heterothallic basidiomycete with a bipolar mating system (McClelland, et al., 2004). The mating loci, *MATa* and *MATα*, are over 100 kb and contain from 24 to 32 genes (Lengeler, et al., 2002). When cells of opposite mating type are in close proximity, *MATα* cells respond to a *MATa* pheromone by developing conjugation tubes (analogous to the shmoo in *S. cerevisiae*) while *MATa* cells become large and swollen (McClelland, et al., 2004); fusion of conjugation tubes to these enlarged cells leads to the formation of heterokaryotic hyphae that subsequently form basidia at their tips. In the basidia, *MATa* and *MATα* nuclei fuse and undergo meiosis to produce chains of haploid spores. Haploid fruiting has also been observed; in this process, cells of one mating type become diploid, allowing them to undergo meiosis and sporulate (Lin, et al., 2005). Several recent reviews provide more detail about sexual reproduction and related processes such as haploid fruiting (Kozubowski and Heitman, 2012; Park and Williamson, 2012).

Mating is typically achieved *in vitro* through nitrogen starvation on V8™ agar medium (Kent, et al., 2008). Strains of opposite mating type are mixed and incubated in a dark, dry place for several weeks at room temperature. Once hyphae are detected (Figure 4D), the haploid spores can be collected and analyzed for recombination by marker or molecular analysis and mating type can be determined by PCR specific for the *MAT* loci or by crossing against tester strains. Because the spores are not contained within an easily-isolated structure (e.g. the ascus of Ascomycetes), strain construction and genetic analysis are slower and more laborious than it is in *S. cerevisiae*, but they are nonetheless powerful tools (Heitman, et al., 1999a; Varma, et al., 1992).

d. Transformation

Several methods for *C. neoformans* transformation have been reported, which vary in efficiency and the fate of the transformed DNA. Early studies used electroporation to introduce an exogenous *URA5* gene into a uracil auxotroph, resulting in phenotypic complementation (Edman and Kwon-Chung, 1990). Although this was a significant advance, targeted genome modification using this method was hampered by low transformation efficiency, instability of transformants, and low frequency of specific recombination with chromosomal sequences.

The soil bacterium *Agrobacterium tumefaciens* has also been used to manipulate *C. neoformans* (Idnurm, et al., 2009; McClelland, et al., 2005). This organism injects a piece of its Ti plasmid into host cells, where it is integrated into the host chromosome. By transforming *A. tumefaciens* cells with an appropriately modified Ti plasmid and then co-incubating them with *C. neoformans*, DNA of interest can reach the fungal genome. While this method does not mediate homologous recombination, it does yield high transformation efficiency, stable integrants, and low perturbation of the host DNA (McClelland, et al., 2005), all useful traits for genetic screens based on random mutagenesis (Idnurm, et al., 2004; Li, et al., 2012; McClelland, et al., 2005).

Biolytic delivery of DNA-coated gold beads, a method used to transform plants, has been particularly effective for manipulation of *Cryptococcus* (Toffaletti, et al., 1993). This approach yields higher rates of transformation and chromosomal integration than electroporation, although at the cost of frequent non-homologous recombination (Nelson, et al., 2003). It has proved a useful tool for chromosomal integration (Davidson, et al., 2002),

typically directed by constructs with significant overlap with the target sequence (300 bp or more (Nelson, et al., 2003)). To increase the efficiency of homologous recombination, strains deleted for Ku proteins (which act in non-homologous end joining of double-stranded DNA breaks) and split-marker strategies have been applied (Fu, et al., 2006; Goins, et al., 2006; Kim, et al., 2012). Despite the potential for non-specific or multiple integration events, the efficiency of the biolistic approach makes it the most commonly used transformation system for *C. neoformans*.

e. Plasmids used to express cryptococcal sequences

Early studies of electroporation revealed that extrachromosomal DNA could be modified with telomeric repeats; addition of these repeats to exogenous DNA increases transformation efficiency and transformant stability (Edman, 1992; Edman and Kwon-Chung, 1990). For plasmid construction such elements are often cloned as inverted repeats, such that linearization of the plasmid by cleaving between them generates a construct with telomeric ends. Many early plasmids also contain a 'STAB' (Varma and Kwon-Chung, 1998) element that was initially thought to have a stabilizing effect, although this was subsequently disproved (Hull and Heitman, 2002). Autonomous replication sequences (ARS) have not been characterized and the likely centromeres are too large (Loftus, et al., 2005) for convenient manipulation, so plasmid copy number remains unpredictable. Nonetheless, plasmids have been used for multiple studies, including protein expression, genomic libraries (Fox, et al., 2003; Vallim, et al., 2005; Varma, et al., 2006), and RNAi (see below).

The first plasmids used in *C. neoformans* were marked by biosynthetic genes (e.g. genes required for synthesis of uracil, adenine, or lysine), which could complement auxotrophic mutants used as DNA recipients (Chang and Kwon-Chung, 1994; Kwon-Chung, et al., 1992; Toffaletti, et al., 1993). This approach has been largely replaced by the use of dominant markers that confer drug resistance to hygromycin B, nourseothricin, G418 (geneticin), or phleomycin (Cox, et al., 1996; Hua, et al., 2000; McDade and Cox, 2001).

Regulatory elements in *Cryptococcus* have not been explored in detail, although both promoter and terminator sequences clearly influence expression levels. Several promoters have been used to drive constitutive gene expression, including those of the glyceraldehyde-3-phosphate dehydrogenase (*GDP*) and actin (*ACT1*) genes (Ory, et al., 2004; Varma and Kwon-Chung, 1999). For regulated expression the most commonly used are *CTR4* (Ory, et al., 2004) and *GAL7* (Baker and Lodge, 2012a; del Poeta, et al., 1999), which are regulated by copper and galactose, respectively. Typical mammalian enhancer sequences have been found to modulate expression of one gene, *LAC1*, which encodes the enzyme that forms melanin (see above). Similar sequences were identified near other virulence-related genes, although these have not been directly tested (Zhang, et al., 1999). DAG and ATF consensus sequences, which are well-characterized transcriptional regulatory regions in higher eukaryotes, have also been found in the promoter region of *APP1*, another virulence-related gene (Tommasino, et al., 2008). Terminators that are commonly used include those of the *TRP1*, *HIS3*, and *CTR4* genes (McDade and Cox, 2001).

Several additional considerations are important in *C. neoformans* vector design. The activity of regulatory sequences may vary considerably depending on their strain of origin (Ory, et al., 2004) and the presence of or absence of native introns may influence gene expression (Goebels, et al., 2013). Finally, despite native flanking sequences and introns, extrachromosomal gene expression may differ from expression at endogenous loci, even beyond the issues of copy number mentioned above (our unpublished observations).

f. Specific gene depletion

Gene deletion is the most definitive way to eliminate gene expression. This is usually achieved by using biolistic transformation to replace a specific gene with a marker via homologous recombination. Several groups have used this approach to generate deletion collections (see below). Because the number of markers is limited, vectors have been generated with loxP sites flanking the resistance cassette, allowing Cre recombinase mediated excision and reuse of the marker for a subsequent round of transformation and selection (Baker and Lodge, 2012b).

Another approach to reducing specific gene expression in *C. neoformans* is RNA interference (Skowrya and Doering, 2012). This was first demonstrated using a plasmid with sense and antisense sequences corresponding to the target gene separated by a spacer sequence: the resulting transcript forms a hairpin with a double-stranded RNA stem that mediates interference (Liu, et al., 2002). Subsequent experiments included the use of constructs with promoters in opposite orientation flanking the sequence of interest, resulting in a RNA duplex that triggers the interference (Bose and Doering, 2011). Studies using these methods also included reporter genes that enabled estimation of the extent of gene suppression and regulatable promoters that allowed transient silencing. Beyond the use of RNAi as an experimental tool in *C. neoformans*, studies in this area have reported endogenous functions for RNAi, including transposon regulation and specific gene-silencing during sexual reproduction or when RNA processing is suboptimal (Dumesic, et al., 2013; Janbon, et al., 2010; Wang, et al., 2010).

g. Imaging

Imaging has been central to the study of *C. neoformans*, starting with the observation of characteristic spaces around cryptococci in infected tissues that were later found to correspond to the space occupied by the polysaccharide capsule. This structure can also readily be detected by negative staining with India ink (Figure 3, top left), which produces a distinctive halo appearance around the cell wall where it excludes the ink particles. Multiple imaging methods typically used for yeasts have also been applied to *C. neoformans*, although they often require methodological modification because of the thick capsule and cell wall. These include thin-section, quick-freeze deep-etch, and scanning electron microscopy; differential interference microscopy; and immunofluorescence imaging. Figure 3 illustrates the application of several imaging methods to visualization of the cryptococcal capsule.

A variety of stains and immunological reagents have been used to image cryptococci. These include cell wall stains (e.g. Lucifer yellow, shown in Figure 2 and (Srikanta, et al., 2011), calcofluor white (Giles, et al., 2009), or eosin Y (Baker, et al., 2007)), anti-capsule antibodies (Belay, et al., 1997; Casadevall, et al., 1994; De Jesus, et al., 2009; Feldmesser, et al., 2000; Kozel and Follette, 1981; Moyrand, et al., 2002; Mukherjee, et al., 1992), and labeled lectins (Botts, et al., 2009; Fonseca, et al., 2013). Several proteins have also been epitope-tagged or fused to fluorescent proteins for visualization. Although these techniques can be successfully applied to *C. neoformans* (Ding, et al., 2013; Haynes, et al., 2011; Liu, et al., 2006; Patel, et al., 2010; Reilly, et al., 2011; Waterman, et al., 2012), such studies are still often confounded by the difficulty of permeabilizing these well-protected cells (our unpublished observations), inadequate expression of vector-encoded proteins (see above), or instability of modified proteins. This situation may be improved by the recent development of several fluorescent proteins that have been codon-optimized for *Cryptococcus* and are robustly expressed (e.g. GFP (Liu, et al., 2006) or mCherry (Waterman, et al., 2012)).

h. Genome-scale analysis

The availability of cryptococcal genome sequence has enabled broad studies of gene expression, mutagenesis, and genetic interactions. Application of gene expression technologies has largely paralleled broader methodological developments in this field. Large-scale transcriptional studies began with serial analysis of gene expression (SAGE), which was applied in *Cryptococcus* to study the effects of temperature, iron concentration, and host tissue environment (Hu, et al., 2008; Lian, et al., 2005; Steen, et al., 2002; Steen, et al., 2003). DNA microarrays, mainly generated through a community effort, have also been used productively to study the transcriptional response of the fungi to CO₂, antifungal compounds, iron deprivation, and oxidative stress (Florio, et al., 2011; Kim, et al., 2010; Upadhyay, et al., 2013), and to discover targets of transcription factors and signaling molecules (Chun, et al., 2011; Cramer, et al., 2006; O'Meara, et al., 2010; Pukkila-Worley, et al., 2005). We have used microarray analysis and RNA Seq to probe capsule regulation, allowing us to reconstruct this complex regulatory network (Haynes, et al., 2011) and discover novel regulators (Maier et al, unpublished data).

Another large-scale resource for studies of *C. neoformans* is a collection of 1,201 gene deletion mutants generated by biolistic transformation, each marked with one of 48 unique DNA sequences (signature tags)(Liu, et al., 2008). This library, which represents about a fifth of the genome, is available through the ATCC and has been used productively for several screens (He, et al., 2012; Liu, et al., 2008; Tseng, et al., 2012). The Madhani group has also used it for cross-species synthetic lethality genetic analysis to uncover the function of novel proteins in *C. neoformans*, based on observed genetic interactions following their expression in the *S. cerevisiae* deletion collection (Brown and Madhani, 2012). A more complete genome deletion collection is currently in progress, and will be a tremendous resource for the research community (Madhani, 2012).

i. Infection models

C. neoformans researchers benefit from multiple models of infection, ranging from cells in culture to vertebrate animals. Since *C. neoformans* is a facultative intracellular pathogen, a major focus has been on its interactions with larger eukaryotic cells that may engulf it. These include free-living microbes like *Acanthamoeba* and *Dictyostelium*, which may be relevant to the fungal life style in the environment (Malliaris, et al., 2004; Neilson, et al., 1978; Steenbergen, et al., 2003), and cells isolated from vertebrate hosts (Alvarez and Casadevall, 2006; Barbosa, et al., 2006; Ma, et al., 2006). Among the vertebrate cells tested are monocytes (Figure 2), macrophages, dendritic cells, and lymphocytes obtained from mouse, rat, and human (Diamond and Bennett, 1973; Goulart, et al., 2010; Qin, 2011). Cell lines have also been valuable for studies of host-pathogen interactions. Phagocytic lines from mouse or human are commonly used (e.g. J774 or THP-1 (Ma, et al., 2006; Ralph, et al., 1975; Srikanta, et al., 2011; Tsuchiya, et al., 1980)), and *Drosophila* S2 cells have been used to assess the role of host factors in the uptake of fungal cells (Qin, et al., 2011). Specialized models are also useful for investigating specific steps of infection, such as interactions with the lung epithelium or the blood-brain barrier (BBB) (Barbosa, et al., 2006; Chen, et al., 2003; Stie and Fox, 2012; Vu, et al., 2009).

A wide spectrum of host models for cryptococcal disease enable studies in intact organisms, including both plants ((Springer, et al., 2010; Warpeha, et al., 2013) and our unpublished data) and animals (Sabiiti, et al., 2012). Invertebrates possess an innate, but not an adaptive, immune system, and offer low cost and rapid infection models with few ethical concerns. For these reasons the soil nematode *C. elegans* (Mylonakis, et al., 2002; Mylonakis, et al., 2003) and the insects *Galleria mellonella* and *Drosophila melanogaster* (Apidianakis, et al., 2004; Mylonakis, 2008; Mylonakis, et al., 2005) have been used to model cryptococcal

disease, although the parallels to mammalian hosts are necessarily limited. Vertebrate animal systems are more commonly used to study cryptococcal pathogenesis (recently reviewed in (Sabiiti, et al., 2012)). Mice are usually the system of choice, because of the variety of genetic backgrounds available (Zaragoza, et al., 2007) and the well-characterized infections that result from intravenous, intranasal, and intratracheal inoculation (Clemens, 2011; Sabiiti, et al., 2012). A rat model that can yield long-term infection has also been reported (Goldman, et al., 1996), and guinea pigs (Kirkpatrick, et al., 2007) have recently been established as a model for antifungal drug testing. Direct CNS infection of immunocompromised (steroid-treated) rabbits is used as a model for meningitis (Perfect, et al., 1980).

VI. Final words

The increasing prevalence of severe *C. neoformans* infections during the last few decades has attracted the attention of researchers, who together have developed tools to increase the tractability of this fascinating system. Application of these tools has led to remarkable progress in the study of cryptococcal virulence and to recognition of the unique biology of this system, which differs in fundamental ways from the most common ascomycete models for basic science (*Saccharomyces cerevisiae*) and fungal pathogenesis (*Candida albicans*).

Significant questions remain to be answered in the realms of both fundamental biology and pathogenesis of *C. neoformans*. Intriguing areas include glycan synthesis, which is considerably more complex in this organism than in model yeast, and the diverse pathways related to sexual reproduction. Numerous questions remain about host: pathogen interactions, ranging from the mechanism of fungal engulfment to the route(s) of central nervous system entry and the specific host responses to infection. The fascinating complexities of capsule regulation are just beginning to be explored (Haynes, et al., 2011; Kumar, et al., 2011; O'Meara, et al., 2013). With powerful tools now in hand to tackle such questions, we can look forward to further discoveries of novel biology, which will potentially improve management of this deadly disease.

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Figure 1.

Comparison of infectious disease deaths globally (total bar height) and in Africa alone (white lines). Cryptococcosis (red bar) kills over half a million individuals annually, mainly in Africa. Numbers shown for cryptococcosis are based on Park *et al* (Park, et al., 2009). Other values are from World Health Organization regional mortality data for 2002 (WHO, 2002); this year was chosen to best match the cohort study data used in (Park, et al., 2009).

Figure 2.
Confocal micrograph of human peripheral blood monocytes (cytosol stained red) with engulfed *Cryptococcus* (cell walls stained green).

Figure 3.

C. neoformans capsule images. Top row: negative stain with India ink; quick freeze deep-etch electron micrograph of a portion of the cell wall with capsule fibers extending to the left); thin section electron micrograph of three cells. Bottom row: immunoelectron micrograph of a portion of a cell (capsule fibers extending upwards) stained with gold-conjugated anticapsule antibody; differential interference contrast micrograph of a budding cell; confocal immunofluorescence micrograph with the capsule stained blue and the cell wall stained green.

Figure 4. Views of *C. neoformans*. A. Colonies grown on medium containing 0.1% L-DOPA to demonstrate melanization. Top, wild type; bottom, laccase mutant. B. Brightfield microscopy. C. Transmission electron microscopy (pseudocolored). D. Mating filaments.