

The Essential Role of Carbon Metabolism in the Virulence of *Cryptococcus neoformans*

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Abstract

Cryptococcus neoformans infections are a major cause of meningoencephalitis in immunosuppressed patients worldwide. Inhaled as spores or desiccated yeast cells, *C. neoformans* can undergo metabolic changes in response to the new host environment that allow it to cross the blood brain barrier and cause deadly central nervous system (CNS) infections. Nutrient acquisition, and specifically carbon metabolism, is critical for survival and proliferation within the host. Notably, efficient carbon metabolism is necessary to produce the polysaccharide capsule, which is arguably *C. neoformans*' most important and well-studied virulence factor. As such, a better understanding of carbon acquisition and regulation is essential for the development of new targeted drug therapies to combat this deadly fungus.

Keywords: *C. neoformans*, capsule, meningoencephalitis

The Essential Role of Carbon Metabolism in the Virulence of *Cryptococcus neoformans***Introduction**

The *Cryptococcus neoformans* species complex is comprised of unicellular encapsulated fungi that can replicate by budding and share many general characteristics with *Saccharomyces cerevisiae* (Aslanyan et al., 2017). An obligate aerobe most often isolated from avian fecal matter, *C. neoformans* is a common environmental fungus found worldwide. Cryptococcal spores are commonly present in the air, and the vast majority of individuals are frequently exposed to this fungus throughout life (Goldman et al., 2001). In healthy individuals, a functioning immune system is able to effectively eliminate spores and yeasts prior to onset of disease with near perfect efficiency.

Cryptococcosis: An Opportunistic Infection

While *C. neoformans* rarely causes long-term complications or systemic disease in immunocompetent individuals, it occasionally results in fungal pneumonia that is resolved by a functioning immune system. However, cryptococcal infections (i.e. cryptococcosis) are a leading cause of meningoencephalitis in immunosuppressed patients worldwide (Dambuza et al., 2018). The highest incidence of disease occurs among individuals with HIV/AIDS, largely due to the lack of CD4⁺ T-helper cells present in the bloodstream. However, any immunocompromised individual can develop cryptococcosis, with at-risk groups including chemotherapy patients, transplant recipients on immunosuppressive anti-rejection medications, and individuals on chronic corticosteroids for autoimmune disorders (Lin, Huang, Mitchell, & Heitman, 2006). Seven species of *Cryptococcus* can cause disease, though *C. neoformans* is the most clinically significant strain with effects ranging from localized cutaneous infections to

widespread systemic disease (Hagen et al., 2015). *C. neoformans* is invasive and difficult to treat, largely due to its ability to cross the blood brain barrier and cause CNS infections. Antifungal drug options are limited and expensive, and while widely available in developed nations, these medications often pose harmful side effects (Fernandes, Martho, Tofik, Vallim, & Pascon, 2015). It is estimated that over 223,000 individuals contract cryptococcal meningitis annually, with prognosis highly dependent on access to sophisticated medical care (Rajasingham et al., 2017). As the epidemiology of this disease is closely linked to the AIDS pandemic, the vast majority of cases are concentrated in sub-Saharan Africa due to the lack of antiretroviral therapy available to patients. Because of its mortality of greater than 50%, scientists are continually working towards a better understanding of *C. neoformans* in order to develop new treatments to combat this disease (Rajasingham et al., 2017).

Pathogenesis

Due to the body's natural immune response, individuals with functioning immune systems are generally able to fend off *C. neoformans* infections prior to systemic disease progression. Studies have shown that immunosurveillance in healthy individuals is very effective, with respiratory epithelial cells playing a critical role. These cells are separated by tight junctions, which helps to provide a physical barrier against invading microbes (Whitsett & Alenghat, 2015). In addition, T-helper 1 (Th1) cells are crucial because they mediate a protective immune response through the production of various cytokines. Specific cell-signaling cytokines that play a role in response against *C. neoformans* include interleukin-2 (IL-2), IL-12, interferon gamma, and tumor necrosis factor alpha

that are produced initially by dendritic cells and macrophages (Whitsett & Alenghat, 2015). While important molecules and proteins have been isolated, the exact mechanism of this response is still an active area of study.

In immunosuppressed hosts, the ability of *C. neoformans* to disseminate systemically before infection is eradicated by the immune response causes serious, and often fatal, disease processes. While this fungus can travel to diverse locations within the body, it has a particular affinity for the CNS. Studies have shown that there are two main pathways for *C. neoformans* dissemination: the intracellular mechanism and extracellular mechanism (Denham & Brown, 2018).

Intracellular mechanism. The intracellular mechanism of *C. neoformans* dissemination uses the machinery of migratory phagocytes, most notably macrophages, to move from the lungs to other areas of the body. Ultimately, this method, also known as the Trojan Horse mechanism, allows the fungal spores to enter into macrophages, where they germinate into yeasts that are carried into the CNS (Charlier et al., 2009; Tseng, et al., 2012). Spores or desiccated cells contact macrophages and dendritic cells in the mucosal layers of the lungs. These macrophages, which can control the infection in healthy individuals, inadvertently facilitate the spread of disease in immunosuppressed hosts. The mechanism is three-fold. First, *C. neoformans* is engulfed by alveolar macrophages in the lungs (Denham & Brown, 2018; Bojarczuk et al., 2016). Because low CD4⁺ helper T cell counts delay the immune response, macrophages in immunosuppressed individuals are able to take up *C. neoformans*, but they have a very difficult time destroying them once they do so.

Secondly, the microbe must be able to survive within the macrophage or phagolysosome for an extended period, allowing enough time for travel to distant organs and body systems. Studies have shown that this is possible due to several key characteristics of *C. neoformans*. This fungus is able to survive at very low pH, which is essential to intracellular survival (Vogel, 1969). This adaptation likely stems from the fact that avian fecal matter, the primary environmental host of *C. neoformans*, is typically quite acidic. Unfortunately, *C. neoformans* is actually able to replicate slightly faster when it is in an acidic environment, making it even more difficult to eliminate in macrophages (Diamond & Bennet, 1973).

The third and final step in intracellular disease progression is the macrophage escape process (Tucker & Casadevall, 2002). In simple terms, *C. neoformans* is able to increase the permeability of the macrophage/phagolysosome by altering phospholipase expression in order to increase permeability; this is accomplished either by physical disruption of membranes or via modulation of cell signaling pathways (Djordjevic, 2010). At the same time, *C. neoformans* transiently alters the host actin network that has a structure resembling a cage. This cage-like apparatus is able to confine the phagosome, but it allows *C. neoformans* to exit via small gaps within the actin network (Johnston & May, 2010). This same mechanism can be used for lateral macrophage transfer or for escape during macrophage division or fusion (Johnston & May, 2010). Through a series of interrelated steps, the Trojan Horse method allows *C. neoformans* to enter the CNS and cause life-threatening meningitis.

Extracellular mechanism. The second mechanism of disease dissemination is

primarily extracellular. It hinges on the process of transcytosis (Chang et al., 2004).

Transcytosis uses vesicular transport to capture a particle or microbe on the luminal side of the cell, transport it through the interior of the cell, and release the particle on the basal side. In the case of inhaled *C. neoformans*, the organism travels through the epithelial cells of the respiratory tract before it is deposited into the bloodstream. From there, *C. neoformans* can travel systemically. GXM, the major polysaccharide in the capsule of *C. neoformans*, is directly responsible for this process (Wang, Aisen, & Casadevall, 1995). GXM mediates the adherence of *C. neoformans* to CD14, a protein found on the cell membrane of epithelial cells (Denham & Brown 2018). Once adhered, *C. neoformans* uses a separate mechanism to enter the epithelial cell and cause damage that allows the cell to pass through. To date, scientists are still determining the exact biochemical interactions that facilitate this process.

Virulence Factors

Among microorganisms, and pathogenic fungi in particular, there are several general indicators of virulence. Specifically for *C. neoformans*, capsule production, melanin production, and the temperature of the host all play a large role (Haynes et al., 2011; Gomez & Nosanchuk, 2003). In a healthy individual, the front-line phagocytes, neutrophils, and dendritic cells of the innate immune response recognize and destroy cryptococcal cells. However, in immunosuppressed patients, these processes are impaired; in the setting of an inefficient or ineffective immune response, *C. neoformans* virulence factors prevent infection control and lead to widespread disease (Campuzano & Wormley, 2018).

Melanin production has long been recognized as a source of pathogenicity for opportunistic fungi such as *C. neoformans* (Gomez & Nosanchuk, 2003). This dark colored pigment is synthesized from a variety of biologic molecules, including some neurotransmitters (Wang & Casadevall, 1994). Because it is comprised of insoluble polymers, melanin cannot be easily degraded by macrophages in the host, making the immune system less effective against cells that produce it (Mednick, Nosanchuk, & Casadevall, 2005). As such, melanization plays a key role in protecting against degradative enzymes, and it has also been shown to protect against phagocytes and reactive oxygen species that are a part of the immune response (Wang & Casadevall, 1994).

While melanin production is often used as a general indicator of fungal pathogenicity, capsule production is often regarded as the most important virulence factor for *C. neoformans*. The polysaccharide capsule, comprised of various polysaccharides, serves both offensive and defensive functions in the host environment (Agustinho, Miller, Li, & Doering, 2018). In vivo, *C. neoformans* can also differentiate into larger titan cells, which have thicker polysaccharide capsules and contain a greater amount of chitin that provides increased strength and protection (Mukaremera et al., 2018). This external cell structure helps to modulate interactions between immune cells while protecting the yeast against phagocytic cells from the host (Garcia-Carnero, Perez-Garcia, Martinez-Alvarez, Reyes-Martinez, & Mora-Montes, 2018). As one of the most important indicators of virulence in *C. neoformans*, capsule production is often used as a marker in determining pathogenicity in experimental studies (Casadevall et al., 2018).

Carbon Metabolism in *C. neoformans*

In order to cause disease in a host organism, a microbe must procure the basic building blocks of life from its surroundings. As a result, one of the largest challenges that any microbe faces is finding efficient metabolic strategies in a rapidly changing environment (Fernandes et al., 2015). Normally, *C. neoformans* lives in soil, decaying wood, and bird feces, where it can procure sufficient amounts of carbon and nitrogen from the environment (Barkal, Walsh, Botts, Beebe, & Hull, 2016). Because the ability to acquire and metabolize nutrients is directly related to a pathogen's fitness in its respective environment, the metabolism of key nutrients, namely carbon, is required for virulence in *C. neoformans* (Ramachandra et al., 2014). Not a member of the normal human flora, *C. neoformans* typically enters the human host via inhalation of desiccated spores, where it must adapt its metabolic strategies prior to systemic dissemination and entry into the CNS (Diamond & Bennett, 1973) and (Guess et al., 2018).

Understanding the metabolism of carbon has long been recognized as an important strategy for developing effective drug targets for specific organisms and disease processes. For example, it has been previously established that cancer cells rely on a functional glycolytic pathway in order to grow, as carbon metabolism allows the cells to proliferate and increase the tumor size (Vander Heiden, Cantley, & Thompson, 2009). As such, researchers have begun to develop glycolysis inhibitors in order to target specific types of cancer cells and limit their growth. While glycolytic enzymes are nearly identical across species and cell types, the slight differences in nucleotide sequence provide potential species-specific targets for drug development. Not only is proliferation

essential to the progression of any malignant disease, but strong similarities are seen between neoplastic processes and eukaryotic pathogens during invasion (Vander Heiden et al., 2009). As such, understanding the complicated role of carbon metabolism in the pathogenesis of *C. neoformans* infections provides valuable insight into potential methods of disease treatment.

Effects of a Changing Microenvironment

Throughout the pathogenesis of cryptococcosis, *C. neoformans* must adapt to a wide range of microenvironments in order to persist and cause disease within the host. As mentioned previously, *C. neoformans* lives and thrives in a diverse array of ecological settings, but it is most commonly isolated from decaying tree bark and soil containing avian fecal matter. In the environment, both atmospheric nitrogen and oxygen are abundant. Additional nutrients such as carbon are easily acquired from decomposing organic material in the soil. While *C. neoformans* is a known human pathogen and the majority of individuals are exposed to this relatively common fungus during childhood, it is not a member of the normal human flora (Perfect, 2006; Diamond & Bennet, 1973).

Metabolism during early pulmonary infection. In addition to facilitating respiration and gas exchange within the human body, the lungs also provide a vital mucosal barrier that serves to protect against the millions of microbes that are found in the air at any given moment. Contact with such microbes is unavoidable, and *C. neoformans* is no exception (Denham & Brown, 2018). As such, cryptococcal infections generally start in the lungs through the inhalation of desiccated cells or spores. After the initial infection, spores can germinate into yeasts that disseminate throughout the body,

exist indefinitely as granulomas in the lungs, or they can be eradicated by the body's immune response.

As the microenvironment of the mammalian lung is fundamentally different from the soil in which *C. neoformans* most commonly resides, this fungus must adapt to the changing availability of nutrients. While nitrogen and oxygen are still present in lung tissue, the availability of elemental carbon is significantly reduced compared to the external environment (Barelle et al., 2006). As such, *C. neoformans* alters the transcription of genes controlling carbon metabolism in a way that shifts the metabolic focus to the procurement of carbon from alternative sources. The glyoxylate shunt, citric acid cycle, and gluconeogenesis are all upregulated at the same time that glycolysis is downregulated (Barelle et al., 2006). While glycolysis is certainly the most efficient way to procure energy from environmental carbon, the upregulation of gluconeogenesis provides *C. neoformans* with an alternative carbon source that it can use in order to survive in the mammalian lung.

Glycolysis and gluconeogenesis during early pulmonary infection. Present in all eukaryotic organisms, the glycolytic pathway is one of the most frequently studied metabolic processes in biology. It plays a central role in carbon metabolism, breaking down glucose into pyruvate that can be shunted into the citric acid cycle. The energy from this process is then used to drive oxidative fermentation in the mitochondria, which eventually uses a proton gradient in order to produce cellular energy in the form of ATP (Hu, Cheng, Sham, Perfect, & Kronstad, 2008). ATP provides energy for numerous cellular processes and is required for any organism to survive, including *C. neoformans*.

However, it has been shown that pyruvate kinase and phosphofructokinase, two enzymes that catalyze irreversible steps in glycolysis, are downregulated in *C. neoformans* cells grown in mammalian lung tissue (Hu et al., 2008). This is suggestive of a nutrient, and specifically a glucose, poor environment. Interestingly, two separate pyruvate kinase and hexokinase knockout strains of *C. neoformans* showed attenuated virulence in the murine inhalation model but satisfactory persistence within the lungs of the host (Price et al., 2011). As these two enzymes function as irreversible steps in the glycolytic pathway, glycolysis is dispensable as a means of carbon acquisition during early pulmonary infection. Additionally, transcription factors known to regulate glycolysis were downregulated during early pulmonary infection, further corroborating this point (Kronstad et al., 2011).

Alternatively, gluconeogenesis, an anabolic process that uses various forms of carbon in order to synthesize glucose, is upregulated (Hu et al., 2008). Gluconeogenesis utilizes numerous precursors depending on the availability of different carbon sources, though some of the most common include lactate, glycerol, and amino acids such as alanine and glutamine. Surfactant (predominantly phosphatidylcholine and phosphatidylglycerol) is also present in the mammalian lung and is a potential substrate for gluconeogenesis. Several different processes are used to form pyruvate from each of these molecules, which accumulates in the mitochondria of hepatocytes and other specialized cell types. In the mitochondria, pyruvate carboxylase converts pyruvate into oxaloacetate; a series of ten additional enzyme-catalyzed reactions then follows, leading to the formation of glucose. While glycolysis and gluconeogenesis are opposite metabolic

processes, three different enzymes in gluconeogenesis are used to catalyze the non-reversible steps in the glycolytic pathway. One such enzyme, phosphoenolpyruvate carboxykinase I (*PCK1*) is the main regulatory step in gluconeogenesis and catalyzes the formation of phosphoenolpyruvate from oxaloacetate. A study looking at the differential expression of *PCK1* transcripts between *C. neoformans* cells grown in mammalian lung tissue versus *in vitro* cultures showed upregulation of this enzyme during early infection (Hu et al., 2008). A later study showed significantly attenuated persistence in a rabbit model of CNS disease, further corroborating that gluconeogenesis plays a central role in the early pathogenesis of cryptococcal infections (Price et al., 2011).

Use of the glyoxylate shunt during early pulmonary infection. Additionally, *C. neoformans* is able to catabolize lipids and other complex carbon compounds to use as alternative energy sources upon initial infection. Fluid collected from mammalian lung tissue by bronchoalveolar lavage is rich in phospholipids (Rooney, Young, & Mendelson, 1994). Transcription profiling shows transiently elevated lipid and fatty acid catabolism genes in *C. neoformans* infected lung cultures when compared to CNS samples or *in vitro* lab cultures (Hu et al., 2008). Combined with the upregulation of *PCK1*, this suggests significant glucose limitation during early pulmonary infection within the host (Hu et al., 2008).

The glyoxylate shunt is a metabolic process similar to the citric acid cycle that plants, protists, and fungi use in order to generate macromolecules from alternative carbon sources. Specifically, this anabolic process uses a series of enzymatic reactions to convert 2-carbon compounds such as acetate into glucose, which can then be used for

various cellular functions. As acetate is one such compound that is produced in the β -oxidation of fatty acids, the glyoxylate shunt is closely linked to the catabolism of lipids. Five citric acid cycle enzymes are used in this process, with the first two steps being identical. Acetate produced by the β -oxidation of fatty acids enters the cycle bound to the thiol group of CoA, where it becomes citrate, cis-aconitate, and then isocitrate. During the glyoxylate shunt, isocitrate is then broken down into glyoxylate and succinate by the enzyme isocitrate lyase. This differs from the citric acid cycle, in which isocitrate dehydrogenase functions to produce α -ketoglutarate while releasing one molecule of carbon dioxide (CO₂). Both processes eventually produce oxaloacetate that can be converted to phosphoenolpyruvate for use in gluconeogenesis.

In short, the benefit of the glyoxylate shunt is that it allows an organism to capitalize on the β -oxidation of fatty acids via a process that bypasses the CO₂ releasing steps in the citric acid cycle. Because carbon, and specifically carbon found in the form of glucose, is essential for many cellular functions, upregulation of the glyoxylate shunt during early pulmonary infection allows *C. neoformans* to produce glucose via gluconeogenesis without sacrificing any carbon in the form of CO₂ (see Figure 1) (Hu et al., 2008). The glyoxylate shunt provides an efficient means of alternative carbon metabolism in *Candida albicans* and *Aspergillus fumigatus* that is necessary for survival (Lorenz & Fink, 2001; Olivas et al., 2008). However, a functional glyoxylate shunt is not necessary for virulence in *C. neoformans*, making this species unique among pathogenic fungi (Rude, Toffaletti, Cox & Perfect, 2002).

Metabolism during systemic infection. In higher eukaryotes such as mammals,

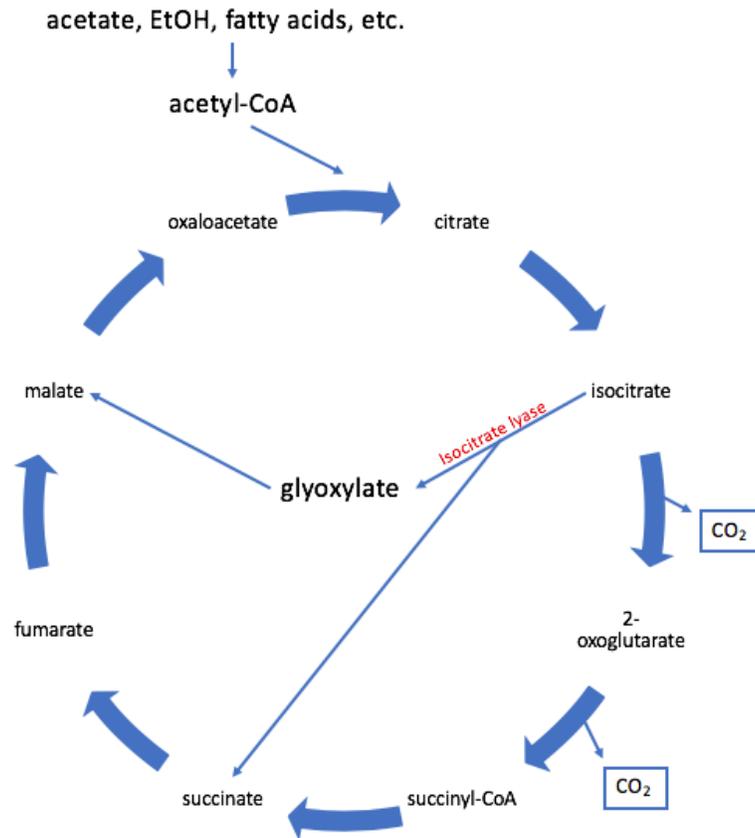


Figure 1. Relationship between the glyoxylate shunt and the citric acid cycle.

By eliminating the steps of the citric acid cycle that produce CO₂, the glyoxylate shunt allows for fatty acid catabolism without carbon loss. Adapted from “Major roles of isocitrate lyase and malate synthase in bacterial and fungal pathogenesis” by M. F. Dunn, J. A. Ramirez-Trujillo, and I. Hernandez-Lucas, 2009, *Microbiology*, 155(10), p. 3167.

the CNS is comprised of the brain and spinal cord. Often thought of as the control center of the human body, the CNS is responsible for receiving incoming neuronal signals from the peripheral nervous system (PNS), interpreting them, and sending signals back to the efferent PNS in order to elicit a response. Because of its vital role in important functions

such as higher-order thinking, controlling reflexes, and maintaining homeostasis, the transport of molecules into and out of the CNS is highly regulated. This poses a new challenge for disseminated cryptococcal cells that have made their way from the lungs to the CNS, as they must adapt to yet another microenvironment in order to survive (Barelle et al., 2006).

While *C. neoformans* faces a nutrient-poor environment upon inhalation in the lungs, oxygen and glucose are more abundant within the CNS and specifically within the brain. However, because CNS cells rapidly consume glucose and oxygen, these resources are not always readily accessible. Because of the high energy demand of actively proliferating cells, the production of ATP via oxidative phosphorylation in *C. neoformans* mitochondria is essential for survival (Ingavale et al., 2008). Indeed, isocitrate dehydrogenase was upregulated in a rabbit cerebrospinal fluid (CSF) model of cryptococcal meningitis; components of NADH dehydrogenase, cytochrome c subunits, and ATP synthase were also upregulated, suggesting heightened energy demand within the CNS (Steen et al., 2003).

Glycolysis and gluconeogenesis. In contrast to the emphasis on gluconeogenesis that is seen in the murine inhalation model of early pulmonary infection, the persistence of *C. neoformans* within the CNS is largely dependent on glycolysis. Again, this was demonstrated using a strategic hexose kinase double-deletion strain (*hvk1Δ hvk2Δ*), which blocks the entry of glucose into the glycolytic pathway (Price et al., 2011). Hexose kinase activity is required not only for initial infection in the lungs, but for persistence in the CNS as well (Price et al., 2011). Additionally, a pyruvate kinase (*PYK1*) knockout

mutant was used to block the exit of glucose from the glycolytic pathway, with the *pyk1Δ* strain exhibiting significantly reduced virulence in the rabbit CSF model. Mice infected with the *pyk1Δ* mutant survived for approximately 6 months, while those infected with the *hvk1Δ hvk2Δ* mutant survived for approximately six weeks (Price et al., 2011). Both strains had an impaired ability to utilize glucose, suggesting that glycolysis is the major player in *C. neoformans*' ability to metabolize carbon once the fungus has reached the CNS (Price et al., 2011). Interestingly, this evidence is corroborated by a similar pattern of central carbon metabolism in *C. albicans*. A *pyk1Δ* strain of *C. albicans* showed significantly attenuated virulence and increased fungal burden in a murine inhalation model of disease, consistent with the attenuated virulence seen in the *C. neoformans* model (Barelle et al., 2006).

While gluconeogenesis is essential for early pulmonary infection and dissemination of cryptococcal cells from the lungs to the CNS, a functional gluconeogenic pathway is not essential for persistence within the CNS itself. Researchers used a real-time PCR-based protocol to determine the expression of *PCK1* in rabbit CSF versus *ex vivo* human CSF and *in vitro* *C. neoformans* cultures. *PCK1* expression was elevated in the *in vivo* rabbit CSF model in comparison to the other samples, suggesting an increased rate of glucose synthesis within the CNS during cryptococcal pathogenesis. However, this same study created a *pck1Δ* knockout strain that retained its virulence despite lacking a functional gluconeogenic pathway. This serves to show that while gluconeogenesis is upregulated in the glucose-poor environment of the CSF, it is not *C. neoformans*' primary means of utilizing alternative carbon sources during CNS infection

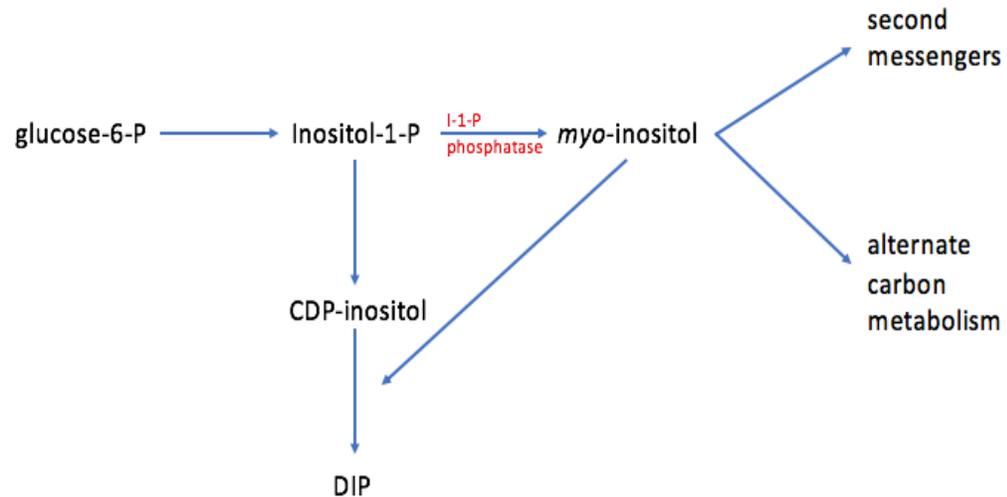
(Price et al., 2011).

Inositol as an alternative carbon source. In addition to glucose, *C. neoformans* is able to use non-preferred carbon sources as a means of obtaining energy and performing cellular functions within the CNS. Inositol, a carbocyclic sugar-alcohol that is made from glucose, serves as the structural basis for numerous important secondary messengers such as inositol triphosphate. As such, this carbon-based compound is present in high concentrations within the human brain, where many second messengers are synthesized and released. Interestingly, *C. neoformans* upregulates inositol monophosphatase in order to provide an alternative means of carbon and energy acquisition in the CNS (see Figure 2) (Steen et al., 2003). Of note, inositol production plays a direct role in intracellular proliferation and melanin production by *C. neoformans* (Luberto et al., 2001; Barnett, 1976).

Carbon Regulation in *C. neoformans*

As previously established, the ability of *C. neoformans* to adapt to a wide variety of host microenvironments is essential for its ability to cause disease within a human host (Barelle et al., 2006). Specifically, this fungus has several molecular mechanisms in place that direct and orchestrate carbon metabolism depending on the nutrients available. In this way, *C. neoformans* is able to survive and proliferate despite environmental stress and change. The CCAAT-binding complex (HAP) in *S. cerevisiae* is a complex of four separate proteins (Hap2/Hap3/Hap4/Hap5) that shifts carbon metabolism from glucose utilization to production when environmental glucose has been depleted. This is

Figure 2. Inositol production via inositol monophosphatase (I-1-P phosphatase).



Inositol monophosphatase catalyzes the conversion of inositol monophosphate to myo-inositol, which can be used in the production of second messengers or as an alternative carbon source for pathogenic fungi residing within the CNS. Adapted from “Cloning and expression of the inositol monophosphatase gene from *Methanococcus jannaschii* and characterization of the enzyme” by L. Chen and M. F. Roberts, 1998, *Applied and Environmental Microbiology*, 64(7), p. 2610.

accomplished by downregulating glycolysis and upregulating genes involved in the citric acid cycle and cytochrome subunits found in the electron transport chain (Nehlin, Carlberg, & Ronne, 1991). As Hap4 is the main regulatory subunit in the Hap Complex of *S. cerevisiae*, an orthologous HapX, was identified in *C. neoformans* (Caza, Hu, Price, Perfect, & Kronstad, 2016).

MIG

Mig1, a transcription factor initially found in *S. cerevisiae*, is a well-studied zinc

finger protein known for its role as a global catabolite repressor. As Mig1p is expressed at high levels when glucose is abundant, the Mig1p complex represses genes involved in alternative carbon acquisition and metabolism. In doing so, Mig1p ensures that glucose is used as the primary carbon source when available. Of note, additional data suggests that Mig1p negatively affects the expression of oxidative phosphorylation and iron transport proteins in glucose-limited environments (Pir et al., 2008). A Mig1p ortholog was identified in *C. neoformans*, with the new candidate gene identified as *MIG1*. It is interesting to note that the regulatory Hap4p subunit in *S. cerevisiae* acts as a transcriptional activator of *MIG1* while the *C. neoformans* ortholog, HapX, functions to repress transcription. Though genome analysis showed lower than expected nucleotide conservation between this gene in *S. cerevisiae* and *C. neoformans*, the zinc-finger domain of the protein was highly conserved (Price et al., 2016). Several different methods were employed to compare the two orthologs, which showed highly conserved functional properties between the two organisms.

A *MIG1* deletion mutant strain (*mig1Δ*) was then analyzed via microarray analysis in an effort to determine the effects of *MIG1* on the transcriptome of *C. neoformans* (Caza et al., 2016). As expected, *mig1Δ* cells exhibited increased expression of genes involved in alternative carbon metabolism when compared to wild type (WT). Interestingly, differential expression was also noted between cells growing on iron-rich and iron-poor media, suggesting a possible link between *MIG1* function and iron availability. Further experimentation linked a functional *MIG1* gene to reactive oxygen species (ROS) resistance, particularly in cultures grown at 37°C (Caza et al., 2016). This

is important to note, as the temperature of the human body generally ranges from 36.1°C to 37.2°C.

A *mig1*Δ strain of *C. neoformans* showed relatively unaffected persistence in a murine macrophage model (Caza et al., 2016). Both the *mig1*Δ strain and the *hapx*Δ strain showed comparable fungal burden in lung tissue when compared to WT cells. However, when lacking both *MIG1* and *HAPX* functions, survival was noticeably decreased, and fungal burden was markedly smaller than WT or *mig1*Δ cells in all examined tissue types except the brain. This data indicates that the interaction between the Hap complex, specifically the HapX subunit, and Mig1 is essential for nutrient sensing and virulence in the pathogenesis of cryptococcus. Importantly, a *MIG1* deletion in *C. neoformans* has been directly linked with increased susceptibility to antifungal compounds such as fluconazole and tetracycline, the current front-line treatments for cryptococcal infections. As such, *MIG1* has been identified as a potential drug target. A strategic drug targeting *MIG1*, when given in conjunction with current treatments such as fluconazole and tetracycline, could positively impact the effectiveness of current treatment options.

SNF1

While Mig1 plays a crucial role in enforcing the preferential use of glucose when available, Snf1 is a well-characterized protein in *S. cerevisiae* that has an orthologous predicted gene (*SNF1*) in *C. neoformans*. As a serine-threonine protein kinase, Snf1 selectively phosphorylates serine or threonine residues on its specific substrates; in this way, Snf1 reverses the glucose-dependent repression of genes involved in the metabolism

of secondary carbon sources (see Figure 3). This allows *C. neoformans* to metabolize alternate carbon sources when necessary, such as in the glucose-poor environment of the mammalian lung upon initial infection. Carbon metabolism is a complex and integrated process, and the glucose-sensing function of the Snf1/AMPK pathway plays a role in regulating the opposing functions of Mig1. As mentioned previously, the Mig1 system is activated in a glucose-rich environment, and alternatively the Snf1/AMPK pathway is activated in nutrient-poor settings (Caza et al., 2016).

A deletion of the *SNF1* gene in *C. neoformans* yielded interesting results. Melanin production was significantly impaired, virulence was decreased, and alternative carbon source utilization was altered. As capsule production is dependent on carbon usage, regulation of this essential virulence factor was also disrupted. Cryptococcal cells were still able to disseminate to the CNS, though they were not able to survive for extended periods of time once they arrived (Yang et al., 2010).

A *SNF1* targeted knockout strain (*snf1Δ*) was created that showed significantly decreased virulence when compared to the WT strain of *C. neoformans*. After being injected with *snf1Δ*, mice had a median survival of 72 days. This was significantly longer than the 20-day median survival time of mice injected with WT *C. neoformans* (Yang et al., 2010). A large volume of data suggests that both Mig1 and Snf1 are essential for carbon acquisition and metabolism in the pathogenesis of cryptococcosis. By guiding the use of glucose and alternative carbon sources, these two proteins allow *C. neoformans* to survive and proliferate in a wide array of microenvironments.

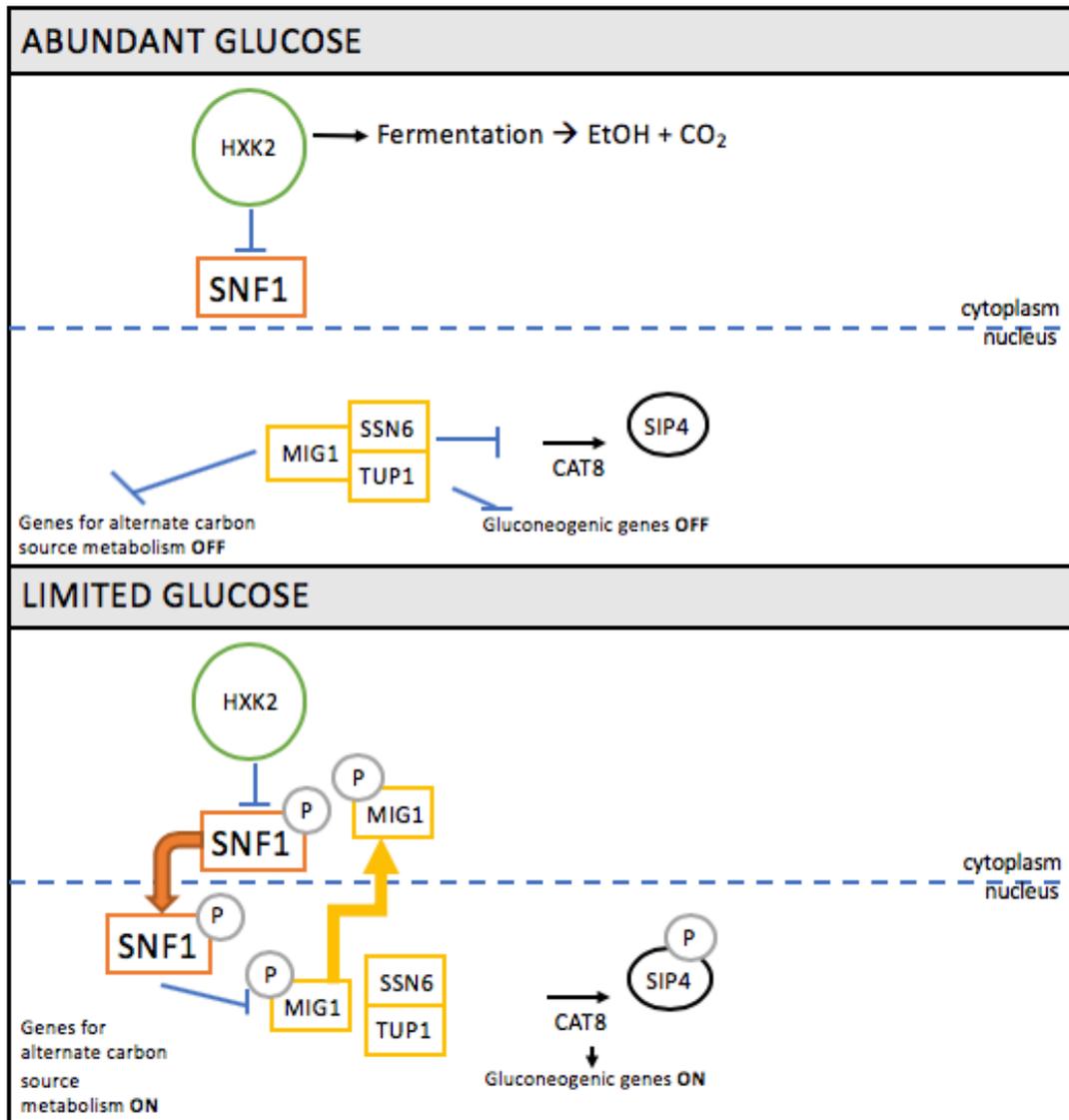


Figure 3. The SNF1/AMPK pathway regulates alternative carbon usage.

When glucose is limited, the SNF1/AMPK pathway is used to upregulate the expression of genes involved in alternative carbon source metabolism. Alternatively, when glucose is available, these same genes are repressed so that the cell can preferentially metabolize glucose. Adapted from “SNF1/AMPK/SnRK1 kinases, global regulators at the heart of energy control?” by C. Polge and M. Thomas, 2007, *Trends in Plant Science*, 12(1), p.

24.

Polysaccharide Capsule

In addition to playing a role in numerous normal cellular functions, glucose metabolism is essential for the creation of *C. neoformans*' characteristic polysaccharide capsule. The capsule is the best studied and most significant virulence factor in this pathogenic fungus, and it can grow to a width that is several times the cell's diameter (See Figure 4; Srikanta, Santiago-Tirado, & Doering, 2014). It also has antiphagocytic properties that prevent the host organism's immune system from recognizing its presence, providing an additional obstacle for clinicians and scientists working to treat cryptococcal infections (Srikanta, Yang, Williams, & Doering, 2011). Additionally, the dynamic nature of this capsule helps *C. neoformans* to evade human immune cells (O'Meara et al., 2010).

The capsule is primarily made of two virulent polysaccharides: glucuronoxylomannan (GXM) and glucuronoxylomannogalactan (GXMGal) (Wang et al., 1995). The monomers are synthesized in the cytoplasm of the cell before they are transported close to the cell surface and formed into long polymers. These polymers are then transported through the cell wall via vesicular transport before they are attached. Additional polymerization can occur outside the cell if the fungus is exposed to inducing conditions. GXM is a large polysaccharide comprised of mainly UDP-mannose subunits and accounts for approximately ninety percent of the capsule. In contrast, the major structural subunit of GXMGal is UDP-galactose. In addition to UDP-mannose and UDP-

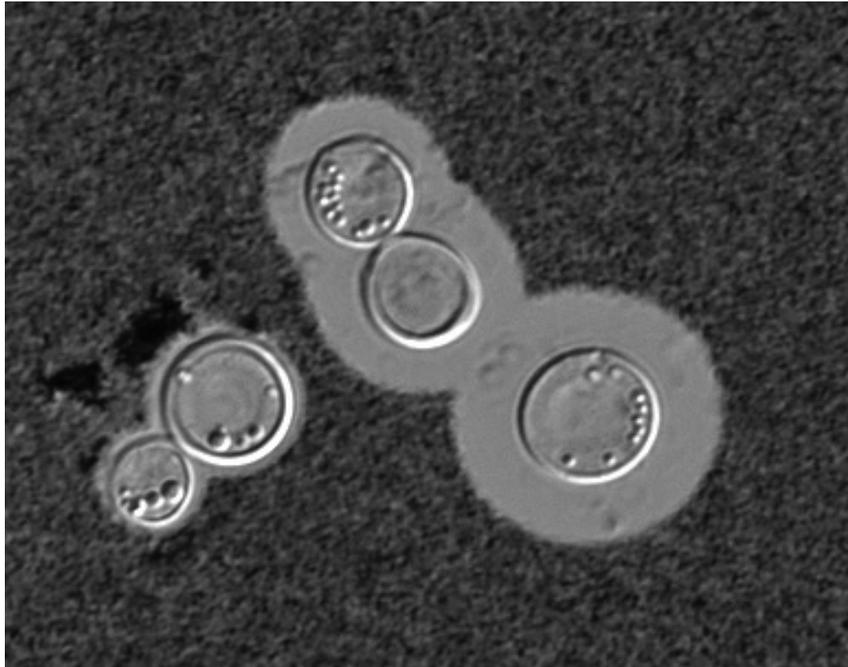


Figure 4. The polysaccharide capsule is a virulence factor of *C. neoformans*.

The polysaccharide capsule of *C. neoformans* grows to several times the cell's diameter and is essential for virulence. This image depicts JEC21, a strain of *C. neoformans*, after exposure to urea as a nitrogen source. Staining with India Ink is a technique commonly used to visualize the large capsule, as is seen in this image (Personal communication, M. S. Price, October 23, 2019).

galactose, UDP-glucuronic acid and UDP-xylose are necessary components of the carbohydrate backbone. Each simple sugar is covalently attached as the polymerization process occurs. Once the backbone structure is complete, it is modified with unique moieties that ensure proper folding and branching (O'Meara & Alspaugh, 2012).

Once specific moieties are added, the capsule is further modified by a variety of

different mechanisms. O-acetylation via the enzyme Cas1 glycosyltransferase occurs exclusively at the mannose and glucuronylated mannose residues (Janbon, Himmelreich, Moyrand, Improvisi, & Dromer, 2001). While their exact function is still under investigation, Pbx1 and Pbx2 are two B-helix proteins that modify the backbone by incorporating glucose residues (Liu, Kelly, Chow, & Madhani, 2007). Additionally, the Csp1 protein is responsible for synthesizing hyaluronic acid, a structural component of the capsule that allows *C. neoformans* to cross the blood brain barrier via facilitated transport. While the exact regulatory mechanism remains unclear, cells that lack hyaluronic acid have a decreased capsule diameter and an altered structure (O'Meara & Alspaugh, 2012; Jong et al., 2007). As dissemination of fungal cells and the ability to cause meningitis is dependent on *C. neoformans*' ability to enter the CNS, proper capsule synthesis is essential for virulence.

Interestingly, studies have shown that while *C. neoformans* is surrounded by a large polysaccharide capsule when taken from *in vivo* samples, *in vitro* cultures show varying degrees of encapsulation (Granger, Perfect, & Durack, 1985). Similarly, this fungus has the ability to alter the composition of its capsule depending on the microenvironment that it is in, and induction of capsule synthesis is often started upon entry into the host organism (O'Meara & Alspaugh, 2012). Once synthesis is induced, the capsule structure remains dynamic as it travels through the host. This dynamic nature of *C. neoformans* allows it to adapt in various organs such as the lungs and brain, which can be seen by the heterogeneous nature of capsules observed from a variety of organs (McFadden, Fries, Wang, & Casadevall, 2007). Additionally, biochemical analysis via

mass spectrometry and NMR showed variable side chain composition of GXM and GXMGal that allowed variable antibody binding depending on the environment. A variety of studies have been performed to better understand the biochemical regulation of capsule synthesis. While it is a very complex process that is still under investigation, several environmental parameters have been identified: iron concentration, CO₂ levels, ambient pH, glucose availability, and nitrogen availability (O'Meara & Alspaugh, 2012). When the cell encounters a less than favorable level of any of these parameters, capsule production is induced.

Studies have shown that mutated strains that are unable to produce a capsule are avirulent, confirming that the polysaccharide capsule is essential to the pathogen's survival in a host (Wilder, Olson, Chang, Kwon-Chung, & Lipscomb, 2002). This has important implications for clinical management, as a capsule inhibitor could serve as a targeted drug therapy when treating this disease.

It is important to note that the capsule of *C. neoformans* currently serves as an important diagnostic tool. Effective treatment of *C. neoformans* depends on early detection and diagnosis, which can be achieved via a highly sensitive antigen test that recognizes common capsule serotypes. Additionally, an India-ink stain can be used to identify encapsulated cells from a sputum culture or from bronchoalveolar lavage fluid.

Conclusion

Cryptococcosis, infection resulting from the *C. neoformans* species complex, kills thousands of immunosuppressed individuals annually. While the majority of these patients have end stage HIV/AIDS, other susceptible populations include transplant

patients on immunosuppressive drugs, cancer patients, and individuals with autoimmune disorders. Severity of the disease can range from localized cutaneous cryptococcus to widespread systemic infection with CNS involvement. Despite aggressive antifungal therapy, the prognosis of patients with *C. neoformans* infections continues to be poor; survival rates hover around fifty percent in patients with systemic disease, even in western countries where patients have access to sophisticated healthcare. Nutrient acquisition, and specifically carbon metabolism, is essential for *C. neoformans* persistence and proliferation in the host. Additionally, carbon is the main structural component in the polysaccharide capsule, the most well studied and arguably most important virulence factor of this devastating fungus. While treatments such as fluconazole do exist, they are expensive and only somewhat effective at treating late-stage and disseminated disease. As such, better understanding the relationship between carbon metabolism and virulence is critical to the development of new targeted drug therapies in the near future.

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