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Aimless mutants of *Cryptococcus neoformans*: failure to disseminate

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Abstract

The pathogenic fungus *Cryptococcus neoformans* exhibits a striking propensity to cause central nervous system (CNS) disease in people with HIV/AIDS. Given that cryptococcal infections are generally initiated by pulmonary colonization, dissemination requires that the fungus withstand phagocytic killing, cross the alveolar-capillary interface in the lung, survive in the circulatory system and breach the blood-brain barrier. We know little about the molecular mechanisms underlying dissemination, but there is a rapidly growing list of mutants that fail to cause CNS disease. These mutants reveal a remarkable diversity of functions and therefore illustrate the complexity of the cryptococcal-host interaction. The challenge now is to extend the analysis of these mutants to acquire a detailed understanding of each step in dissemination.

Keywords

fungal pathogens; blood brain barrier; invasion

1. Introduction

The basidiomycete yeast *Cryptococcus neoformans* causes life-threatening pulmonary infections and meningoencephalitis in immunocompromised individuals. Cryptococcal meningitis is usually fatal if left untreated, and it is estimated that > 600,000 deaths occur globally from the disease each year (Park *et al.*, 2009). Infections are generally initiated by inhalation of *C. neoformans* spores or yeast cells, and the fungus must adapt to nutritional and physical conditions in the host including the intracellular environment of phagocytic cells, and the extracellular environment of the lung, bloodstream and central nervous system (CNS) (Heitman, 2011; Kronstad *et al.*, 2011a). The fungus must also withstand the innate and adaptive immune responses.

The fungal factors that contribute to pathogenesis include elaboration of a polysaccharide capsule, production of the antioxidant pigment melanin in the cell wall, and the export of enzymes such as phospholipase B, urease and superoxide dismutase (Kronstad *et al.*, 2011b). The roles of these factors in interactions with the host are understood with varying levels of detail. However, there is a rapidly expanding list of mutants with defects in other

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functions that affect dissemination and colonization of the CNS and cerebral spinal fluid (CSF). This review summarizes the pathway of dissemination for *C. neoformans* and highlights selected mutants to emphasize key findings as well as experimental approaches that shed light on dissemination.

2. The path from the lung to the brain

During initial infection, desiccated cryptococcal cells or spores that are sufficiently small (3 μm) to reach the alveoli encounter a nutrient-limited environment and innate defenses such as alveolar macrophages (Geuntes-Boyer *et al.*, 2009; Giles *et al.*, 2007; Hu *et al.*, 2008a; Kronstad *et al.*, 2011b) (Fig. 1). Activation of alveolar macrophages through opsonization results in the phagocytosis of fungal cells, and these cryptococci can survive and proliferate in the acidic environment of the phagolysosome (Feldmesser *et al.*, 2000). *C. neoformans* is a facultative, intracellular pathogen and, remarkably, the fungus can move out of or between phagocytic cells by a process of expulsion (Alvarez and Casadevall, 2006; Alvarez and Casadevall, 2007; Ma *et al.*, 2006; Ma *et al.*, 2007). These capabilities may facilitate fungal movement out of the lung environment via residence in macrophages and monocytes, with subsequent escape. In addition, the fungus adapts to lung conditions through the formation of a subpopulation of enlarged cells (called titan or giant cells) that resist phagocytosis (Okagaki *et al.*, 2010; Zaragoza *et al.*, 2010).

Cryptococcal cells must traverse the pneumocytic cell layer of the alveolar wall to escape the lung for hematogenous or lymphatic spread (Schaible *et al.*, 2010). The alveolar-capillary interface, also known as the blood-air barrier is formed by the pneumocytes of the alveolar wall, the endothelial cells of the capillaries and type IV collagen between the two layers (Schaible *et al.*, 2010). It also contains a battery of immune defenses such as defensins, cathelicidins, lysozyme and lactoferrin (Schaible *et al.*, 2010). The mechanisms by which cryptococcal cells breach the barrier are not well understood but presumably involve transit within phagocytic cells, and interactions with epithelial cells that involve invasion and host cell damage. The characterization of interactions with lung epithelial cells reveals that capsule polysaccharide participates in cryptococcal attachment to these cells, interacts with the CD14 receptor, and influences the expression of intercellular adhesion molecule-1 (ICAM-1) and interleukin-8 (IL-8) (Barbosa *et al.*, 2006; Barbosa *et al.*, 2007; Merkel and Scofield, 1997).

Very little is known about the ability of *C. neoformans* to survive in blood and the lymphatic system. Blood is a nutrient-rich environment but the extent of persistent bloodstream infection (fungemia) during cryptococcosis is unclear (Sahn, 2007; Thompson and Keir, 1990; Waugh and Allison, 2007). Experimental infections in mice and comparisons with human cases of cryptococcosis indicate that the fungus can persist in the bloodstream (Chretien *et al.*, 2002; Dromer *et al.*, 2007; Lortholary *et al.*, 1999). For example, following tail vein infection in mice, the level of *C. neoformans* in blood correlates with the level of colonization of brain, lung and spleen at day 1, but only with lung and spleen at a later time (day 8). This suggests that the brain responds differently to cryptococcal infection and that fungal levels in the blood may be a good indicator of lung and spleen colonization (Lortholary *et al.*, 1999). Cryptococcal cells also are found both free and in association with peripheral blood mononuclear cells in the blood of infected mice (Chretien *et al.*, 2002; Lortholary *et al.*, 1999). These cells can subsequently become lodged in brain capillaries and cross the blood-brain barrier (BBB) to initiate infection of the CNS (Charlier *et al.*, 2009; Santangelo *et al.*, 2004) (Fig. 1).

The BBB is a structural and functional barrier formed by brain microvasculature and endothelial cells held together by tight junctions, as well as by astrocytes and pericytes

which support the tissues of the CNS and prevent apoptosis of endothelial cells (Kim, 2000; Kim, 2008) (Fig. 1). *C. neoformans* is thought to traverse the BBB by three mechanisms: a Trojan horse strategy, transcytosis and paracellular traversal. The Trojan horse approach involves the phagocytosis of fungal cells in the blood or, proximal to brain vasculature endothelial cells, by phagocytes which transport the pathogen to the parenchyma (Casadevall, 2010; Dromer *et al.*, 2007). Alternatively, cryptococcal cells have been shown to enter and exit brain microvascular endothelial cells (BMECs) through fungal hyaluronic acid interactions with the host endothelial CD44 protein for PKC-dependent internalization, which occurs without disruption of tight junctions between endothelial cells by a process of transcytosis (Chen *et al.*, 2003; Jong *et al.*, 2008). This mode of invasion has been observed for many bacterial pathogens and the fungal pathogen *Candida albicans* (Bonazzi *et al.*, 2009a; Bonazzi *et al.*, 2009b; Jong *et al.*, 2001; Kim, 2000; Nizet *et al.*, 1997; Zink *et al.*, 1996). Lastly, microbial penetration between barrier cells with/without tight junction disruption (paracellular traversal) has been shown to occur in mouse models in which fungal urease promotes invasion without involvement of capsule or host CD44 (Olszewski *et al.*, 2004). Additionally, a possible role for host plasma proteases in CSF invasion has been suggested by increased *in vitro* invasion of BMECs by plasminogen-coated *C. neoformans* (Stie *et al.*, 2009; Stie and Fox, 2011). Once in the brain, *C. neoformans* encounters a new nutritional and physical environment with differences in pH, glucose and oxygen availability (Fig. 1).

3. Genetic requirements for virulence and dissemination

Numerous studies have employed random insertional and targeted mutagenesis, in combination with animal models of infection, to identify genes associated with cryptococcal virulence and/or dissemination to the CNS (see Idnurm *et al.*, 2009; Liu *et al.*, 2008) (Table 1). These studies usually employ infection models that include intracisternal or intrathecal inoculation of rabbits to measure proliferation in the CSF, inoculation of mice via inhalation, intratracheal (IT) instillation or intravenous (IV) injection, as well as assays of survival/proliferation in CSF and during interactions with phagocytic and other host cells *in vitro*. Additional information can also be obtained by analyzing a time course or competition between mutant and wild-type strains during infection. In general, these approaches provide different types of information about dissemination, and often more than one assay is needed to distinguish poor *in vivo* growth from defects in colonization and survival in specific organs. Here we consider the phenotypes of selected mutants in light of the application of these models and focus on those that exhibit virulence defects, differential brain colonization/persistence, or survival in CSF. The emerging picture is that a wide variety of functions influence virulence and dissemination (Fig. 2, Table 1).

Mutations affecting melanization

C. neoformans produces melanin in the presence of phenolic substrates and deposits the pigment in the cell wall. Melanin provides protection against host-derived oxidative and nitrosative stresses, contributes to defense against antimicrobial peptides and antifungal drugs, and interferes with protective T-cell responses and antibody-mediated phagocytosis (for further information about melanin, see reviews by Liu and Nizet, 2009; Panepinto and Williamson, 2006; Zhu and Williamson, 2004). Salas *et al.*, 1996 showed that deletion of *LAC1* (encoding laccase) leads to albino mutants with attenuated virulence after IV infection of mice. Noverr *et al.* (2004) also found that a *lac1* mutant was attenuated for virulence upon IT infection. However, no differences in pulmonary clearance were found for the mutant compared to a *LAC1* strain, although higher numbers of the melanized cells were found in the brain, spleen and lung-associated lymph nodes (Noverr *et al.*, 2004). A closer examination of dissemination, using IV inoculation of mice, revealed that similar numbers of laccase-positive and laccase-negative cells were detected in the brain and spleen. This

result indicates that laccase is not required for survival in sites outside of the lung and that the enzyme must play a role in escape. Subsequent studies indicated that localization of laccase to the cell wall is influenced by pH, interactions with macrophages and tissue location (Waterman *et al.*, 2007a).

A variety of functions that influence melanin production, such as transcription factors, signaling components, protein sorting and secretion factors, and sphingolipid production, also contribute to virulence and dissemination. For example, the transcription factor Cuf1 regulates genes for copper acquisition in *C. neoformans* and also controls the copper-induced transcription of *LAC1* (Jiang *et al.*, 2009). Waterman *et al.* (2007b) showed that a *cuf1* mutant is attenuated for virulence upon IV inoculation in mice and that the mutant proliferates as well as wild type in the lung after intranasal or IV infection. However, a time course of dissemination after IV infection revealed reduced growth of the *cuf1* mutant in the brain, suggesting that copper may be limiting in CNS infection. Another transcription factor (Sre1) that is involved in melanin expression, sterol production and adaptation to hypoxia showed a virulence defect and reduced lung colonization after IV or intranasal inoculation in a competitive infection with wild-type cells (Chun *et al.*, 2007). The *sre1* mutant also had a moderate defect in colonization of spleen and brain (Chun *et al.*, 2007). Chang *et al.* (2007) also characterized the *SRE1* gene and found that an *sre1* mutant was attenuated for virulence and failed to accumulate in the brain after tail vein injection. The mutant reached the brain at a similar level as the wild-type strain at 5 hours but was much less abundant by 48 hours suggesting that the mutant had a defect in growth in the CNS. The approach of using a time course to assess colonization of the brain and persistence has also been employed to examine the role of the Snf1 kinase in virulence in an inhalation model of infection (Hu *et al.*, 2008a). Loss of Snf1, which controls virulence, alternative carbon source utilization, melanin formation and the response to stress, results in mutants that are unable to persist in the brain despite high fungal burden in the lung (Hu *et al.*, 2008a; Yang *et al.*, 2010).

Mutants with defects in components of vesicular transport and export also show reduced melanin production and attenuated virulence after IV infection of mice. For example, Sec6 is an essential component of the exocyst protein complex involved in the fusion of vesicles with the plasma membrane (Novick *et al.*, 1980). Reduced *SEC6* expression by RNAi resulted in a complete absence of extracellular vesicle production, with a loss of extracellular laccase, urease and polysaccharide secretion. However, the strains with reduced *SEC6* expression retained the ability to produce capsule and secrete phospholipase, suggesting the existence of different secretion pathways (Panepinto *et al.*, 2009). Inoculation of the strains into mice (IV) demonstrated that they were less virulent, although mutant cells could be recovered from the brain. A defect in another transport component, Vps34 (phosphatidylinositol 3-kinase), resulted in no melanin production, loss of virulence in both an IV and intranasal infection models, and rapid clearance from the lungs (Hu *et al.*, 2008b; Lee *et al.*, 2010).

Melanin is also influenced by inositol-phosphoryl ceramide synthase 1 (Ipc1), an enzyme that is essential for the formation of complex sphingolipids and diacylglycerol (DAG) (Shea and Del Poeta, 2006). Regulated expression to lower *ICP1* transcript levels caused reduced melanization, attenuated virulence and decreased CSF survival of *C. neoformans* in the brains of infected rabbits (Luberto *et al.*, 2001).

Mutations affecting capsule

The capsule is the major virulence factor of *C. neoformans* and it is composed of two polysaccharides: glucuronoxylomannan (GXM) and galactoxylomannan (GalXM) (Zaragoza *et al.*, 2009). While the mechanisms of capsule synthesis have not been elucidated in detail, the deletion of genes that cause an acapsular phenotype (e.g., *CAP* genes) causes attenuated

virulence upon IV or IT inoculation of mice (Chang and Kwon-Chung, 1994; Chang *et al.*, 1996; Chang and Kwon-Chung, 1998; Chang and Kwon-Chung, 1999; Chun and Madhani, 2010; Garcia-Rivera *et al.*, 2004; Moyrand *et al.*, 2004; Wilder *et al.*, 2002). As an example of the influence on dissemination, Wilder *et al.*, (2002) infected mice IT with an acapsular strain (*cap64*) or an encapsulated strain (*CAP64*), and followed the fungal burden in the lung and brain. The acapsular strain was cleared from the lung faster than the encapsulated strain and also failed to appear in the brain. Similarly, a defect in the gene *USX1* encoding a UDP-xylose synthase involved in the xylosylation of capsule GXM caused loss of virulence and failure to accumulate in the brains of mice (Moyrand *et al.*, 2002). A study of the other capsule polysaccharide, GalXM, focused on the genes *UGE1* (UDP-glucose epimerase) and *UGT1* (UDP-galactose transporter) (Moyrand *et al.*, 2007; 2008). Deletion of these genes results in strains which are GXM-positive but GalXM-negative, and which are unable to colonize mouse brains upon IV injection. While these mutants also exhibited temperature sensitivity, suppressor mutants that were able to grow at 37°C and were GalXM negative still could not colonize the brain. The re-introduction of *UGT1* and *UGE1* restored a normal organ colonization pattern and GalXM production. This study raises the possibility that GXM mutants readily expose GalXM at the cell surface so that traditional “acapsular mutants” should in fact be considered “GalXM exposed” mutants (Moyrand *et al.*, 2007). This complication should be considered in studies that examine the influence of capsule polysaccharides on dissemination.

Capsule may indirectly impact dissemination to the brain by influencing phagocytosis. An example of this phenomenon comes from a study by Shea *et al.* (2006) on the influence of inositol sphingolipid metabolism on virulence and dissemination. Specifically, a mutant lacking inositol phosphosphingolipid-phospholipase C1 (*Isc1*) was more sensitive than the wild-type strain to oxidative and nitrosative stress and showed mild attenuation of virulence. However, the mutant also produced an enlarged capsule during pulmonary infection of mice and was found predominantly in the extracellular environment rather than in macrophages. The mutant showed a reduced ability to reach the brain, and this defect was ablated by depleting macrophages by pharmacological means or by using a mouse strain in which macrophages were not efficiently activated. It is possible that the larger capsule on the mutant interfered with passage across the BBB, although other mutants with enlarged capsules can cross the BBB (e.g., the *pkrl* mutant described below) (Shea *et al.*, 2006).

Mutants with defects in capsule and melanin

Mutants that fail to produce both melanin and capsule due to metabolic or signaling defects are often pleiotrophic. For example, deletion of the genes encoding ATP-citrate lyase (*ACL1*) or a peroxisomal β -oxidation enzyme (*MFE2*) exhibit reduced dissemination to the brain in a murine inhalation model, as well as defects in carbon source utilization, capsule and melanin production and susceptibility to azole antifungals (E.G., M.K. and J. K., unpublished results). The influence of a signaling defect is nicely illustrated by deletion of *PKA1* encoding the catalytic subunit of cAMP-dependent protein kinase (PKA); loss of this gene attenuates virulence, reduces mating, and influences the response to stress (D’Souza *et al.*, 2001). The CSF of rabbits injected intrathecally with a *pka1* mutant showed complete clearance of fungal cells by day 10 post infection, indicating a marked growth defect in the brain which correlated with attenuated virulence in a murine inhalation model (D’Souza *et al.*, 2001). In contrast, a mutant lacking the regulatory subunit of PKA (*Pkr1*) produced an enlarged capsule in infected tissue, was hypervirulent in mice, accumulated at higher numbers than the wild-type strain in the CNS and survived well in the rabbit CSF model of infection (D’Souza *et al.*, 2001).

The connection between a pleiotrophic function and CNS colonization is also illustrated by the recent characterization of the WASP protein, *Wsp1* (Shen *et al.*, 2011). WASP proteins

are multimodular proteins that contain an N-terminal WASP homology (WH1), a proline-rich domain (PR), a basic amino acid domain (B) as well as other modules. The *WSP1* gene in *C. neoformans* is not essential, but a *wsp1* mutant has defects in growth, cytokinesis, chitin distribution, mating, endocytosis and exocytosis. The mutant is also unable to produce capsule and urease, has reduced melanin production and is more readily phagocytosed by macrophages. Additionally, the *wsp1* mutant was detected at a 10–100 fold lower level than wild type in the brains of infected mice upon IV injection suggesting a problem with crossing the BBB or proliferating in the brain (Shen *et al.*, 2011). Interestingly, Wsp1 is known to interact with phosphatidylinositol biphosphate (PIP2) through the B domain, and also with Cdc42 and Cin1 through other domains (Shen *et al.*, 2011). Cdc42 is a Rho-like GTPase and a *cdc42* mutant fails to disseminate in a mouse inhalation model (Ballou *et al.*, 2010). Cin1 contains a RhoGEF domain and a *cin1* mutant had pleiotropic effects on growth, cytokinesis, intracellular transport and the production of several virulence factors (e.g., capsule or melanin) (Shen *et al.*, 2010). The effects of these mutants may be connected in a number of ways by influences on intracellular transport and cell polarity. Another protein involved in cell polarity and microtubule dynamics, the protein kinase Kin1, also contributes to dissemination to the brain upon tail vein injection in mice (Mylonakis *et al.*, 2004).

A more direct role in capsule formation and interactions with endothelial cells has been established for the hyaluronic acid synthase Cps1 (Jong *et al.*, 2007). Deletion of *CPS1* results in modification of an ultrastructural layer between the cell wall and the capsule, and leads to temperature sensitivity, elimination of virulence in a mouse model and loss of cell surface hyaluronic acid. Importantly, a *cps1* mutant has a reduced ability to associate with human brain membrane endothelial cells *in vitro* (Jong *et al.*, 2007; Lee *et al.*, 2010). Although colonization of the CNS has not been reported, loss of surface hyaluronic acid may reduce interactions with host CD44 and prevent traversal of the BBB. A *cps1* mutant also has impaired growth in CSF *in vitro* (Lee *et al.*, 2010).

Urease and Phospholipase B

The deletion of genes encoding two key enzymes, urease and phospholipase B results in a failure of fungal cells to disseminate efficiently to the brain, thus attenuating virulence. Deletion of the *URE1* gene encoding urease results in a mutant that is able to persist as well as wild-type cells in CSF upon direct inoculation into the brains of rabbits (Cox *et al.*, 2000). However, the *ure1* mutant is attenuated for virulence upon IV or intranasal inoculation in mice (Cox *et al.*, 2000). A detailed examination of organ burden upon IT, IV and intracranial inoculations in mice supports the model that urease facilitates blood-to-brain invasion by enhancing fungal sequestration within microcapillary beds (Olszewski *et al.*, 2004). This idea is supported by recent intravital microscopic examination of mouse brain capillaries upon infection with a *ure1* mutant (Shi *et al.*, 2010).

The enzyme phospholipase B (Plb1) plays a critical role in pulmonary cryptococcosis and egress from the lung (Djordjevic, 2004). During initial interactions in the lung, the enzyme hydrolyzes surfactant lipids and facilitates adherence of cryptococcal cells to epithelial cells. The hydrolysis of membrane phospholipids may also release fatty acids as a potential energy source. Plb1 is known to promote the replication of *C. neoformans* in macrophage-like cell lines *in vitro* and in alveolar macrophages *in vivo* (Cox *et al.*, 2001; Noverr *et al.*, 2003; Santangelo *et al.*, 2004). Santangelo *et al.*, (2004) also showed that the enzyme is required for entry into the blood and for lymphatic spread from the lung to lymph nodes. In a thorough study, they demonstrated a defect in the ability of a *plb1* mutant to reach interstitial macrophages following IT inoculation. Although the mechanism is not yet known, this finding demonstrates that specific functions are needed for *C. neoformans* to move out of the lung. Plb1 is not required to establish a CNS infection but Santangelo *et al.* (2004) did

demonstrate that mononuclear phagocytes could serve as vehicles for dissemination of *C. neoformans* in the bloodstream, a result later supported by Charlier *et al.* (2009).

Plb1 also contains lysophospholipase transacetylase activity that is essential for the incorporation of macrophage-derived arachidonic acid into cryptococcal lipids during cryptococcus-phagocyte interactions. The enzyme plays a role during intracellular proliferation in macrophages and in facilitating the exit of the fungus from the phagolysosome (Alvarez and Casadevall, 2006; Alvarez and Casadevall, 2007; Chayakulkeeree *et al.*, 2011; Ma *et al.*, 2006; Ma *et al.*, 2007). Secretion of Plb1 is dependent on Sec14, a homolog of a phosphatidylinositol transfer protein that regulates trans-golgi export pathways in *Saccharomyces cerevisiae* (Chayakulkeeree *et al.*, 2011). A mutant lacking *SEC14* in *C. neoformans* still produces melanin and capsule but was attenuated for virulence and dissemination to the CNS.

Additional factors influencing virulence and dissemination

A variety of other mutants with virulence defects and/or reduced dissemination have also been identified, and some of these are unaffected for capsule and melanin production. The array of functions identified by these mutants include transport, metabolism, cell wall synthesis, signaling, regulation, protein sorting/degradation, secretion and the response to stress. Here we focus on selected mutants to illustrate representative phenotypes. Many genes involved in the response to host temperature and oxidative and nitrosative stress have been characterized in *C. neoformans* and the related pathogenic species *C. gattii* (Ngamskulrungraj *et al.*, 2009; Petzold *et al.*, 2006). For example, the trehalose synthesis pathway for synthesis of the disaccharide trehalose plays a role in virulence and stress tolerance during cryptococcal infections. In *C. neoformans*, the genes encoding trehalose-6-phosphate synthase (*TPS1*) and trehalose-6-phosphate phosphatase (*TPS2*) of the trehalose pathway are required for growth at 37°C and glycolysis, and a *tps2* mutant also accumulates the toxic intermediate trehalose-6-phosphate (Ngamskulrungraj *et al.*, 2009; Petzold *et al.*, 2006). Importantly, a *tps1* mutant was attenuated for virulence and failed to persist in the lungs and brains of mice. The mutant was also rapidly cleared from the CSF following intracisternal injection into rabbits (Petzold *et al.*, 2006). Wild type levels of melanin and capsule formation were found for the *tps1* and *tps2* mutants in *C. neoformans*, but the equivalent mutants were defective for these traits in *C. gattii*. However, as with *C. neoformans*, the *tps1* and *tps2* mutants of *C. gattii* were both unable to persist in the lungs and brains of infected mice (Ngamskulrungraj *et al.*, 2009; Petzold *et al.*, 2006). Other mutants with growth defects at host temperature also fail to persist in CSF in the rabbit model of cryptococcal virulence. A well-characterized example is the *ras1* mutant that is rapidly cleared from the CSF. In *C. neoformans*, the small GTP-binding protein Ras1 regulates mating and filamentation as well as growth at high temperature (Alspaugh *et al.*, 2000).

Reactive oxygen species (ROS), including the superoxide anion, hydrogen peroxide and hydroxyl radicals are produced by immune effector cells as part of the oxidative burst during infection. Superoxide dismutases are the first line of antioxidant defence and disruption of SOD genes in *C. neoformans* and *C. gattii* generally attenuates virulence (Cox *et al.*, 2003; Narasipura *et al.*, 2005). With regard to dissemination, loss of *SOD2* in *C. gattii* results in attenuated virulence in an IV model of mouse infection and mutant cells are found at a 1000-fold lower level in the brain compared to the wild-type strain (Narasipura *et al.*, 2005). Thus, Sod2 in *C. gattii* contributes to crossing the BBB and/or persistence in the CNS, although the protein did not provide protection against PMN killing, nor was it required for the production of other virulence factors (Narasipura *et al.*, 2005).

As an alternative approach to animal infections, a library of signature-tagged and site-directed mutants was screened for *in vitro* growth defects in CSF (Lee *et al.*, 2010). This approach identified a number of functions including *Ena1*, a P-type ATPase, *PIK1*, a 1-phosphatidylinositol 4-kinase, *Swe1*, a MYT1 kinase involved in regulation of the cell cycle, and *Pxa2*, an ABC transporter. Mutants of *ena1* exhibited cell death after 36hr of incubation in CSF, poor survival in macrophages, increased sensitivity to high pH and attenuation of virulence upon intracisternal injection in rabbits. A *pik1* mutant similarly exhibited decreased survival in macrophages and attenuation in the rabbit model, but to a lesser extent than the *ena1* mutant. Interestingly, melanin production was enhanced in the *pik1* and *swe1* mutants. The assay in CSF also revealed a defect for a mutant with a deletion in *Hrd1*, the synoviolin E3 ligase involved in ER associated degradation. The mutant was unaltered in capsule, melanin and temperature sensitivity yet failed to grow in CSF. Other novel mutants with poor growth in CSF identified defects in *Vam6*, a Rab guanyl-nucleotide exchange factor, *Csn1201*, a transcription-associated recombination protein, *Ckb1*, a casein kinase involved in the regulation of the cell cycle, *Rub1*, a ribosomal chaperone, and *Vps25*, an ESCRT-II complex trafficking protein (Lee *et al.*, 2010).

Finally, we note that recent studies have identified contributions to virulence and dissemination for factors involved in metal homeostasis, signaling, and glucosylceramide synthesis. In the case of metal homeostasis, the high affinity iron permease, *Cft1*, is required for iron acquisition from transferrin *in vitro*, for full virulence in mice and for dissemination to the CNS (Jung *et al.*, 2008). Similarly, a mutant in the copper-dependent transcription factor, *Cuf1*, also influences virulence and dissemination, as mentioned above (Waterman *et al.*, 2007a).

For signaling components, a recent study showed that *Rdi1*, a Rho family signaling protein, was required for establishing cell and vacuole morphology, cell polarity, cytokinesis, intracellular survival in macrophages and virulence in an IV murine model (Price *et al.*, 2008). In a separate study, deletion of *SKN7*, a two-component stress response regulator, resulted in a mutant with attenuated virulence and reduced invasion of human umbilical-vein endothelial cells (Coenjaerts *et al.*, 2006). As mentioned earlier, signaling functions such as the cAMP-dependent protein kinase play a role in regulating the major virulence factors. The *CRG1* gene encodes a regulator of G-protein signaling in the pheromone response pathway and, interestingly, a *crg1* mutant is hypervirulent and shows enhanced melanin production (Whittington and Wang, 2011; Xue *et al.*, 2008). In contrast, loss of *Crg2*, another regulator of G-protein signaling (in the cAMP and pheromone pathways) resulted in attenuated virulence and a dissemination defect in a mouse inhalation model (Whittington and Wang, 2011; Xue *et al.*, 2008). However, *Crg2*, appeared to mainly participate in establishment of a pulmonary infection.

Glucosylceramide is an antigenic membrane glycosphingophospholipid that elicits an antibody response during cryptococcosis (Rodrigues *et al.*, 2000). This lipid is synthesized by glucosylceramide synthase (*Gcs1*) (Kechichian *et al.*, 2007), and it is essential for growth in the alveolar space and the bloodstream (neutral/alkaline pH), but not within the acidic phagolysosome. A *gsc1* mutant is avirulent in an intranasal infection in mice and the fungus was found in the lungs and brains of the mice (Kechichian *et al.*, 2007). IV inoculation resulted in delayed disease progression but the mice showed signs of CNS disease and the fungus was able to disseminate to the brain. An examination of the inflammatory response in mice with intranasal versus IV inoculation indicated that the former method resulted in containment of the mutant in the lung compared with dissemination seen with the IV approach (Rittershaus *et al.*, 2006).

4. Conclusions

The mechanisms for egress of cryptococcal cells from the lung, transport through the blood/lymphatic system and traversal across the blood brain barrier are just now being dissected through the use of mutants and a variety of assays. As illustrated in this review, *C. neoformans* mutants with defects in a wide variety of functions are attenuated for virulence in animal models, and a subset of these exhibit diminished capacity for dissemination to and/or persistence in the brain or CSF. It would be difficult to test each mutant in the full battery of assays necessary to examine dissemination in detail. However, for selected mutants it would be justified to examine the fungal burden in lung and brain following both intranasal and IV inoculations of mice. If warranted, follow up experiments could examine the ability of mutants to exit the lung and appear in interstitial macrophages, blood, and the lymphatic system, as demonstrated by the careful study of Santangelo et al. (2004). Similarly, the ability of mutants to cross the BBB can be tested with *in vitro* models of the BBB and by intravital microscopy (Jong et al., 2008; Shi et al., 2010; Vu et al., 2009). In addition, the assays developed by John Perfect's group for examining proliferation and survival in the CSF of infected rabbits or *in vitro* in CSF are valuable for determining the fate of mutants once they cross the BBB (Lee et al., 2010; Perfect et al., 1980). Not surprisingly, we know much about the role of the enzymes urease and phospholipase B through the application of many of these dissemination assays. Perhaps the analysis of additional enzymes (e.g., proteases) will prove useful in identifying factors that function at specific steps in the dissemination pathway.

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References

- Alspaugh JA, Cavallo LM, Perfect JR, Heitman J. *RAS1* regulates filamentation, mating and growth at high temperature of *Cryptococcus neoformans*. Mol Microbiol. 2000; 36:352–365. [PubMed: 10792722]
- Alvarez M, Casadevall A. Phagosome extrusion and host-cell survival after *Cryptococcus neoformans* phagocytosis by macrophages. Curr Biol. 2006; 16:2161–2165. [PubMed: 17084702]
- Alvarez M, Casadevall A. Cell-to-cell spread and massive vacuole formation after *Cryptococcus neoformans* infection of murine macrophages. BMC Immunol. 2007; 8:16. [PubMed: 17705844]
- Ballou ER, Nichols CB, Miglia KJ, Kozubowski L, Alspaugh JA. Two *CDC42* paralogues modulate *Cryptococcus neoformans* thermotolerance and morphogenesis under host physiological conditions. Mol Microbiol. 2010; 75:763–780. [PubMed: 20025659]
- Barbosa FM, Fonseca FL, Holandino C, Alviano CS, Nimrichter L, Rodrigues ML. Glucuronoxylomannan-mediated interaction of *Cryptococcus neoformans* with human alveolar cells results in fungal internalization and host cell damage. Microbes Infect. 2006; 8:493–502. [PubMed: 16293436]
- Barbosa FM, Fonseca FL, Figueiredo RT, Bozza MT, Casadevall A, Nimrichter L, Rodrigues ML. Binding of glucuronoxylomannan to the CD14 receptor in human A549 alveolar cells induces interleukin-8 production. Clin Vaccine Immunol. 2007; 14:94–98. [PubMed: 17093102]
- Bonazzi M, Lecuit M, Cossart P. *Listeria monocytogenes* internalin and E-cadherin: from bench to bedside. Cold Spring Harb Perspect Biol. 2009a; 1:a003087. [PubMed: 20066101]
- Bonazzi M, Lecuit M, Cossart P. *Listeria monocytogenes* internalin and E-cadherin: from structure to pathogenesis. Cell Microbiol. 2009b; 11:693–702. [PubMed: 19191787]

- Casadevall A. Cryptococci at the brain gate: break and enter or use a Trojan horse? *J Clin Invest.* 2010; 120:1389–1392. [PubMed: 20424319]
- Chang YC, Kwon-Chung KJ. Complementation of a capsule-deficient mutation of *Cryptococcus neoformans* restores its virulence. *Mol Cell Biol.* 1994; 14:4912–4919. [PubMed: 8007987]
- Chang YC, Penoyer LA, Kwon-Chung KJ. The second capsule gene of *Cryptococcus neoformans*, *CAP64*, is essential for virulence. *Infect Immun.* 1996; 64:1977–1983. [PubMed: 8675296]
- Chang YC, Kwon-Chung KJ. Isolation of the third capsule-associated gene, *CAP60*, required for virulence in *Cryptococcus neoformans*. *Infect Immun.* 1998; 66:2230–2236. [PubMed: 9573112]
- Chang YC, Kwon-Chung KJ. Isolation, characterization, and localization of a capsule-associated gene, *CAP10*, of *Cryptococcus neoformans*. *J Bacteriol.* 1999; 181:5636–5643. [PubMed: 10482503]
- Chang YC, Jong A, Huang S, Zervas P, Kwon-Chung KJ. *CPS1*, a homolog of the *Streptococcus pneumoniae* type 3 polysaccharide synthase gene, is important for the pathobiology of *Cryptococcus neoformans*. *Infect Immun.* 2006; 74:3930–3938. [PubMed: 16790766]
- Chang YC, Bien CM, Lee H, Espenshade PJ, Kwon-Chung KJ. Sre1p, a regulator of oxygen sensing and sterol homeostasis, is required for virulence in *Cryptococcus neoformans*. *Mol Microbiol.* 2007; 64:614–629. [PubMed: 17462012]
- Charlier C, Nielsen K, Daou S, Brigitte M, Chretien F, Dromer F. Evidence of a role for monocytes in dissemination and brain invasion by *Cryptococcus neoformans*. *Infect Immun.* 2009; 77:120–127. [PubMed: 18936186]
- Chayakulkeeree M, Sorrell TC, Siafakas AR, Wilson CF, Pantarat N, Gerik KJ, Boadle R, Djordjevic JT. Role and mechanism of phosphatidylinositol-specific phospholipase C in survival and virulence of *Cryptococcus neoformans*. *Mol Microbiol.* 2008; 69:809–826. [PubMed: 18532984]
- Chayakulkeeree M, Johnston SA, Oei JB, Lev S, Williamson PR, Wilson CF, Zuo X, Leal AL, Vainstein MH, Meyer W, Sorrell TC, May RC, Djordjevic JT. *SEC14* is a specific requirement for secretion of phospholipase B1 and pathogenicity of *Cryptococcus neoformans*. *Mol Microbiol.* 2011; 80:1088–1101. [PubMed: 21453402]
- Chen SH, Stins MF, Huang SH, Chen YH, Kwon-Chung KJ, Chang Y, Kim KS, Suzuki K, Jong AY. *Cryptococcus neoformans* induces alterations in the cytoskeleton of human brain microvascular endothelial cells. *J Med Microbiol.* 2003; 52:961–970. [PubMed: 14532340]
- Chretien F, Lortholary O, Kansau I, Neuville S, Gray F, Dromer F. Pathogenesis of cerebral *Cryptococcus neoformans* infection after fungemia. *J Infect Dis.* 2002; 186:522–530. [PubMed: 12195380]
- Chun CD, Liu OW, Madhani HD. A link between virulence and homeostatic responses to hypoxia during infection by the human fungal pathogen *Cryptococcus neoformans*. *PLoS Pathog.* 2007; 3:e22. [PubMed: 17319742]
- Chun CD, Madhani HD. Ctr2 links copper homeostasis to polysaccharide capsule formation and phagocytosis inhibition in the human fungal pathogen *Cryptococcus neoformans*. *PLoS One.* 2010; 5
- Coenjaerts FE, Hoepelman AI, Scharringa J, Aarts M, Ellerbroek PM, Bevaart L, Van Strijp JA, Janbon G. The Skn7 response regulator of *Cryptococcus neoformans* is involved in oxidative stress signalling and augments intracellular survival in endothelium. *FEMS Yeast Res.* 2006; 6:652–661. [PubMed: 16696662]
- Cox GM, Mukherjee J, Cole GT, Casadevall A, Perfect JR. Urease as a virulence factor in experimental cryptococcosis. *Infect Immun.* 2000; 68:443–448. [PubMed: 10639402]
- Cox GM, McDade HC, Chen SC, Tucker SC, Gottfredsson M, Wright LC, Sorrell TC, Leidich SD, Casadevall A, Ghannoum MA, Perfect JR. Extracellular phospholipase activity is a virulence factor for *Cryptococcus neoformans*. *Mol Microbiol.* 2001; 39:166–175. [PubMed: 11123698]
- Cox GM, Harrison TS, McDade HC, Taborda CP, Heinrich G, Casadevall A, Perfect JR. Superoxide dismutase influences the virulence of *Cryptococcus neoformans* by affecting growth within macrophages. *Infect Immun.* 2003; 71:173–180. [PubMed: 12496163]
- D'Souza CA, Alspaugh JA, Yue C, Harashima T, Cox GM, Perfect JR, Heitman J. Cyclic AMP-dependent protein kinase controls virulence of the fungal pathogen *Cryptococcus neoformans*. *Mol Cell Biol.* 2001; 21:3179–3191. [PubMed: 11287622]

- Djordjevic JT. Role of phospholipases in fungal fitness, pathogenicity, and drug development - lessons from *Cryptococcus neoformans*. *Front Microbiol.* 2010; 1:125. [PubMed: 21687772]
- Dromer F, Mathoulin-Pelissier S, Launay O, Lortholary O. Determinants of disease presentation and outcome during cryptococcosis: the CryptoA/D study. *PLoS Med.* 2007; 4:e21. [PubMed: 17284154]
- Fan W, Kraus PR, Boily MJ, Heitman J. *Cryptococcus neoformans* gene expression during murine macrophage infection. *Eukaryot Cell.* 2005; 4:1420–33. [PubMed: 16087747]
- Feldmesser M, Kress Y, Novikoff P, Casadevall A. *Cryptococcus neoformans* is a facultative intracellular pathogen in murine pulmonary infection. *Infect Immun.* 2000; 68:4225–4237. [PubMed: 10858240]
- Garcia-Rivera J, Chang YC, Kwon-Chung KJ, Casadevall A. *Cryptococcus neoformans* CAP59 (or Cap59p) is involved in the extracellular trafficking of capsular glucuronoxylomannan. *Eukaryot Cell.* 2004; 3:385–392. [PubMed: 15075268]
- Geunes-Boyer S, Oliver TN, Janbon G, Lodge JK, Heitman J, Perfect JR, Wright JR. Surfactant protein D increases phagocytosis of hypocapsular *Cryptococcus neoformans* by murine macrophages and enhances fungal survival. *Infect Immun.* 2009; 77:2783–2794. [PubMed: 19451250]
- Giles SS, Batinic-Haberle I, Perfect JR, Cox GM. *Cryptococcus neoformans* mitochondrial superoxide dismutase: an essential link between antioxidant function and high-temperature growth. *Eukaryot Cell.* 2005; 4:46–54. [PubMed: 15643059]
- Giles SS, Zaas AK, Reidy MF, Perfect JR, Wright JR. *Cryptococcus neoformans* is resistant to surfactant protein A mediated host defense mechanisms. *PLoS One.* 2007; 2:e1370. [PubMed: 18159253]
- Heitman J.; Kozel, TR.; Kwon-Chung, KJ.; Perfect, JR.; Casadevall, A. *From Human Pathogen to Model Yeast.* Washington, D.C: ASM Press; 2011.
- Hu G, Cheng PY, Sham A, Perfect JR, Kronstad JW. Metabolic adaptation in *Cryptococcus neoformans* during early murine pulmonary infection. *Mol Microbiol.* 2008a; 69:1456–1475. [PubMed: 18673460]
- Hu G, Hacham M, Waterman SR, Panepinto J, Shin S, Liu X, Gibbons J, Valyi-Nagy T, Obara K, Jaffe HA, Ohsumi Y, Williamson PR. PI3K signaling of autophagy is required for starvation tolerance and virulence of *Cryptococcus neoformans*. *J Clin Invest.* 2008b; 118:1186–1197. [PubMed: 18259613]
- Idnurm A, Walton FJ, Floyd A, Reedy JL, Heitman J. Identification of ENA1 as a virulence gene of the human pathogenic fungus *Cryptococcus neoformans* through signature-tagged insertional mutagenesis. *Eukaryot Cell.* 2009; 8:315–326. [PubMed: 19151325]
- Jiang N, Sun N, Xiao D, Pan J, Wang Y, Zhu X. A copper-responsive factor gene *CUF1* is required for copper induction of laccase in *Cryptococcus neoformans*. *FEMS Microbiol Lett.* 2009; 296:84–90. [PubMed: 19459959]
- Jong AY, Stins MF, Huang SH, Chen SH, Kim KS. Traversal of *Candida albicans* across human blood-brain barrier in vitro. *Infect Immun.* 2001; 69:4536–4544. [PubMed: 11401997]
- Jong A, Wu CH, Chen HM, Luo F, Kwon-Chung KJ, Chang YC, Lamunyon CW, Plaas A, Huang SH. Identification and characterization of *CPS1* as a hyaluronic acid synthase contributing to the pathogenesis of *Cryptococcus neoformans* infection. *Eukaryot Cell.* 2007; 6:1486–1496. [PubMed: 17545316]
- Jong A, Wu CH, Shackleford GM, Kwon-Chung KJ, Chang YC, Chen HM, Ouyang Y, Huang SH. Involvement of human CD44 during *Cryptococcus neoformans* infection of brain microvascular endothelial cells. *Cell Microbiol.* 2008; 10:1313–1326. [PubMed: 18248627]
- Jung WH, Sham A, Lian T, Singh A, Kosman DJ, Kronstad JW. Iron source preference and regulation of iron uptake in *Cryptococcus neoformans*. *PLoS Pathog.* 2008; 4:e45. [PubMed: 18282105]
- Kechichian TB, Shea J, Del Poeta M. Depletion of alveolar macrophages decreases the dissemination of a glucosylceramide-deficient mutant of *Cryptococcus neoformans* in immunodeficient mice. *Infect Immun.* 2007; 75:4792–4798. [PubMed: 17664261]
- Kim KS. *E. coli* invasion of brain microvascular endothelial cells as a pathogenetic basis of meningitis. *Subcell Biochem.* 2000; 33:47–59. [PubMed: 10804851]

- Kim KS. Mechanisms of microbial traversal of the blood-brain barrier. *Nat Rev Microbiol.* 2008; 6:625–634. [PubMed: 18604221]
- Klutts JS, Doering TL. Cryptococcal xylosyltransferase 1 (Cxt1p) from *Cryptococcus neoformans* plays a direct role in the synthesis of capsule polysaccharides. *J Biol Chem.* 2008; 283:14327–14334. [PubMed: 18347023]
- Kronstad J, Saikia S, Nielson ED, Kretschmer M, Jung W, Hu G, Geddes JM, Griffiths EJ, Choi J, Cadieux B, Caza M, Attarian R. Adaptation of *Cryptococcus neoformans* to mammalian hosts: integrated regulation of metabolism and virulence. *Eukaryot Cell.* 2011a; 11:109–118. [PubMed: 22140231]
- Kronstad JW, Attarian R, Cadieux B, Choi J, D'Souza CA, Griffiths EJ, Geddes JM, Hu G, Jung WH, Kretschmer M, Saikia S, Wang J. Expanding fungal pathogenesis: *Cryptococcus* breaks out of the opportunistic box. *Nat Rev Microbiol.* 2011b; 9:193–203. [PubMed: 21326274]
- Lee A, Toffaletti DL, Tenor J, Soderblom EJ, Thompson JW, Moseley MA, Price M, Perfect JR. Survival defects of *Cryptococcus neoformans* mutants exposed to human cerebrospinal fluid result in attenuated virulence in an experimental model of meningitis. *Infect Immun.* 2010; 78:4213–4225. [PubMed: 20696827]
- Liu OW, Chun CD, Chow ED, Chen C, Madhani HD, Noble SM. Systematic genetic analysis of virulence in the human fungal pathogen *Cryptococcus neoformans*. *Cell.* 2008; 135:174–188. [PubMed: 18854164]
- Liu GY, Nizet V. Color me bad: microbial pigments as virulence factors. *Trends Microbiol.* 2009; 17:406–413. [PubMed: 19726196]
- Lortholary O, Improvisi L, Nicolas M, Provost F, Dupont B, Dromer F. Fungemia during murine cryptococcosis sheds some light on pathophysiology. *Med Mycol.* 1999; 37:169–174. [PubMed: 10421848]
- Luberto C, Toffaletti DL, Wills EA, Tucker SC, Casadevall A, Perfect JR, Hannun YA, Del Poeta M. Roles for inositol-phosphoryl ceramide synthase 1 (*IPC1*) in pathogenesis of *C. neoformans*. *Genes Dev.* 2001; 15:201–212. [PubMed: 11157776]
- Luberto C, Martinez-Mariño B, Taraskiewicz D, Bolaños B, Chitano P, Toffaletti DL, Cox GM, Perfect JR, Hannun YA, Balish E, Del Poeta M. Identification of App1 as a regulator of phagocytosis and virulence of *Cryptococcus neoformans*. *J Clin Invest.* 2003; 112:1080–94. [PubMed: 14523045]
- Ma H, Croudace JE, Lammas DA, May RC. Expulsion of live pathogenic yeast by macrophages. *Curr Biol.* 2006; 16:2156–2160. [PubMed: 17084701]
- Ma H, Croudace JE, Lammas DA, May RC. Direct cell-to-cell spread of a pathogenic yeast. *BMC Immunol.* 2007; 8:15. [PubMed: 17705831]
- Merkel GJ, Scofield BA. The in vitro interaction of *Cryptococcus neoformans* with human lung epithelial cells. *FEMS Immunol Med Microbiol.* 1997; 19:203–213. [PubMed: 9453390]
- Moyrand F, Klaproth B, Himmelreich U, Dromer F, Janbon G. Isolation and characterization of capsule structure mutant strains of *Cryptococcus neoformans*. *Mol Microbiol.* 2002; 45:837–849. [PubMed: 12139628]
- Moyrand F, Chang YC, Himmelreich U, Kwon-Chung KJ, Janbon G. Cas3p belongs to a seven-member family of capsule structure designer proteins. *Eukaryot Cell.* 2004; 3:1513–1524. [PubMed: 15590825]
- Moyrand F, Fontaine T, Janbon G. Systematic capsule gene disruption reveals the central role of galactose metabolism on *Cryptococcus neoformans* virulence. *Mol Microbiol.* 2007; 64:771–781. [PubMed: 17462022]
- Moyrand F, Lafontaine I, Fontaine T, Janbon G. UGE1 and UGE2 regulate the UDP-glucose/UDP-galactose equilibrium in *Cryptococcus neoformans*. *Eukaryot Cell.* 2008; 7:2069–2077. [PubMed: 18820075]
- Mylonakis E, Idnurm A, Moreno R, El Khoury J, Rottman JB, Ausubel FM, Heitman J, Calderwood SB. *Cryptococcus neoformans* Kin1 protein kinase homologue, identified through a *Caenorhabditis elegans* screen, promotes virulence in mammals. *Mol Microbiol.* 2004; 54:407–419. [PubMed: 15469513]

- Narasipura SD, Chaturvedi V, Chaturvedi S. Characterization of *Cryptococcus neoformans* variety *gattii* *SOD2* reveals distinct roles of the two superoxide dismutases in fungal biology and virulence. *Mol Microbiol.* 2005; 55:1782–1800. [PubMed: 15752200]
- Ngamskulrungron P, Himmelreich U, Breger JA, Wilson C, Chayakulkeeree M, Krockenberger MB, Malik R, Daniel HM, Toffaletti D, Djordjevic JT, Mylonakis E, Meyer W, Perfect JR. The trehalose synthesis pathway is an integral part of the virulence composite for *Cryptococcus gattii*. *Infect Immun.* 2009; 77:4584–4596. [PubMed: 19651856]
- Nizet V, Kim KS, Stins M, Jonas M, Chi EY, Nguyen D, Rubens CE. Invasion of brain microvascular endothelial cells by group B streptococci. *Infect Immun.* 1997; 65:5074–5081. [PubMed: 9393798]
- Noverr MC, Cox GM, Perfect JR, Huffnagle GB. Role of *PLB1* in pulmonary inflammation and cryptococcal eicosanoid production. *Infect Immun.* 2003; 71:1538–1547. [PubMed: 12595473]
- Noverr MC, Williamson PR, Fajardo RS, Huffnagle GB. *CNLAC1* is required for extrapulmonary dissemination of *Cryptococcus neoformans* but not pulmonary persistence. *Infect Immun.* 2004; 72:1693–1699. [PubMed: 14977977]
- Novick P, Field C, Schekman R. Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. *Cell.* 1980; 21:205–215. [PubMed: 6996832]
- Okagaki LH, Strain AK, Nielsen JN, Charlier C, Baltes NJ, Chretien F, Heitman J, Dromer F, Nielsen K. Cryptococcal cell morphology affects host cell interactions and pathogenicity. *PLoS Pathog.* 2010; 6:e1000953. [PubMed: 20585559]
- Olszewski MA, Noverr MC, Chen GH, Toews GB, Cox GM, Perfect JR, Huffnagle GB. Urease expression by *Cryptococcus neoformans* promotes microvascular sequestration, thereby enhancing central nervous system invasion. *Am J Pathol.* 2004; 164:1761–1771. [PubMed: 15111322]
- Panepinto JC, Williamson PR. Intersection of fungal fitness and virulence in *Cryptococcus neoformans*. *FEMS Yeast Res.* 2006; 6:489–498. [PubMed: 16696645]
- Panepinto J, Komperda K, Frases S, Park YD, Djordjevic JT, Casadevall A, Williamson PR. Sec6-dependent sorting of fungal extracellular exosomes and laccase of *Cryptococcus neoformans*. *Mol Microbiol.* 2009; 71:1165–1176. [PubMed: 19210702]
- Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. *Aids.* 2009; 23:525–530. [PubMed: 19182676]
- Perfect JR, Lang SD, Durack DT. Chronic cryptococcal meningitis: a new experimental model in rabbits. *Am J Pathol.* 1980; 101:177–194. [PubMed: 7004196]
- Petzold EW, Himmelreich U, Mylonakis E, Rude T, Toffaletti D, Cox GM, Miller JL, Perfect JR. Characterization and regulation of the trehalose synthesis pathway and its importance in the pathogenicity of *Cryptococcus neoformans*. *Infect Immun.* 2006; 74:5877–5887. [PubMed: 16988267]
- Price MS, Betancourt-Quiroz M, Price JL, Toffaletti DL, Vora H, Hu G, Kronstad JW, Perfect JR. *Cryptococcus neoformans* requires a functional glycolytic pathway for disease but not persistence in the host. *MBio.* 2011; 2:e00103–11. [PubMed: 21652778]
- Price MS, Nichols CB, Alspaugh JA. The *Cryptococcus neoformans* Rho-GDP dissociation inhibitor mediates intracellular survival and virulence. *Infect Immun.* 2008; 76:5729–5737. [PubMed: 18779335]
- Rittershaus PC, Kechichian TB, Allegood JC, Merrill AH Jr, Hennig M, Luberto C, Del Poeta M. Glucosylceramide synthase is an essential regulator of pathogenicity of *Cryptococcus neoformans*. *J Clin Invest.* 2006; 116:1651–1659. [PubMed: 16741577]
- Rodrigues ML, Travassos LR, Miranda KR, Franzen AJ, Rozental S, de Souza W, Alviano CS, Barreto-Bergter E. Human antibodies against a purified glucosylceramide from *Cryptococcus neoformans* inhibit cell budding and fungal growth. *Infect Immun.* 2000; 68:7049–7060. [PubMed: 11083830]
- Sahn S. The value of pleural fluid analysis. *Am J Med Sci.* 2007; 335:7–15. [PubMed: 18195577]
- Salas SD, Bennett JE, Kwon-Chung KJ, Perfect JR, Williamson PR. Effect of the laccase gene *CNLAC1*, on virulence of *Cryptococcus neoformans*. *J Exp Med.* 1996; 184:377–386. [PubMed: 8760791]

- Santangelo R, Zoellner H, Sorrell T, Wilson C, Donald C, Djordjevic J, Shounan Y, Wright L. Role of extracellular phospholipases and mononuclear phagocytes in dissemination of cryptococcosis in a murine model. *Infect Immun*. 2004; 72:2229–2239. [PubMed: 15039347]
- Schaible B, Schaffer K, Taylor CT. Hypoxia, innate immunity and infection in the lung. *Respir Physiol Neurobiol*. 2010; 174:235–243. [PubMed: 20709192]
- Shea JM, Del Poeta M. Lipid signaling in pathogenic fungi. *Curr Opin Microbiol*. 2006; 9:352–358. [PubMed: 16798065]
- Shea JM, Kechichian TB, Luberto C, Del Poeta M. The cryptococcal enzyme inositol phosphosphingolipid-phospholipase C confers resistance to the antifungal effects of macrophages and promotes fungal dissemination to the central nervous system. *Infect Immun*. 2006; 74:5977–5988. [PubMed: 16988277]
- Shen G, Wang YL, Whittington A, Li L, Wang P. The RGS protein Crg2 regulates pheromone and cyclic AMP signaling in *Cryptococcus neoformans*. *Eukaryot Cell*. 2008; 7:1540–1548. [PubMed: 18658258]
- Shen G, Whittington A, Song K, Wang P. Pleiotropic function of intersectin homologue Cin1 in *Cryptococcus neoformans*. *Mol Microbiol*. 2010; 76:662–676. [PubMed: 20345666]
- Shen G, Whittington A, Wang P. Wsp1, a GBD/CRIB domain-containing WASP homolog, is required for growth, morphogenesis, and virulence of *Cryptococcus neoformans*. *Eukaryot Cell*. 2011; 10:521–529. [PubMed: 21357479]
- Shi M, Li SS, Zheng C, Jones GJ, Kim KS, Zhou H, Kubes P, Mody CH. Real-time imaging of trapping and urease-dependent transmigration of *Cryptococcus neoformans* in mouse brain. *J Clin Invest*. 2010; 120:1683–1693. [PubMed: 20424328]
- Stie J, Bruni G, Fox D. Surface-associated plasminogen binding of *Cryptococcus neoformans* promotes extracellular matrix invasion. *PLoS One*. 2009; 4:e5780. [PubMed: 19492051]
- Stie JT, Fox DS. Blood-brain barrier invasion by *Cryptococcus neoformans* is enhanced by functional interactions with plasmin. *Microbiol*. 2011 in press.
- Thompson EJ, Keir G. Laboratory investigation of cerebrospinal fluid protein. *Ann Clin Biochem*. 1990; 27:425–435. [PubMed: 1704202]
- Vu K, Weksler B, Romero I, Couraud PO, Gelli A. Immortalized human brain endothelial cell line HCMEC/D3 as a model of the blood-brain barrier facilitates in vitro studies of central nervous system infection by *Cryptococcus neoformans*. *Eukaryot Cell*. 2009; 8:1803–1807. [PubMed: 19767445]
- Waterman SR, Hacham M, Hu G, Zhu X, Park YD, Shin S, Panepinto J, Valyi-Nagy T, Beam C, Husain S, Singh N, Williamson PR. Role of a *CUF1/CTR4* copper regulatory axis in the virulence of *Cryptococcus neoformans*. *J Clin Invest*. 2007a; 117:794–802. [PubMed: 17290306]
- Waterman SR, Hacham M, Panepinto J, Hu G, Shin S, Williamson PR. Cell wall targeting of laccase of *Cryptococcus neoformans* during infection of mice. *Infect Immun*. 2007b; 75:714–722. [PubMed: 17101662]
- Waugh MS, Nichols CB, DeCesare CM, Cox GM, Heitman J, Alsbaugh JA. Ras1 and Ras2 contribute shared and unique roles in physiology and virulence of *Cryptococcus neoformans*. *Microbiology*. 2002; 148:191–201. [PubMed: 11782511]
- Waugh, A.; Allison, G. *Anatomy and Physiology in Health and Illness*. Churchill Livingstone Elsevier; 2007.
- Whittington A, Wang P. The RGS protein Crg2 is required for establishment and progression of murine pulmonary cryptococcosis. *Med Mycol*. 2011; 49:263–275. [PubMed: 20818923]
- Wilder JA, Olson GK, Chang YC, Kwon-Chung KJ, Lipscomb MF. Complementation of a capsule deficient *Cryptococcus neoformans* with *CAP64* restores virulence in a murine lung infection. *Am J Respir Cell Mol Biol*. 2002; 26:306–314. [PubMed: 11867339]
- Wormley FL Jr, Heinrich G, Miller JL, Perfect JR, Cox GM. Identification and characterization of an *SKN7* homologue in *Cryptococcus neoformans*. *Infect Immun*. 2005; 73:5022–5030. [PubMed: 16041017]
- Xue C, Hsueh YP, Chen L, Heitman J. The RGS protein Crg2 regulates both pheromone and cAMP signalling in *Cryptococcus neoformans*. *Mol Microbiol*. 2008; 70:379–395. [PubMed: 18761692]

- Yang J, Li D, Liu X, Pan J, Yan B, Zhu X. Regulation of virulence factors, carbon utilization and virulence by *SNF1* in *Cryptococcus neoformans* JEC21 and divergent actions of *SNF1* between cryptococcal strains. *Fungal Genet Biol.* 2010; 47:994–1000. [PubMed: 20719250]
- Zaragoza O, Rodrigues ML, De Jesus M, Frases S, Dadachova E, Casadevall A. The capsule of the fungal pathogen *Cryptococcus neoformans*. *Adv Appl Microbiol.* 2009; 68:133–216. [PubMed: 19426855]
- Zaragoza O, Garcia-Rodas R, Nosanchuk JD, Cuenca-Estrella M, Rodriguez-Tudela JL, Casadevall A. Fungal cell gigantism during mammalian infection. *PLoS Pathog.* 2010; 6:e1000945. [PubMed: 20585557]
- Zhu X, Williamson PR. Role of laccase in the biology and virulence of *Cryptococcus neoformans*. *FEMS Yeast Res.* 2004; 5:1–10. [PubMed: 15381117]
- Zink S, Nass T, Rosen P, Ernst JF. Migration of the fungal pathogen *Candida albicans* across endothelial monolayers. *Infect Immun.* 1996; 64:5085–5091. [PubMed: 8945550]

C. neoformans causes life-threatening meningoencephalitis in people with HIV/AIDS.

Dissemination of the fungus from the lung to the brain is poorly understood.

A variety of diverse functions influence virulence and the dissemination process.

Animal models and in vitro assays are critical for evaluating dissemination factors.

Cell wall melanin and specific enzymes are key factors in egress from the lung.

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Figure 1.

Dissemination of *Cryptococcus* across the lung and brain barriers. Cryptococcal cells infiltrate alveoli, passing through the interstitial space and capillary endothelial cells into the blood either as free cells or within alveolar macrophages. Cryptococcal cells traverse the blood-brain barrier to infect the central nervous system within macrophages by a Trojan horse mechanism, transcellularly by crossing through cells or by paracellular traversal between endothelial cells.

Figure 2.

Interconnections between functions that influence virulence and/or dissemination in *C. neoformans*. The diversity of factors is shown to illustrate the involvement lipid and sugar metabolism, and trafficking and signaling components, as well as influences on the elaboration of virulence-related functions. For example, glucose is used to produce trehalose as well as acetyl-CoA, an important intermediate of lipid production. Lipid metabolism supports the formation of diacyl glycerol (DAG) which both affects the transcription of the antiphagocytic protein gene *APP1* (Luberto *et al.*, 2003), as well as a cascade of signaling molecules leading to the expression of proteins required for high temperature growth, cell wall integrity and melanin production, as well as other functions. The details for each function are provided in the text and in Table 1.

Table 1

Mutants of *Cryptococcus neoformans* with altered dissemination to and/or persistence in the brain.

A) Mutants with a defect in melanin production.							
Functional category	Gene	Function	Gene number	Growth at 37°C	Infection model	Macrophage interaction	Reference
Signaling/transcription factors	<i>SRE1</i>	Regulator of oxygen sensing and sterol homeostasis	CNAG_04804	Yes	Inhalation and IV		Chun <i>et al.</i> , 2007 Chang <i>et al.</i> , 2007
	<i>CKB1</i>	Casein kinase II beta chain	CNAG_03590	Slightly reduced	CSF <i>in vitro</i>		Lee <i>et al.</i> , 2010
	<i>CUF1</i>	Copper-related transcription factor	CNAG_07724	Yes	Inhalation and IV		Waterman <i>et al.</i> , 2007a; Jiang <i>et al.</i> , 2009
	<i>SNF1</i>	Protein kinase for alternative carbon source utilization	CNAG_01612	No/39°C	Inhalation		Hu <i>et al.</i> , 2008a; Yang <i>et al.</i> , 2010
Protein sorting/degradation	<i>RUB1</i>	Ribosomal chaperone	CNAG_02827	Yes	Intrathecal rabbit	Reduced survival	Lee <i>et al.</i> , 2010
	<i>CSN1201</i>	Transcription- associated recombination protein	CNAG_01697	Yes	CSF <i>in vitro</i>		Lee <i>et al.</i> , 2010
	<i>VAM6</i>	RabGEF guanyl- nucleotide exchange factor activity	CNAG_05395	Yes	CSF <i>in vitro</i>		Lee <i>et al.</i> , 2010
	<i>VPS34</i>	Class III PI3K	CNAG_02953	Yes	Inhalation and IV	Reduced survival	Hu <i>et al.</i> , 2008b
Virulence	<i>LAC1</i>	Laccase for melanin production	CNAG_03465	Yes	IV		Noverr <i>et al.</i> , 2004; Salas <i>et al.</i> , 1996
Stress	<i>IPC1</i> #	Inositol- phosphoryl ceramide synthase 1	CNAG_04453	Yes	Intrathecal rabbit	Reduced growth	Luberto <i>et al.</i> , 2001
	<i>SEC6</i>	Exocytosis	CNAG_01911	Yes	IV		Panepinto <i>et al.</i> , 2009

B) Mutants with defects in capsule production.							
Functional category	Gene	Function	Gene number	Growth at 37°C	Infection model	Macrophage interaction	Reference
Metabolism	<i>MFE2</i>	Peroxisomal bifunctional β -oxidation protein	CNAG_05721	Yes	Inhalation		M.K. and J.W.K. (unpublished data)
Protein sorting/degradation	<i>VPS25</i>	ESCRT-II complex subunit	CNAG_04863	Yes	CSF <i>in vitro</i>		Lee <i>et al.</i> , 2010
Virulence	<i>USX1</i> #	UDP-xylose synthase	CNAG_03322	No data	Inhalation		Moyrand <i>et al.</i> , 2002
	<i>CAP59</i>	GMX export	CNAG_00721	Yes	IV		Chang and Kwon-Chung, 1994; García-Rivera <i>et al.</i> , 2004; Chun and Madhani, 2010
	<i>CAP6</i> #	Capsule synthesis	CNAG02885	Yes	IV		Chang <i>et al.</i> , 1996; Wilder <i>et al.</i> , 2002; Chun and Madhani, 2010

B) Mutants with defects in capsule production.						
Functional category	Gene	Function	Gene number	Growth at 37°C	Infection model	Macrophage interaction
	<i>CAP10#</i>	Capsule synthesis	CNAG_07554	Yes	IV	
	<i>CAP6#</i>	Mannosyl transferase	CNAG_00600	Yes	IV	
						Chun and Madhani, 2010; Chang and Kwon-Chung, 1999; Chang and Kwon-Chung, 1998; Chun and Madhani, 2010

C) Mutants impaired in capsule and melanin production.						
Functional category	Gene	Function	Gene number	Growth at 37°C	Infection model	Macrophage interaction
Metabolism	<i>ACLI</i>	ATP-citrate lyase	CNAG_04640	Yes	Inhalation	
Cell wall	<i>CPS1#</i>	Hyaluronic acid synthase	CNAG_04320	Reduced	IV	
Signaling/transcription factors	<i>PKA1</i>	Catalytic subunit of PKA	CNAG_00396	Yes	IV	Compromised
	<i>WSP1</i>	CRIB/GBD domain, Wiskott-Aldrich syndrome (WAS) protein (WASP)	CNAG_07718	No	IV	Higher uptake
Secreted virulence factors	<i>PLC1</i>	Phosphatidyl- inositol-specific phospholipase C for release of PLB1	CNAG_02867	No	Inhalation	
						Chayakulkeeree et al., 2008

D) Mutants with intact capsule and melanin formation.						
Functional category	Gene	Function	Gene number	Growth at 37°C	Infection model	Macrophage interaction
Transporter	<i>ENA1</i>	ENA P-type ATPase1 involved in cation transport	CNAG_00531	Yes	Intrathecal rabbit	82% less survival after 48h
	<i>PAX2</i>	Adrenoleuko- dystrophy protein; ABC transporter	CNAG_02764	Yes	CSF <i>in vitro</i>	
	<i>CFT1</i>	High-affinity iron permease	CNAG_06242	Yes	Inhalation	
Metabolism	<i>GSC1</i>	Glucosyl- ceramide synthase	CNAG_05583	Yes	Inhalation	Like wild type
	<i>TPS1</i>	trehalose-6- phosphate (T6P) synthase	CNAG_05292	No	Intrathecal rabbit and mouse inhalation	Like wild type
Signaling/transcription factors	<i>PIK1</i>	1-Phosphatidyl- inositol 4-kinase	CNAG_07744	Yes	Intrathecal rabbit	89% less survival after 48h
	<i>SWE1</i>	MYT1 kinase involved in regulation of cell cycle	CNAG_03171	yes	CSF <i>in vitro</i>	
						Lee et al., 2010

D) Mutants with intact capsule and melanin formation.							
Functional category	Gene	Function	Gene number	Growth at 37°C	Infection model	Macrophage interaction	Reference
	<i>KIN1</i>	Serine/threonine protein kinase	CNAG_01938	Yes	Inhalation and IV	Increased binding	Mylonakis <i>et al.</i> , 2004
	<i>RDH1</i>	Rho-GDP dissociation inhibitors	CNAG_03316	Yes	Inhalation and IV	87% reduced survival after 24h	Price <i>et al.</i> , 2008
	<i>SKN7</i>	Two-component stress response regulator	CNAG_03409	Yes	IV	Like wild type	Wormley <i>et al.</i> , 2005
	<i>PKR1</i>	Regulatory subunit of PKA	CNAG_00570	Yes	IV		D'Souza <i>et al.</i> , 2001
	<i>RAS1</i>	Small guanine nucleotide- binding protein	CNAG_01672	No	IV rabbit		Alsbaugh <i>et al.</i> , 2000; Waugh <i>et al.</i> , 2002
	<i>CDC42</i>	Rho-like GTPase	CNAG_05348	No	Inhalation		Ballou <i>et al.</i> , 2010
Protein sorting/degradation	<i>HRD1</i>	Synoviolin like Ubiquitin ligase activity for ER associated protein degradation	CNAG_05469	Yes	CSF <i>in vitro</i>		Lee <i>et al.</i> , 2010
Virulence	<i>UGE1</i>	UDP-glucose epimerase	CNAG_00697	No	IV		Moyrand <i>et al.</i> , 2008
	<i>UGT1</i>	Golgi UDP transporter	CNAG_05139	No	IV		Moyrand <i>et al.</i> , 2007
	<i>CXT1</i> #	Xylosyl- transferase	CNAG_05148	Yes	Inhalation		Kluttis and Doering, 2008
	<i>ISC1</i> #	Phosphosphingo- lipid- phospholipase C	CNAG_06085	No data	Mouse inhalation and rabbit IV	Decreased	Shea <i>et al.</i> , 2006
Secreted virulence factors	<i>PLB1</i>	Phospholipase B	CNAG_06085	Yes	Mouse inhalation and rabbit IV	Decreased	Cox <i>et al.</i> , 2001
	<i>SEC14</i>	Phosphatidyl- inositol transfer protein	CNAG_03153	Yes	Inhalation	Decreased survival	Chayakulkeeree <i>et al.</i> , 2011
	<i>URE1</i>	Urease involved in hydrolysis of urea to ammonia and carbamate	CNAG_05540	Yes	Inhalation and intrathecal	Decreased	Cox <i>et al.</i> , 2000
	<i>APP1</i>	Antiphagocytic protein	CNAG_06574	Yes	Inhalation	Increased uptake	Luberto <i>et al.</i> , 2003
Stress	<i>SOD2</i>	Mitochondrial superoxide dismutase	CNAG_04388	No	Inhalation		Giles <i>et al.</i> , 2005
	<i>PYK1</i>	Pyruvate kinase	CNAG_01820	Yes	Mouse inhalation and rabbit IV	Reduced survival	Price <i>et al.</i> , 2011
	<i>CRG2</i>	Regulators of G protein signalling	CNAG_01371	Yes	Inhalation	Like wild type	Shen <i>et al.</i> , 2008 Xue <i>et al.</i> , 2008 Whittington and Wang, 2011

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Information about capsule, melanin formation, macrophage interaction or growth at 37°C is not available for every mutants. If information for capsule or melanin production was not available, it was considered to be like wild type. If no macrophage interaction is indicated, the data were not available.