Non-Capsular Virulence Factors of *Cryptococcus neoformans*

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Abstract

Cryptococcus neoformans is an emerging pathogen that kills hundreds of thousands every year, especially in underdeveloped areas with little access to modern medical care. New treatments for the disease are needed to shorten treatment and decrease the side effects and costs associated with the drugs currently in use. Many C. neoformans genes have been identified that are necessary for full virulence in the host. Cir1, a regulatory protein associated with iron regulation, and Zip1, a surface zinc transporter, are both necessary for full virulence in the host. Anti-fungals targeted at these proteins or the proteins produced by other genes discussed in this paper could help treat this AIDS-defining illness more effectively.

Non-Capsular Virulence Factors of *Cryptococcus neoformans*

Introduction

Cryptococcus neoformans is a fungal pathogen that mainly infects immunocompromised individuals and is an AIDS defining illness. The death rates for patients who complete the full course of treatment are extremely low, however, if the full course of treatment cannot be carried out the prognosis is poor. A very high percentage of *C. neoformans* infections in immunocompromised individuals in sub-Saharan Africa result in death, even when treatment with fluconazole is performed (Mwaba et al., 2001; Oumar, Dao, Ba, Poudiougou, & Diallo, 2008). This disease is the fourth most deadly in the sub-Saharan region, where it kills more than 500,000 people per year (Park et al., 2009). Alternative methods for the reduction of both the virulence of the pathogen and the length of the course of treatment are needed to improve the survival rate of those without easy access to medical care.

Cryptococcosis has spread concurrently with HIV in Africa and India, and has become a much higher priority illness to treat in recent years. A *C. neoformans* infection requires a much longer treatment course than that required by other prevalent African diseases, such as malaria or other parasitic infections. Current treatment indicates an 8-week course of amphotericin B and flucytosine, followed by daily fluconazole for a year. This treatment plan is very effective and has a high success rate. However, for those without easy access to medical services, a yearlong course of fluconazole may well be out of reach, contributing to the high rate of mortalities to the disease observed in sub-Saharan Africa. The need for regularly administered antifungals as well as the toxicity of those medicines make completing a full course of treatment very difficult. Amphotericin

B in particular is extremely toxic and can cause blurred vision and weakness. Flucytosine also has relatively severe side effects, ranging from diarrhea and jaundice to headaches and hallucinations. An ongoing shortage of flucytosine also contributes to the difficulties associated with obtaining appropriate treatment in developing countries.

The side effects from these drugs are often severe, and further increase the economic barriers preventing patients from being able to complete a full course of treatment. The Amphotericin B necessary at the start of treatment alone costs more than the average annual income of a resident of sub-saharan Africa. When combined with the necessary length of treatment and the need for maintenance anti-fungals for extended periods following initial treatment, it is no surprise that mortality rates are elevated in this region. New treatment options are badly needed; especially drugs that can treat *Cryptococcus* infection more rapidly or completely eliminate it from the patient, preventing the need for maintenance fluconazole.

C. neoformans is an encapsulated yeast from the phylum Basidiomycota, which also includes the mushrooms, rusts, and smuts. *Cryptococcus* is also the only known genera of fungi to have a polysaccharide capsule. It tends to proliferate in the cerebrospinal fluid, among other locations, metabolizing the glucose in the CSF as its main carbon source. This proliferation can lead to meningitis (swelling of the meninges) and encephalitis (inflammation of the brain) due to immune response to the pathogen. Both conditions are serious and potentially life threatening. The organism can go dormant for extended periods of time, in some cases for ten years or more, only reappearing when the host becomes immunocompromised (Garcia-Hermoso, Janbon, & Dromer, 1999). *C. neoformans* also produces melanin, a darkening pigment required for virulence, in much

the same manner that the human body does. The production of melanin in the capsule

helps protect the yeast from attacks by host immune cells. Once in the body, the yeast

must also be able to grow at 37ºC in order to thrive in the host. Many of the mutant forms

of *C. neoformans* exhibit temperature sensitivity at 37°C, attenuating their virulence or

rendering them completely harmless.

There are several strain variants with different capsule structures with at least one

substituting xylose for the mannose backbone in the matrix. Two main serotypes of

Cryptococcus have been characterized in the literature, as well as an interstrain hybrid

variant (Enache-Angoulvant et al., 2007; Hagen et al., 2015). *C. neoformans* var.

neoformans is comprised of serotype D, whereas *C. neoformans* var. *grubii* is also known

as serotype A, while *C. gatii* contain both serotypes B and C. *C. gattii* was formerly

considered a subspecies of *C. neoformans* (Hagen et al., 2015). The H99 clinical strain of

C. neoformans is serotype A, while the JEC21 laboratory strain is a serotype D

strain(Loftus et al., 2005). A serotype AD strain has also been identified and is the most

widespread of the hybrid serotypes. More serotypes are presumed to exist, but have not

yet been characterized (Banerjee, Datta, & Casadevall, 2004).

Pathology

Initial infection by *C. neoformans* is alveolar, as the pathogen in its virulent form

is airborne. Basidiospores, or sexually-produced reproductive spores, are believed to be

the initial source of the infection; the vegetative cells are too large to enter the lung

parenchyma. After entrance to the lung, the now-active cells are absorbed by the alveolar

macrophages present in the lung tissue. However, phagocytosis by immune cells does not

kill the yeast; it is able to survive inside of the macrophage due to its unique capsule,

where it is shielded from interaction with the immune system. Inside the macrophage, *C. neoformans* can grow and reproduce, creating a phagosome filled with new cells inside of the macrophage. After reproducing and combining with other phagosomes, *C. neoformans* is capable of triggering its own release from the macrophage (Alvarez & Casadevall, 2006; H. Ma, Croudace, Lammas, & May, 2006). The phagosome is extruded by a process known as vomocytosis, in which the intracellular actin used to hold the phagosome in place is degraded (Alvarez & Casadevall, 2006; Nicola, Robertson, Albuquerque, Derengowski Lda, & Casadevall, 2011). This process is similar to that observed in *Mycobacterium tuberculosis*, which also use the privileged environment of a macrophage to survive intracellularly for extended periods of time (Pieters & Gatfield, 2002). Non-lytic expulsion of the new yeast cells means that less inflammation occurs, reducing the local immune response, and enabling the infection to progress.

Many genes in *C. neoformans* exhibit large increases in expression when engulfed by a macrophage. These genes encode proteins necessary for DNA repair, free radical elimination, and anti-nitrosative processes, although the yeast is less able to deal with nitrogen radicals than reactive oxygen species (Alspaugh & Granger, 1991). The cell wall of *C. neoformans* contains molecules which trigger the M2 activation of macrophages, which are less effective against the yeast than M1-activated macrophages (Martinez, Helming, & Gordon, 2009). This unequal effectiveness of response is one mechanism by which *C. neoformans* increases its rate of survival in macrophages. M1 macrophage activation requires release of inflammatory cytokines, which the intracellular reproduction method of *C. neoformans* reduces. M1 activated macrophages can

successfully phagocytose and digest *C. neoformans* cells due to the reactive nitrogen species produced by M1 activation (Alspaugh & Granger, 1991).

In case of a successful M1 macrophage activation, granulomas will be formed around the *Cryptococcus* cells in the lung parenchyma and degrading enzymes are released (Majka, Kasimos, Izzo, & Izzo, 2002). This reaction is heavily dependent on the immunocompetency and genetics of the host, and is similar to the body's response to infection with tuberculosis (Qualls & Murray, 2016). If macrophages are depleted, then not enough will be recruited to encapsulate the *Cryptococcus* infection and it will persist (Huffnagle, Yates, & Lipscomb, 1991). Natural killer cells can also bind directly to the *C. neoformans* cells and eliminate them by use of perforin (Levitz, Dupont, & Smail, 1994; L. L. Ma et al., 2004). T-helper cells exhibit a fungistatic effect against *C. neoformans* cells (Levitz et al., 1994). The lungs of AIDS patients are lacking in T-cells and macrophages, and therefore afford little protection against cryptococcal sepsis (Shibuya et al., 2005).

After invasion of the lungs and bloodstream, *C. neoformans* must cross the blood-brain barrier (BBB). The exact mechanisms used by *C. neoformans* to cross this barrier are still being investigated, but the yeast must pass through the endothelial lining of the capillaries, the tight junctions surrounding those capillaries, and the astrocytes that protect the neural tissue (Tseng et al., 2015). Monocytes have been shown to be a necessary for successful crossing of the BBB by *C. neoformans* cells, indicating a trojan horse effect (Charlier et al., 2009). Other modes of crossing have also been observed, however, including a transcellular mechanism that does not require monocytes (Y. C. Chang et al., 2004).

Once the BBB has been crossed, the yeast is free to proliferate in the harsh but isolated environment of the cerebrospinal fluid (CSF), as well as brain tissue. This ability to thrive in the CSF is not common to all infectious yeasts (Lee et al., 2010). Multiple abscesses are formed at the meninges and throughout the brain parenchyma, causing fever and headache (Bicanic & Harrison, 2004; Y. C. Chang et al., 2004). Intracranial pressure is also frequently increased during cryptococcosis, presumably due to physical blockage of the arachnoid villi with yeast cells (Bicanic & Harrison, 2004). The mechanism by which *C. neoformans* is able to survive in the CSF is currently unknown, although several mutations that affect survival in the CSF have been identified (Lee et al., 2010).

C. neoformans' response to resistance by the host immune system is complex. Sialic acid is incorporated into the cell wall and released from the cell. This chemical has been shown to have a protective effect against phagocytosis by macrophages in the host (Rodrigues et al., 1997). Sialic acid also helps to repel other cells by making the cell wall of *C. neoformans* more negative. However, the capsule of *C. neoformans* is the most effective defense against the host immune system. The capsule is a polysaccharide with a mannose backbone. Glucuronic acid and xylose are both incorporated into the matrix of the capsule as well (Doering, 2009).

The extracellular polysaccharide matrix has several unique functions. When IgG antibodies bind to the *C. neoformans* cell, they frequently disappear beneath the surface of the capsule, preventing them from aiding the macrophages in detection and phagocytosis. Free fragments of the GXM capsule (glucuronic acid, xylose, and mannose) have been shown to severely inhibit the effectiveness of the host immune

response by essentially appearing as false positives all over the body, inhibiting the response to actual sites of infection. Coat proteins on the capsule also reduce the host response to the infection by reducing the number of co-stimulatory molecules produced (Martinez et al., 2009).

The carbon cycle in *C. neoformans* is heavily focused on the use of glucose, much like other eukaryotes. The Warburg effect suggests that *C. neoformans* tends to use glycolysis, followed by lactic acid fermentation, to produce energy; this means that demand for glucose will be extremely high for *C. neoformans* to successfully invade and proliferate in a host (Vander Heiden, Cantley, & Thompson, 2009). This process is not ordinarily energetically favorable, as the electron transport system can generate far more ATP per glucose than aerobic glycolysis does. However, proliferating cells also need a large variety of organic molecules that are energetically expensive to make from smaller carbon molecules. By halting catabolism after production of pyruvate, the cell can directly use the carbon from fermentation products at a lower energetic cost than would be incurred by using the ATP produced by the citric acid cycle to construct entirely new molecules, such as amino acids and NADPH (Vander Heiden et al., 2009). When this central carbon utilization pathway is disrupted, severe attenuation of virulence results.

Numerous methods are being investigated to reduce the required length of treatment to a manageable level for those without easy access to the required drugs for extended treatment periods. This research is made more necessary by the difficulty in obtaining flucytosine. This shortfall increases the need for alternative modes of treatment for Cryptococcosis that can be delivered to areas where conventional medical care may

be more difficult to obtain. A better understanding of the carbon usage of *C. neoformans* could lead to new ways to inhibit its growth and aid recovery.

Regulation of *C. neoformans* virulence

Much of the genome of any species of any organism is made up of proteins that solely affect the transcription of other genes. *C. neoformans* has many transcription factors which are necessary for virulence, or at least for full virulence. These intracellular targets are somewhat more difficult to access than a membrane-bound protein, but their effects are widespread and profound.

GAT201 is a key regulator of virulence which has been shown to impart resistance to macrophage function against *C. neoformans* based on factors other than capsule size. This gene regulates over 1000 other genes, or roughly one sixth of the genome of *C. neoformans*. *GAT201* has also been shown to be active under conditions in which macrophages would be present (Chun, Brown, & Madhani, 2011).

Overexpression of *GAT201* has been shown to dramatically increase virulence in the murine inhalation model (Liu et al., 2008). Overexpression mutants show increased melanin production, especially on L-DOPA media, and can induce capsule formation under conditions not normally associated with encapsulation (Liu et al., 2008). Conversely, *gat201Δ* strains have shown drastically increased rates of phagocytosis by bone macrophages, with approximately a five-fold increase in association between macrophages and *C. neoformans* cells, as compared to strains with gross capsule defects (Liu et al., 2008).

The majority of the effect on virulence of *GAT201* seems to be caused by its regulatory effects on two other genes: *GAT204*, a transcriptional regulator, and *BLP1*, which encodes a signal peptide (Chun et al., 2011). *gat204Δblp1Δ* double-knockouts show very high levels of macrophage association when co-cultured, approaching a significant percentage of that of the *gat201*Δ strain (Chun et al., 2011). However, knockouts of these two regulated genes show no effect on capsule size, indicating the source of protection from macrophages is mediated by other factors.

Blp1 was identified as a signal peptide which controls functions that have not yet been elucidated; although, the presence of a Barwin-like domain indicates that it could bind to polysaccharides (Svensson et al., 1992). There is some evidence that either *GAT204* is a repressor of *BLP1*, or that *BLP1* transcription is increased when *GAT204* is not present, as in a *gat204Δ* knockout. *blp1*Δ strains show only slightly lower resistance to phagocytosis compared to wild type *C. neoforman*s. Its effect on virulence appears to be largely synergistic with, and dependent on, the presence of a defective *GAT204* gene.

Gat201 regulates two pathways in *C. neoformans*: the Gat204/Blp1 pathway and an uncharacterized pathway that is not controlled by *GAT204* or *BLP1* (Chun et al., 2011). This uncharacterized pathway also protects the cell from phagocytosis by alveolar macrophages beyond the effect conferred by the Gat204/Blp1 pathway alone. *C. neoformans'* survival in the lung is severely impaired by the loss of *GAT201*, and its infectivity is therefore greatly reduced as well. Further research is needed on the exact functions of both Blp1 and Gat204 as it is currently unknown what the specific effects of these two proteins are; although, the general functions have been inferred by homology (Chun et al., 2011).

SRE1 is a highly conserved gene among eukaryotes which regulates response to low oxygen conditions (Hughes, Todd, & Espenshade, 2005). It was identified in *C. neoformans* by homology to a similar gene in *Schizosaccharomyces pombe.* This gene encodes a protein known as sterol regulatory element binding protein, which is believed to be a membrane-bound transcription factor similar to mammalian *SREBP* (Bien, 2010). The *SRE1* gene appears to influence transcription of about 100 genes in *C. neoformans* when under hypoxic ($<1\%$ O_2) conditions (Yun C. Chang, Bien, Lee, Espenshade, & Kwon-Chung, 2007).

As the mammalian brain is a relatively low oxygen environment, *C. neoformans* must transition from its normal biosynthetic pathways to ones better suited to low oxygen levels (Erecinska & Silver, 2001). *SRE1* and *SREBP* are believed to be essential in oxygen sensing in *C. neoformans* and are key regulators of virulence in both the inhalation and tail vein injection murine models (Bien, 2010).

Ergosterol is a membrane associated lipid with properties similar to cholesterol which is common among fungi and some protists. It is believed to serve a similar function to cholesterol in membranes: regulating membrane fluidity. Because mammals do not produce this lipid and it is localized at the cell membrane, ergosterol makes an excellent target for antifungal drugs. Amphotericin B and the azole drugs target this molecule. *SRE1* is heavily involved in regulating synthesis of ergosterol at several key points in the biosynthetic process (Yun C. Chang et al., 2007). Ergosterol synthesis requires large amount of oxygen to synthesize, so its production is downregulated under hypoxic or anoxic conditions in many organisms (Hughes et al., 2005). Unlike many

other non-capsular virulence regulating genes, *SRE1* has no detectable effect on survival in alveolar macrophages (Yun C. Chang et al., 2007).

Iron and copper metabolism is also regulated by *SRE1*, with at least 6 different iron or copper uptake proteins differentially upregulated under the effects of a functional gene (Yun C. Chang et al., 2007). *sre1Δ* mutant strains showed greatly reduced growth on limited iron medium, with the generational time increasing roughly four-fold (Yun C. Chang et al., 2007). Restoration of the gene by complement at least partially restored growth on low-iron media.

The transcription factor *MIG1* is involved in regulation of the HAP complex, transitioning the transcriptome of S. *cerevisiae* away from the use of alternative carbon sources in the presence of glucose (Nehlin, Carlberg, & Ronne, 1991). A homologue of *MIG1* in *C. neoformans* shows similar traits, reducing the transcription of genes necessary for galactose, raffinose, and maltose metabolism (Caza, Hu, Price, Perfect, & Kronstad, 2016). Loss of MIG1 has a variety of effects, including repression of both *HAPX* and *CIG1*, a putative heme-binding protein (Caza et al., 2016). The loss of *CIG1* has been linked to a decrease in virulence (Cadieux et al., 2013). This decrease in virulence is presumably caused by the loss of iron sourced from hemoglobin. MIG1 is also linked with repression of genes necessary for the TCA cycle, including the genes responsible for complexes I and III of the electron transport chain (Caza et al., 2016). MIG1 is also required for resistance to reactive oxygen species, especially at 37*C (Caza et al., 2016).

Deletion of MIG1 causes increased susceptibility to fluconazole, to tetracycline, and to both anti-fungals combined (Caza et al., 2016). The *mig1Δ*-induced sensitivity to

the combination of fluconazole and tetracycline is especially pronounced. Deletion of MIG1 has the opposite effect on sensitivity to caffeine and rapamycin. Survival on media with these compounds was enhanced in *mig1*Δ mutants compared to wild type H99 *C. neoformans* (Caza et al., 2016). Testing with high-salt media also showed reduced growth for the mig1Δ strain compared to wild type.

Despite the sensitivities induced by deletion of MIG1, the mig1Δ mutant strain did not show significantly reduced survival in murine macrophages (Caza et al., 2016). However, deletion of HAPX in addition to MIG1 significantly reduced the rate of survival in macrophages. Mig1Δ mutants were also able to establish an infection in the murine inhalation model at a level similar to that of wild type H99 *C. neoformans*. The mutant strain also showed much higher levels of tissue fungal burden in the blood, brain, spleen, and liver at time of death compared to wild type. Deletion of HAPX in addition to MIG1 significantly reduced infectivity, and reduced fungal burden in the previously mentioned tissues to roughly the same level observed in mice infected with wild type H99.

Mig1 seems to regulate sensitivity to a broad range of compounds. Deletion of MIG1 causes an increase in sensitivity to fluconazole and tetracycline, but a decrease in sensitivity to rapamycin. Rapamycin is not a viable candidate for anti-fungal treatment in humans, as it is an immunosuppressant (Abraham & Wiederrecht, 1996). Those suffering from a *C. neoformans* infection are already immunosuppressed, and rapamycin would only cause further problems. The increased sensitivity to tetracycline and fluconazole makes this gene an appealing target for further research on potential anti-fungals, as these drugs are already widely used. Increasing their effectiveness could only be beneficial.

Iron metabolism is a key pathway for invading pathogens. Extracellular iron levels are normally maintained at a very low level in the human body to reduce its availability to pathogens (Jung & Kronstad, 2008). CIR1 is an iron sensing protein in *C. neoformans* which regulates many other genes related to iron metabolism (Jung, Sham, White, & Kronstad, 2006). Interestingly, the effects exerted by CIR1 do not appear to be dependent on regulation of its expression due to changing iron concentrations (Jung & Kronstad, 2011b). Intracellular iron has been found to stabilize the Cir1 regulatory protein, which can then bind to its target genes and reduce transcription. CIR1 has also been linked to changes in mating and pheromone production, to capsule and melanin production, to cell wall proteome, and to other metal ion homeostasis pathways (Jung & Kronstad, 2011a, 2011b). Deletion of CIR1 also reduces ergosterol synthesis by reducing expression of SRE1 (Jung et al., 2006).

Deletion of CIR1 reduces virulence drastically, with nearly all major virulence markers missing (Jung et al., 2006). The cir1Δ mutant strain is more sensitive to antibiotics and was unable to establish an infection in the murine inhalation model. CIR1 should make an excellent target for potential anti-fungals, as it appears to have homologs in many other yeasts (Jung et al., 2006). Its effects on cellular metabolism and homeostasis are extremely pronounced, and even small reductions in the efficacy of its regulatory function could shorten treatment and improve clearance of a cryptococcal infection.

SNF1 increases the ability of *C. neoformans* to grow on non-glucose media, by activating numerous glucose repressed genes, and is a key regulator in reversing the glucose effect when glucose levels are low. The presence of Snf1 is necessary for growth

on galactose, sucrose, and maltose media in JEC21, although it is not essential for growth

of H99 on those media. This represents a divergence in carbon usage between the H99

and other yeasts (Celenza & Carlson, 1984, 1989). The function of wild-type *SNF1* is to

reverse the repression of glucose-repressible enzymes and allow the cell to switch to a

secondary carbon source. This is achieved by increasing production of invertase, which

splits sucrose into glucose and fructose (Neumann & Lampen, 1967). Removal of this

gene by deletion made the JEC21 strain largely unable to grow on alternative carbon

sources.

Disruption of the gene *SNF1*, a key regulator of cryptococcal carbon usage,

results in defects in carbon usage, melanin production, and capsule formation (Yang et

al., 2010). Stress response regulation is also a function of this gene. *C. neoformans* strain

JEC21 *snf1Δ* mutants showed reduced growth on glycerol, galactose, and especially

sucrose media, indicating that the gene is responsible for the same functions in *C.*

neoformans as it is in *S. cerevisiae*. Reconstituted JEC21 showed restoration of normal

growth on those media. *SNF1* has also been linked to regulation of growth at body

temperature. *snf1Δ* mutants are far less robust at 37°C compared to wild-type JEC21.

snf1Δ mutants from this strain also showed reduced ability to grow in media with high

cation concentration, as well as reduced overall virulence.

Given the wide variety of stress responses in which *SNF1* is a vital component, it

is no surprise that *snf1Δ* mutants have reduced virulence. Mice injected with *snf1Δ* strains

of JEC21 survived for 72 days, on median, while those infected with the wild type strain

lasted only 20 (Yang et al., 2010). It is beyond the scope of this paper to infer whether the

defects in melanin and capsule production were the source of the reduced virulence, but

the capsule is the major virulence factor of *C. neoformans*, so it is difficult to separate the effects of metabolic defects from those caused solely by changes to the capsule.

       snf1Δ strains show an unexplained temperature sensitivity at 45°C for two hours or 35°C for two days. The presence of a functional *SNF1* allele was necessary for survival. Complemented and wild type JEC21 were both resistant to the heat treatment. H99, again, was largely unaffected by the deletion of *SNF1* for this test, further emphasizing the divergent functions of *SNF1* in the two strains. The role of *SNF1* in heat shock response is unclear, but it seems to have a regulatory effect on Hsf1, although it is only one of several proteins that can participate in Hsf1 activation (Verghese, Abrams, Wang, & Morano, 2012).

       JEC21 also requires a functional *SNF1* allele to survive stress induced by high concentrations of cations. The *snf1Δ* strain of JEC21 was abnormally sensitive to high concentrations of NaCl and hygromycin B. However, high sorbitol concentrations did not elicit the same sensitivity, indicating that high osmolarity is not the source of the sensitivity (Yang et al., 2010). *snf1Δ* H99 did not show increased sensitivity to the tested ion concentrations. Nevertheless, the mutant H99 strain did show increased sensitivity to amphotericin B, while the mutant JEC21 strain did not.

       As mentioned previously, regulatory proteins make enticing targets for new anti-fungals due to their broad effects on numerous virulence factors of *C. neoformans*. Other types of targets exist, however, and the digestive and transport enzymes of the cell also have the potential to be effective targets for interference. The transport proteins in particular are ideal because a potential drug targeted at the protein does not necessarily have to make it through the cellular defenses and into the cytoplasm or nucleoplasm.

However, metabolic enzymes tend to be much more central to the function of the organism and are also worth investigating.

Pyruvate Kinase is a key enzyme in glycolysis, the central carbon utilization pathway for most non-photosynthetic organisms. Successful acquisition of carbon is a vital step in the infection process for pathogens, and *C. neoformans* is no exception. Reduced ability to obtain biochemically useful carbon substrates can reduce ATP production and impede production of other key molecules from the pentose phosphate pathway. The CSF contains glucose that can be used by an invading pathogen that is able to cross the blood brain barrier. Any reduction in the ability of *C. neoformans* to use this glucose would reduce virulence and aid in clearing of the yeast from the host (Price et al., 2011). Alternative pathways for carbon utilization exist, but an organism that cannot use glucose is severely reduced in virulence.

Deletion of *PYK1* from *C. neoformans* results in a strain that shows no defects in capsule or melanin production, although it does display a temperature sensitivity at 37°C (Price et al., 2011). However, this strain is prevented from growing on alternative media, such as lactate or glycerol, by carbon catabolite repression. Even though the cell cannot use the glucose, the presence of glucose still reduces the ability of the cell to obtain carbon from other sources. Deletion of *MIG1*, the major carbon catabolite repressor transcription factor, in the *pyk1*Δ background enables the strain to grow at near-wild type levels on glycerol or lactate media, which do not require a functional pyruvate kinase enzyme to enter oxidative phosphorylation. However, the *mig1*Δ *pyk1*Δ mutant also displayed lower growth rates than those of wild type *C. neoformans* in permissive media. The double-deletion strain also exhibits complete avirulence in the murine inhalation

model, although the strain was extremely persistent in the lung with very little clearance

by macrophages observed (Price et al., 2011).

Additionally, production of ATP was drastically reduced in the *pyk1*Δ mutant,

which would explain its decreased survival relative to wild type *C. neofo*rmans in ex vivo

CSF(Price et al., 2011). The *mig1*Δ *pyk1*Δ double-mutant recovers ATP production in

CSF, although production is still below that of wild type. Presumably, the limited pool of

lactate accessible in the CSF results in lower ATP production after glucose repression is

removed (Price et al., 2011).

Drugs targeting the glycolytic pathway have not been used to treat fungal

infectious diseases yet, but the work by Price and colleagues suggests that an

appropriately targeted compound could exert a fungistatic effect on *C. neoformans* even

after it has crossed the blood-brain barrier and begun to colonize the meninges.

Presumably, a drug that targets Pyk1 would not also target Mig1 and intracellular ATP

levels could be drastically reduced, allowing more time for treatment by traditional

means or for clearance by the immune system.

Sterylglucosides are glycolipids which are produced by *C.* neoformans and are

present on its surface, but are digested by another enzyme to prevent their accumulation

(Watanabe et al., 2015). When this enzyme, known as EGCrP2, has been deleted from

the genome, these sterylglucosides have been shown to accumulate on the exterior of the

cell and trigger an appropriate immune response by the host. EGCrP2, which is an

endoglycoceramidase that destroys sterylglucosides on the exterior of the cell, is

necessary for full virulence of *C.* neoformans, and is encoded by the *SGL1* gene (Rella et

al., 2015). Deletion of *SGL1* does not affect other known virulence factors such as

melanin production or capsule size, indicating that a novel protective effect is exerted by expression of this gene.

Triggering of the T_H2 immune response (described below) has been linked with decreased effectiveness of immune response and increased overall susceptibility to cryptococcosis. Infection with a sgl1Δ strain created by knockout from H99 *C. neoformans* via the murine inhalation model resulted in null virulence (Rella et al., 2015). Infection with the sgl1Δt strain also conferred complete immunity against further infection by both the *sgl1Δ* strain and the wild-type H99 strain. This vaccine effect also did not appear to be mediated by CD4+ T-cells, as mice depleted of CD4+ cells prior to infection also survived infection with the *sgl1Δ* strain and received the immunity from future infections previously observed. This is an exciting discovery, indicating that it is very possible to create a vaccine against *C. neoformans* which could potentially be effective even in patients with HIV/AIDS. While introduction of an attenuated strain of a pathogen always carries the risk of a reversion mutation, this strain provides a potential method to significantly reduce susceptibility to cryptococcosis.

A key component of phagocytic attack by macrophages is the use of oxygen radicals to damage engulfed cells. Many organisms use the enzyme superoxide dismutase to metabolize these peroxides and split them into less reactive components. Wild-type *C. neoformans* has a functional superoxide dismutase gene; other enzymes with a similar function further increase the ability of the yeast to deal with radicals (Cox et al., 2003).

TSA1 is a gene that has been identified by homology to an *S. cerevisiae* gene as a 2-cys peroxiredoxin, meaning that is has both an n-terminal and c-terminal cysteine that form crosslinks (Missall, Pusateri, & Lodge, 2004). This enzyme confers additional

protection against free radicals and significantly enhances *C. neoformans'* ability to survive phagocytosis. Under conditions of free radical stress, TSA1 production is increased nearly four-fold in wild type *C. neoformans* (H99), indicating that this gene used to fulfill its homology-hypothesized function (Missall et al., 2004). Production of TSA1 is also upregulated at higher temperatures. This effect has been hypothesized to be a result of changes in metabolism at 37°C which increase the amount of internally produced oxygen radicals (Missall et al., 2004). Regardless of its control mechanisms, TSA1 has been shown to be a vital part of the process of clearing free radicals.

Knockout mutants of TSA1 show drastically reduced survival in media containing small amounts of hydrogen peroxide and t-butylhydroperoxide at 30°C (Missall et al., 2004). Reconstitution of the gene resulted in a return to wild-type phenotype when grown under oxidative conditions and virulence approaching that of H99 *C. neoformans*. This data is consistent with the hypothesis that secondary peroxidases are a necessary component for survival post-engulfment. Virulence in the murine inhalation model was essentially zero for `infection to occur by the normal method. Injection of *tsa1Δ C. neoformans* into the tail resulted in significantly increased virulence, although less than that of H99 wild-type in an inhalation-infection model.

Echinocandins are a more recent class of anti-fungal than those traditionally used to treat cryptococcosis: fluconazole and amphotericin B (Denning, 2003). This new class of drugs is effective against many forms of fungi, including *Aspergillus* and *Candida* species. However, these drugs are not effective at clinical doses against *Cryptococcus* infections. The mechanism for this resistance was recently determined to be a lipid flippase that is homologous with *S. cerevisiae* gene *CDC50* (Huang et al., 2016).The

gene responsible for resistance to the echinocandin anti-fungal drug caspofungin was determined by growing a mutagenesis library of KN99 *C. neoformans* mutants on media inoculated with the antibiotic (Huang et al., 2016).

Several mutants showed decreased growth under the above conditions, but the *cdc50Δ* strain showed the greatest decrease in growth from the effects of caspofungin alone. The flippase encoded by CDC50 could prove to be a potential target for new drugs, as it is a protein not found in humans. The combination of a drug targeted at this protein and an echinocandin antifungal could allow for a much shorter and less toxic course of treatment than the current standard of care.

Zinc is also necessary for many functions within the cell. It is a component of alkaline phosphatase, zinc-finger domains of many proteins including Mig1, and other enzymes (Lulloff, Hahn, & Sohnle, 2004). The regulatory and uptake systems for zinc in *S. cerevisiae* has been well characterized, but only recently have the plasma membrane zinc transporters for *C. neoformans* been identified (Do, Hu, Caza, Kronstad, & Jung, 2016). The *ZIP* family of zinc transporters are the main family of proteins responsible for maintaining intracellular zinc levels. The Zip1 transporter has been linked with zinc uptake under low-zinc conditions, and Zip2 with intake under normal or zinc-replete conditions. Zip1 is an N-glycosylated protein localized to the plasma membrane with 8 transmembrane domains. Zip2 is also a membrane-localized zinc transporter, but its effect on virulence is negligible.

Deletion of *ZIP1* resulted in decreased growth on low-zinc media, while deletion of *ZIP2* had little effect at any concentration of zinc (Do et al., 2016). Significantly higher zinc levels were required for optimal growth for the *zip1Δ* mutant compared to the

zip2Δ mutant, indicating that *ZIP1* is responsible for high-affinity zinc transport. Deletion of *ZIP1* significantly reduced survival in macrophages, as well as virulence in the murine inhalation model. Deletion of *ZIP2* alone had little effect on either of these two tests, further illustrating the dependence of *C. neoformans* on Zip1 for obtaining zinc. Deletion of both *ZIP1* and *ZIP2* showed effects on virulence and macrophage survival similar to those of the *zip1Δ* strain. The *zip1Δ* mutant strain also showed no change in melanin or capsule formation compared to H99 wild type *C. neoformans*. The reduction in virulence is therefore linked to the decreased ability of the cell to obtain zinc, rather than on other mediating factors. Although deletion of *ZIP1* does not cause a complete attenuation of virulence, the relative ease of access to a membrane protein compared to an intracellular target makes Zip1 an appealing target for new drugs. More research needs to be done to determine whether deletion of *ZIP1* causes increased susceptibility to other antifungals or compounds.

Another active area of *C. neoformans* research is on the formation of titan cells, which are large, polyploid cells (Okagaki & Nielsen, 2012). These titan cells can reach 100 microns in diameter, much larger than the normal vegetative *C. neoformans* cells that are ≤ 10 microns in diameter. There are also numerous changes in the capsule of the cell when a titan cell is formed, including increased cross-linking and decreased permeability. Internal transformation also occurs in these cells, including increased amounts of DNA and increased formation of vacuoles. As mentioned previously, these cells are polyploid. Vegetative *C. neoformans* cells are haploid, but titan cells can be tetraploid, octoploid, and may even be hexadecaploid (i.e. having 16 copies of the genome in a nucleus). However, progeny of these titan cells are still haploid. The mechanism by which

polyploid cells are produced from haploid cells is believed to be mitotic, but it is currently unknown how polyploid titan cells are able to produce haploid progeny. Movement of titan cells across membranes is also hampered by the large size of these cells, meaning that it is unlikely they will ever leave the lung but may instead serve as a source of dissemination.

Titan cell formation can be triggered by the presence of macrophages or other phagocytic cells, such as amoebae. It has been postulated that sensing of phospholipids commonly found in phagocytic cell plasma membranes is the trigger for titan cell formation. Production of titan cells has been demonstrated to be controlled by multiple intracellular pathways, including adenyl cyclase/cAMP control and G-protein coupled receptors (Okagaki et al., 2011; Zaragoza et al., 2010). Titan cells are too large to be engulfed, and therefore provide a relatively untouchable source of *C. neoformans* cells in the alveolar tissue (Crabtree et al., 2012). Similar to the immune response to other parasites, eosinophils are recruited to the lung, potentially indicating an alternative activation of the immune system that is less effective at dealing with the non-titan vegetative *C. neoformans* cells (Crabtree et al., 2012). The progeny of titan cells are more resistant to oxidative and nitrosative stress than standard *C. neoformans* cells (Okagaki et al., 2010). The method by which this protection from free radicals is conferred by the titan cell is currently unknown.

Several genes have been identified as regulators of titan cell formation. Gpr5 is a membrane protein that has been shown to be necessary for titan cell formation, and may be a sensor that triggers the transition (Crabtree et al., 2012). It is unknown whether this is the sensor that regulates response to coat lipids of macrophages. Ste3a is also believed

to be a regulator for the titan cell formation process and is believed to be involved with mating type a pheromone response (Okagaki et al., 2010). Both of these regulatory proteins are believed to interact with Gpa1, which is another intermediate in the regulatory pathway (Choi, Vogl, & Kronstad, 2012). *RIM101* is also involved with control of titan cells, and is necessary for titan cell formation (O'Meara et al., 2010). Deletion of these genes and the resulting decrease in titan cell formation showed significantly lowered virulence in the murine inhalation model (Crabtree et al., 2012).

It is believed that the effects on virulence of the titan cells that are not mediated by the changes to the capsule are due to alternative activation of the immune system (Crabtree et al., 2012). Similar to how *C. neoformans* cells cause M2 activation of macrophages, activation of the T_H2-type immune response is maladaptive. Alterative activation of the immune system to deal with the large titan cells tends to reduce response to the normally-sized vegetative cells, allowing them to proliferate while eosinophils attack the titan cells. Titan cells are extremely resistant to this type of attack, however, and can remain in the lung for months (Crabtree et al., 2012). Mice which showed T_H2 type immune activation had much higher rates of dissemination across the blood-brain barrier, and also showed reduced recruitment of T and B-cells to the lungs (Garcia-Barbazan et al., 2016).

Conclusion

As would be expected from any complex organism, the list of genes that are necessary for full virulence is long and varied. Many of the genes discussed in this paper would make good candidates for new drugs, and could substantially increase survival if their target genes or proteins could be inhibited. Resistance to antibiotics is always a

concern with any long-lasting infection, and the current treatment plans for a *C. neoformans* infection very extended in length and form a fertile breeding ground for resistant phenotypes. Fortunately, the fungal genome is relatively resistant to change, and complete resistance to the azole drugs is very rare.

Other methods could also be used to control the spread of *C. neoformans*. Pigeons have been documented as carriers of this disease, and their droppings are believed to be the most common way the illness spreads. Population control of wild pigeons could therefore be used to limit spreading of the disease. Similarly, in Australia, a type of Eucalyptus tree has been shown to be a reservoir of *C. gattii* in the wild. Finding ways to remove or disinfect the trees could be a potential way to reduce to effects of cryptococcosis on the population.

Ultimately, however, as long as AIDS is present in the world, cryptococcal infections will occur. The organism is ubiquitous, and extremely hardy and long lasting. Programs designed to preserve innate immunity will be more effective in the long run than trying to replace the functionality of an immune system with more drugs.

Knowledge obtained about the regulatory pathways and cellular defenses of *C. neoformans* against the immune system can also be used to create treatments for *C. gattii*, which does not require that the host lack a functional immune system. This emerging disease could cause massive damage if it became resistant to current antifungals, as the infection is very difficult to treat, and is usually not detected until the patient experiences symptoms related to encephalitis or meningitis.

The Zip1 transporter should be a relatively safe target for new antifungals. The CSF is a relatively isolated environment, so any drugs introduced to that reservoir have many fewer potential human cells that could be negatively impacted. Care must be taken to ensure that neural tissue is not harmed by the introduction of drugs to the CSF, but presumably at least one of the proteins discussed above will be completely unique to fungi and cause few side effects.

For the borderline immunocompetent, finding ways to stimulate M1 activation of macrophages rather than the M2 activation encouraged by *C. neoformans* is probably the best option. Stopping the infection in the alveolar tissue of the lung is obviously preferable to treating the disease after infection of the CSF.

New antifungals are constantly in development, and it remains to be seen how effective the latest class of azoles will be against *C. neoformans*. Isavuconazole, developed for treatment of infection by *Aspergillus* species, targets the production of ergosterol. Ergosterol, as mentioned above, has been shown to be necessary for virulence in *C. neoformans*, so this drug has the potential to be useful in treating cryptococcosis. However, another azole drug will most likely not be sufficient to significantly reduce the burden of treatment for cryptococcosis. Some method of allowing the immune system to appropriately respond to the yeast would be ideal, preferably by enabling CD8+ lymphocytes, which are not depleted by infection with HIV, to successfully phagocytize and destroy the pathogen (Daniel et al., 2001). A drug that could potentially shut down a key regulator such as Cir1 could reduce the metabolic capabilities of the cell and make it unable to defend against the use of superoxide radicals inside of the macrophage.

Like alternative M2 macrophage activation, triggering of T_H2-type immune response by *C. neoformans* titan cells decreases the effectiveness of macrophages and increases dissemination across the blood-brain barrier. If proper stimulation of the immune system can be achieved, treatment is far more likely to be successful and short. As before, the ideal solution is to encourage the immune system to respond appropriately rather than to necessitate extended use of antifungals to prevent reinfection.

C. neoformans and *C. gattii* are emerging pathogens that have seen a drastic increase in infection as the frequency of AIDS infection has increased. While cryptococcosis kills thousands every year, it is currently still treatable by standard antifungal drugs with a high survival rate. However, difficulties in obtaining the necessary antibiotics, as well as the length of treatment prevent members of isolated or low-income areas from being able to complete a full course of treatment. Finding new drugs that target the pathways discussed herein should be a priority for the medical research community.

As with other common microbial infections, the development of strains that are resistant to the current drugs of choice is a concern. If more antifungals can be created that are effective against *C. neoformans*, the emergence of highly resistant strains can be delayed by allowing rotation of antifungals and for antibiotics of last resort to remain effective. Again, like other microbial infections, early detection is the key to preventing loss of life from the disease. Regular screening of those with HIV/AIDS for *C.* neoformans infection could help save lives without the introduction of new treatment methods.

The possibility of a viable vaccine is also being explored, and has the potential to revolutionize the treatment of cryptococcosis in a way that reactive treatment can never hope to match. Research on vaccination against *C. neoformans* is still at the very beginning stages, but this approach holds considerable promise for those who may become infected with *C. neoformans* in the future.

The recent outbreaks of *C. gattii* are also concerning, and highlight further the need for better research on *Cryptococcus*, as well as more drugs that are effective against this hardy pathogen. Several of the mutations described in this paper have the potential, if targeted, to allow current and newly created antifungals to be more effective. Whether the method of attack is to synergize with existing drugs or to create entirely new courses of treatment, the result will be shortened treatment times and increased effectiveness of treatment. These two accomplishments would make full and successful treatment a possibility for many who are currently unable to afford or find appropriate treatment.

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