Review

Transcription Factors Control the Virulence-Associated Metabolic Enzymes and Membrane Proteins in Response to Nutrient Bioavailability in Cryptococcal Infection

Olufemi Samuel Folorunso * and Olihile Moses Sebolai *

Posted Date: 19 June 2023

doi: 10.20944/preprints202306.1372.v1

Keywords: Cryptococcus; biofilm; metabolic enzymes; virulence factors; transcription factors; membrane permeases; membrane transporters; catabolite repression

Review

Transcription Factors Control the Virulence-Associated Metabolic Enzymes and Membrane Proteins in Response to Nutrient Bioavailability in Cryptococcal Infection

Olufemi S Folorunso [1,2,*] and Olihile M Sebolai [2,*]

[1] Schepens Eye Research Institute of Mass Eyes and Ears Infirmary, Harvard Medical Teaching School, Department of Ophthalmology, Boston, MA USA; OSFolorunso@meei.harvard.edu

[2] Pathogenic Yeast Research Unit, Department of Biochemistry and Microbiology, University of the Free State, Bloemfontein, South Africa; SebolaiOM@ufs.ac.za

* Correspondence: SebolaiOM@ufs.ac.za; Tel.: +27-51-401-2004; 2014061341@ufs4life.ac.za; Tel.: +1-781-957-7963

Abstract: *Cryptococcus neoformans* is a pathogenic fungus that causes cryptococcosis, a significant secondary infection in immunocompromised individuals with immune-suppressive related symptoms from HIV infection and organ transplants. Fungaemia occurs via the respiratory route and spreads by tissue invasion into other body parts. Enzymes and membrane-associated permeases/transporters are accessory proteins deployed to enhance the survival, adaptation, and infection caused by *Cryptococcus* species. The nutrient bioavailability determines the functional turnover rate of these proteins in the immediate fungal environment. Low oxygen levels in the internal organs, essential micro-elements locked in the storage proteins, catabolite repression, phagolysosomal low glucose level and pH, tight junction, and extracellular matrixes are challenges to the survival of this pathogen in the host. This review identifies key hydrolytic and metabolic enzymes and permeases/transporters as essential weapons of virulence in addition to survival, tolerance, resistance, adaptation, and infection in humans. Under the regulation of different transcription factors, these proteins are released in response to nutrient sensors designed to siphon the host nutrients and induce infection in predisposed individuals. The extracellular secretory vesicles called "exosomal virulence bag" also harbour cryptococcal urease, laccase, phosphatase, and capsular components as additional secretory protein weapons for immune evasion, tissue invasion and persistence.

Keywords: *Cryptococcus*; biofilm; metabolic enzymes; virulence factors; transcription factors; membrane permeases; membrane transporters, catabolite repression; mutants; extracellular vesicles

1. Introduction

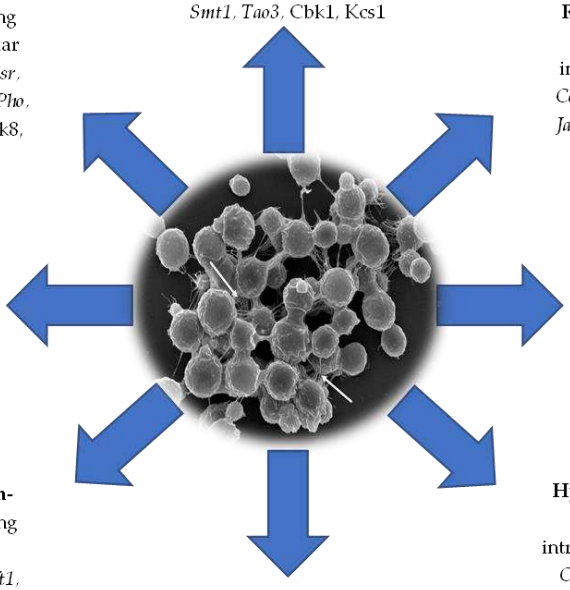
Cryptococcus neoformans is an opportunistic environmental yeast found within the shrub trees and humous soil contaminated with Aves dropping [1-3]. As an encapsulated and obligate aerobe, it becomes pathogenic and infects, invades, and colonises various animal and human organ systems [4]. Being an environmental saprophytic fungus, the internalisation of extracellular nutrients is necessary to survive. Following extracellular digestion, numerous membrane-associated proteins (permeases, transporters, and pumps) help with nutrient internalisation.

The assimilation of extracellularly digested nutrients and micro-nutrients is a complex metabolic system that requires enzyme sequestrations. In *C. neoformans,* membrane-bound proteins obtain ions such as Cu^{2+}, Fe^{2+}, and Zn^{2+} for membrane potentials. Pumps, such as Na^{+}/K^{+}-ATPase and P-type Na^{+}-ATPase (Ena1), are used to maintain membrane polarisation, while ABC (Afr1) and ABD-type

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 June 2023 doi:10.20944/preprints202306.1372.v1

multidrug homologue pumps (Pdr5, Yor1, and Snq1) are deployed for drug resistance. Several transporters and permeases like Ca^{2+} transporters (Cch1, Eca1, and Vcx1), Na^{+}/H^{+} antiporter (Nha1), aquaporin (Aqp1), phosphate affinity permeases (Pho) are implemented for cellular homeostasis, tolerance, resistance, antioxidation, and environmental stress response (oxidative and osmotic stress) [5,6].

One of the attributes of cryptococcal cells is the ability to form a biofilm, which is called cryptococcomas [7]. This is an essential phenotype of invasive pathogenic fungi and bacteria that involves a consortium of microorganisms adhering to one another in a polyfilm extracellular matrix of complex polymeric substances. Cells in this form come together for colonisation, extracellular matrix modelling, quorum sensing, resistance, protection, adhesion, survival, adaptation, communication, sharing, maturation, filamentation, and invasion [8-13]. Biofilm formation in *C. neoformans* is metabolically regulated, induced by unfavourable conditions and capsular components [14]. *In vivo* or *in vitro*, *C. neoformans* and *C. gattii* can form a biofilm, even on surfaces and invasive clinical instruments [14-19]. Redox process, anaerobic metabolism, substrate-level phosphorylation, proteolysis, and antiosmotic processes are upregulated during biofilm formation, but general metabolic processes, replication, transcription, translation, transportation, permeases, and pump systems are generally repressed [20]. Further work showed that cryptococcal cells are more resistant to antifungal and more virulent in the biofilm state due to elevated expression of *Cap59*, *Lac1*, and *Ure1* [15], **Figure 1**.



Adhesion, Invasion, and Colony morphology: Regulated under the following induced *transcription factors* and intracellular proteins –*Afr1*, *Amt1*, *Amt2*, *Cap60*, *Cps1*, *Gcs1*, *Ndh1*, *Pcl12*, *Pgi1*, *Ptp1*, *Ptp2*, *Sog2*, *Smt1*, *Tao3*, Cbk1, Kcs1

Cell wall, Membrane integrity, stability, potential, & trafficking, and Ergosterol metabolism: Regulated under the following induced *transcription factors* and intracellular proteins – *Atg8*, *Ccc1*, *Cda*, *Cdc*, *Cft1*, *Chs*, *Csr*, *Ctr1*, *Ena1*, *Gcs1*, *Erg*, *Grx4*, *Ipc1*, *Kre*, *Nha1*, *Pho*, *Pmm1*, *Rho1*, *Sec*, *Skn1*, *Smt1*, *Vph1*, *Vps*, Cdk8, Ire1, Kcs1, Mpk1, Pkc1

Redox: Regulated under the following induced *transcription factors* and intracellular proteins – *Ald5*, *Cat3*, *Cir1*, *Coq11*, *Ctr1*, *Cuf1*, *Dre2*, *Ena1*, *Grx6*, *Isa2*, *Jac1*, *Mcr1*, *Mir1*, *Mrs3/Mrs4*, *Nha1*, *Sat4*, *Sod1*, *Tah18*, *Yah1*

Thermotolerance and Heat-stress: Regulated under the following induced *transcription factors* and intracellular proteins – *Ada2*, *Apn2*, *Aro80*, *Atf1*, *Cdc24*, *Cdc42*, *Cdc43*, *Erg11*, *Ert1*, *Fzc31*, *Gat6*, *Gre2*, *Grx4*, *Hap1*, *Hsp10*, *Hsp12*, *Hsp60*, *Hsp70*, *Hsp90*, *Hsp122*, *Lhp1*, *Mbs2*, *Miz1*, *Puf4*, *Sp1*, *Rac1*, *Ras1*, *Sks2*, *Sre1*, *Usv101*, Bck1, Kcs1, Kic1, Pos5, Swe102, Vrk1

Filamentation: Regulated under the following induced *transcription factors* and intracellular proteins – *Asp1*, *Cam1*, *Cdc42*, *Cdc420*, *Cbp1*, *Cin1*, *Cna1*, *Cnb1*, *Crz1*, *Cuf1*, *Mat2*, *Pbx1*, *Pbx2*, *Pho4*, *Rac1*, *Ras1*, *Ras2*, *Ssn801*, *Sxi1*, Arg1, Cdk8, Kcs1, Ste12/Cph1, Ste20

Lighting, UV exposure, and Quorum-sensing: Regulated under the following induced *transcription factors* and intracellular proteins – *Bwc1*, *Bwc2*, *Cft1*, *Cqs1/Qsp1*, *Cqs2/Qsp2*, *Cwc1*, *Cwc2*, *Gat201*, *Gat204*, *Liv3*, *May1*, *Opt1*, *Pum1*, *Tup1*, *Rad17/Ddc1*, *Rad24*, *Ssn8*, *Uve1*, Crk1

Hypoxia: Regulated under the following induced *transcription factors* and intracellular proteins – *Alh302*, *Aox1*, *Lys10*, *Ctr3*, *Ergs*, *Fet3*, *Fre7*, *Gat1*, *Hsp78*, *Scp1*, *Sre1*, *Suc2*, *Sur2*, Tco1

Antifungal resistance: Regulated under the following induced *transcription factors* and intracellular proteins – *Aca1*, *Cac1*, *Cap59*, *Cuf1*, *Ena1*, *Fzc6*, *Grx4*, *Gat204*, *Hog1*, *Hsp12*, *Hxl1*, *Lac1*, *Mbs1*, *Nrg1*, *Pdr802*, *Ptp2*, *Ras1*, *Rim101*, Ure1, Arg1, Mpk1, Kcs1, Sch9, Ssk1, Ste12

Figure 1. Cryptococcal cells can form a matured biofilm unit with numerous fungi enveloped within a thin film coat of extracellular protective sheath. The sheath comprises polymeric protein molecules, chitins, nucleic acids, β-1,3-glycans, capsular components, and lipids derivatives. Cryptococcal cell latent activities in a biofilm are majorly associated with metabolic systems, mating, and growth; however, transcription factors involved in the virulence, resistance, tolerance, morphology, quorum-sensing, survival, and adaptation processes are also induced. The arrows indicated the extension of the protective sheath via which cells communicate. These connective sheaths are complex conjugated

 doi:10.20944/preprints202306.1372.v1

compounds of polysaccharides and structural proteins. The biofilm image was adapted from Martinez et al. [21].

Environmental abiotic factors (like pH, temperature, light, and CO_2) and predatory biotic interaction with the soil amoeba are challenges to *C. neoformans* that require adequate cellular taxis. Amoeboid protozoans can "*catch*" and "*kill*" internalised cryptococcal cells in a similar way to mammalian macrophages that phagocytose invading pathogens [22]. Surprisingly, cryptococcal cells can be engulfed yet can survive, replicate, and become more virulent intracellularly within the soil amoeba, an unconducive condition that may kill amoeba [23,24]. Morphologically, the engulfed *C. neoformans* usually display a pleiotropic phenotype forming pseudohyphal or yeast and lacking capsule or melanin with a high-impact mutation in genes like *Opt1, Pkr1,* and *Cpk2* [25,26]. In immunocompromised patients, the immune response against cryptococcal infection is weak and may lead to a life-threatening condition, meningoencephalitis.

Based on their immediate interaction, cryptococcal cells could be environmental or clinical. Both showed similar pathogenicity in animal models [27] because of the similarity in the virulence factors – capsule, melanin, extracellular secretory hydrolytic enzymes (like urease, phosphatase, DNase, lipase, protease, glycosidase), and membrane-associated enzymes (like laccase, IPC synthase, chitin synthase, catalase, and superoxide dismutase) [28]. Via the nutrient sensors like cAMP/Pka, Gpr1, Gpr4, Aap, Gpp2, and Hxt1, *C. neoformans* reprogram gene expressions to survive inadequate nutrient bioavailability and nutrient adaptation [29]. Nutrient shortages enhance membrane transporter and enzyme upregulations that ensure carbon intermediates shuttling from alternative pathways such as β-oxidation (mitochondria function) and glyoxylate cycle (peroxisome function) when phagocytosed by amoeba/macrophage. These two pathways fetch cryptococcal cells acetyl-CoA [30], which is converted via gluconeogenesis into various sugars needed for the cell wall components [31]. In addition, *C. neoformans* resorts to anabolic pathways to generate resourceful intermediates to synthesise melanin and capsule. This is observed with increased activity of fructose-2,6-bisphosphatase (encoded by *Fbp1*) when phagocytosed by amoeba and increased phosphoenolpyruvate carboxykinase activity (encoded by *Pck1*) when phagocytosed by macrophages [30,32].

Besides, several other upregulated transcription genes during phagocytosis have been reported. These include genes for nutrient absorption (amino acids, copper, and iron), antioxidative stress genes, and genes involved in autophagy, apoptosis, and mating [33]. In extreme cases, the redox process, substrate-level phosphorylation, and anaerobic decarboxylation can be employed to generate energy for cellular function and growth [20]; however, the lack of glucose or other utilisable sugars generally impairs cryptococcal proliferation, virulence, persistence, and tissue invasion as displayed by *Δpyk1, Δpyk1Δmig1, Δmig1, Δpck1, Δhxk1, Δhxk2,* and *Δhxk1Δhxk2* mutants in the presence of glucose, glycerol, or lactate [34]. The *Δacl1* mutants showed delayed growth in media with glucose with attenuated virulence and increased antifungal and macrophage susceptibility [35]. In addition, *Δpck1* mutants failed to carry out gluconeogenesis and attenuated for virulence in the murine inhalation model of cryptococcosis; however, *Δpyk1, Δhxk1,* and *Δhxk2* failed to utilise glucose and severely attenuated for virulence and CNS persistence [34].

Though similarities occur in the responses of *C. neoformans* to soil amoeba and mammalian macrophages based on their intracellular attack but the ecological niches surrounding the amoeba are quite different from the mammalian cells. Interaction of *C. neoformans* with the soil amoeba is usually at the ambient temperature (28 – 30°C) in a slightly acidic environment, while with the macrophage is at the physiological temperature of 37°C and a near-neutral pH range (7.2 – 7.4). By equilibrating the natural environment of *Acanthamoeba castellanii* and murine macrophage to *C. neoformans,* Derengowski et al. showed massive similarities in the modulation of several enzymes and membrane transporters involved in all significant metabolic systems with at least 2-fold increase as compared to the non-phagocytosed *C. neoformans*; though only a few numbers of these proteins were differentially modulated [30].

So, deploying several membrane transporters, permeases, and extracellular proteins for cellular nutrient balance and pathogenesis of *C. neoformans* is critical. We review several of these proteins and

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 June 2023 doi:10.20944/preprints202306.1372.v1

discuss their functional relevance to cryptococcal survival and adaptation in nutrient repletion or depletion and as weapons in human infections. Careful consideration of their consequences to mammalian cells is, however, needed if any of these proteins is considered a target for antifungal formulation against invasive cryptococcal infections in humans.

2. Environmental Nutrient Sensors determine metabolisms in *Cryptococcus* with Appropriate Release of Transcription Factors

The environmental transition of *C. neoformans* enables this fungus to cause infection in a susceptible host. During systemic infection, macrophage engulfs cryptococcal cells and restricts access to nutrients in the phagosomes. For these fungi to survive, alternative routes of nutrient acquisition are needed for adaptation, immune evasion, and virulence. A futile cycle is avoided in *C. neoformans* by switching on the essential and inevitable transcription factors, while some, considered redundant, are temporarily switched off. When phagocytosed, gene expression for membrane transporters, secretomes, cell wall formation, anti-oxidative and anti-nitrosative stress, anti-autophagy and peroxisome functions, intracellular metabolism, mating, and genomic repair are highly expressed. In contrast, the ribosomal metabolism and translational process are repressed even as the infection progresses [33]. Thus, *C. neoformans* possess a highly classical environmental sensor, robust enough to help decipher the micro-conditional changes and subsequently activate the fort of transcription factors for nutrient acquisition that translate into cellular morphology for infections and immune evasion.

Numerous environmental sensors are part of the membrane and cell wall integrity of *C. neoformans.* These sensors can associate directly or get involved in the signalling event induced by nutrient or environmental factors. With Rim21-Rim8 complex, *C. neoformans* responds to ΔpH; Cam1 senses the ΔpH and temperature; Tco1, Tco2, Ssk2, Ras1, phosphatidylinositol, Pdk1, and Rho-factors are various stress sensors; Can2 senses change in CO_2, and Gpr4 can be induced by methionine for Gpa1-Cac1 activation that produces cAMP/Pka [29].

2.1. Metabolism in C. neoformans is influenced by cAMP/Pka

C. neoformans possess arrays of metabolic enzymes that re-wire carbon metabolites to favour the sugar-conjugate products and metabolic intermediates such as acetyl-CoA used for capsule and melanin biosynthesis. In addition, the *in vitro* and *in vivo* analysis showed numerous membrane transporters specific for each nutrient within their environment [36]. *C. neoformans* secrete hydrolytic enzymes such as peptidases, esterase lipases, and glycosidases to release amino acids, fatty acids, and sugars, respectively. The plethora of enzymes involved in these metabolic activities is influenced by cAMP signalling pathways. The cAMP-deficient mutants are poor in sensing glucose (via Hxtp – a sugar/hexose transporter and G-protein coupled receptor, like Gpr1p) and amino acids (via the Gpr4p) because these metabolites provide precursors needed for growth, mating, and virulence which are constantly under the regulation of cAMP/Pka signalling factors [37,38]. The *Δgpr4* mutants are very poor in sensing certain amino acids, such as Met, which triggers receptor internalisation of Gpr4p and subsequent activation of the cAMP/Pka pathway [29]. However, *Δgpr4* mutants showed a normal response to glucose, unlike a poor glucose response from *Δhxt* mutants. Despite a good response to glucose, the *Δgpr4* mutants still displayed impaired capsule formation and mating defect, and both conditions are recoverable when cAMP is supplied exogenously [37].

The significant role of cAMP as a secondary messenger in metabolic regulation is invaluable, coupling environmental sensing to metabolism, virulence, cell differentiation, mating, and growth. Metabolisms generally begin with activating corresponding transporters, permeases, carriers, couplers, and pumps. This enables the endocytic movement of nutrients into the cell for metabolism. The transcriptome of *Δpka1* and *Δpkr1* mutants have been characterised by higher-level gene transcripts involved in different metabolic pathways [39] (**Figure 2**). Many of these pathways are activated via the cAMP/Pka pathways and regulated by Pkr1 expression.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 June 2023 doi:10.20944/preprints202306.1372.v1

C. neoformans is sensitive to glucose limitation, and phagolysosome does this by constantly maintaining acidic pH to minimise glucose availability to pathogens. To circumvent this, gluconeogenesis is promoted in *C. neoformans* to supply metabolic sugars needed for titanisation and capsule formation. The interdependence of peroxisome, glyoxysome and cytoplasm to break down assimilated fatty acids (*β*-oxidation) to generate acetyl-CoA is essential in driving the glyoxylate cycle that generates oxaloacetate used in gluconeogenesis.

Contrary to *C. albicans*, which showed up-regulated genes for key enzymes of gluconeogenesis [40], phagocytosed *C. neoformans* showed no upregulation of such enzymes [33]. However, transcriptional response and analysis of *C. neoformans* ingested by macrophages and amoeba showed that key enzymes like phosphoenolpyruvate carboxykinase and fructose-1,6-bisphosphatase were respectively upregulated [30]. The *Icl1* (encoding isocitrate lyase), *Mls1* (encoding malate synthase in glyoxylate cycle), and genes for *β*-oxidation enzymes are upregulated in *C. neoformans* recovered from murine macrophage [33], but *Mdh2* (encoding malate dehydrogenase) and genes for ergosterol biosynthesis were downregulated in the phagocytosed *C. neoformans* [30]. The findings of Rude et al. showed that though the Icl1p and the glyoxylate shunt are essential for ATP production during fungal infection, still *Δicl1* mutants failed to display any apparent virulent defect in animal studies and produced a *wt* level of capsule and melanin without any weakness in macrophage survival or phagocytic index [41].

2.2. Involvement of NADPH in Cryptococcus metabolism

In most biological reactions, reducing equivalents, such as NADPH, are highly required for redox processes. Hypervirulence-associated protein 1 (encoded by *Hva1*) has been identified to be associated with NADPH to regulate *C. neoformans* metabolisms [42]. An exogenous supply of NADPH alone promotes an animal tissue burden of *Δhva1* mutant in the spleen and brain than in the lungs [42]; Derengowski et al. discovered a multiple-fold increase in transcript level of *Hva1* in amoeba-phagocytosed cryptococcal cells [30]. The absence of Hva1p unexpectedly increased the level of phosphoenolpyruvate kinase but impaired the TCA due to reduced activity of 2-ketoglutarate dehydrogenase complex activity. This condition may attenuate mitochondria oxidative reaction but favours sudden energy increased from preponderant cytoplasmic NADPH via alternative pathways to ATP production. This metabolic energy production shift favours cell proliferation and growth, hence the hypervirulence trait of *Δhva1* mutants in the murine model but not in the moths or worms [42], making the *Δhva1* virulence traits temperature dependent. Comparing *Δhva1* to *Δhva1+Hva1* reconstituted mutants, there was no significant difference in the animal, moth, and worm virulence models, as well as in capsule size and structure, melanin production, GXM content, phospholipase and urease activity, growth and doubling time under stressful conditions, survival and fungal burden in macrophage [42]. Therefore, Hva1p plays essential metabolic roles that may support tissue invasion; however, *Hva1* is dispensable for cryptococcal virulence and infection.

2.3. Iron regulators induced iron-dependent metabolic enzymes

The exocyst complex vesicles (guided by *Sec* genes), enzymes (such as glutamate dehydrogenase, *p*-aminobutyrate aminotransferase, proteasomes, ubiquitin ligase, RNA-processing enzymes, and *m*RNA splicing proteins), and transporters/permeases (for allantoate, urea, ammonia, and purine-cytosine, involving in nitrogen and amino acid metabolism) have been observed to be elevated under the iron-enriched media [43]. Not only these, but iron also influences several of the amino acid metabolism (anabolism and catabolism).

C. neoformans is among the pathogenic fungi that utilise other carbon sources, including alcohol. Inexpediently, mutants lacking *Hap3* and *Hap5* showed weaker growth in sucrose, acetate, and ethanol and are more sensitive to 0.01% SDS [44]. In contrast, the *ΔhapX* mutant and the *wt* showed similar growth in the glucose, sucrose, acetate, and ethanol, even in 0.01% SDS [44]. Although *Hap* genes are iron-dependent regulatory genes, surprisingly, supplementing the media with hemin failed to improve the weak growth in *hap* mutants [44]. Because of this, iron-enrich media specifically improved

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 June 2023 doi:10.20944/preprints202306.1372.v1

gene expression encoding several iron-dependent enzymes in the glycolytic pathway, TCA cycle, functional oxidative pathways, and mitochondrial redox processes, but gene expression targeting the pentose phosphate pathway was scarcely tagged in the SAGE analysis [43]. Apart from these elevated key enzymes, other accessory proteins such as ubiquinone, prohibitin (a membrane-bound chaperone stabilising the mitochondria proteins), and ATPases are equally pre-eminent [43]. The expression of TCA-targeting enzymes such as aconitase and succinate dehydrogenase further confirmed the importance of the heme and Fe-S redox proteins to the upkeep of *C. neoformans* metabolic systems. Aconitase identified in *C. neoformans* appears as paralogous encoding genes that possibly enhance its extracurricular activities, such as *m*RNA stability and translation – functions known for iron regulatory proteins (Irp1). By speculation, *C. neoformans* might regulate excessive iron by converting this Irp1p to cytosolic aconitase, which fails to stabilise *m*RNA but promotes Irp2 ubiquitination and proteasomal degradation to avoid iron toxicity [45].

In *S. cerevisiae,* iron may orchestrate the binding of α-isopropylmalate to homodimeric DNA-binding protein Leu3 to regulate branched-chain amino acid biosynthesis, amino acid permeases (Aap), nitrogen, and carbohydrate metabolisms [46,47]. The expression of *Lys4* for Lys biosynthesis and *Leu1* for Leu biosynthesis is iron-dependent because of the presence of conserved aconitase repeating motifs that bind Fe-S [48], and possibly other amino acids biosynthesis proteins such as Met2p, Met3p, and Met6p for Met; Ilv2p for Ile and Val may have aconitase repeating motifs. In *C. neoformans,* mutants generated from these transcription factors are primarily auxotrophs, avirulent or attenuated for virulence in the murine infection model of cryptococcosis, and highly sensitive to antifungals [49-51]. This iron-coordinated virulence is centred on the interplay of Hap3 and HapX proteins.

Further into the regulatory roles of Hap3 and HapX, these two transcription factors negatively modulate the expression of the orthologue *Lys4* (encoding homoaconitase that converts homocitrate to homoisocitrate in lysine biosynthesis) in the LIM. This was confirmed by low detection of *Lys4* transcripts in *ΔhapX* and *Δhap3* mutants cultured with 100 μM $FeCl_3$, but in the absence of $FeCl_3$, the expression of *Lys4* is well induced in *Δhap3* and *ΔhapX* mutants [52]. Relative to the *wt*, the expression of *Lys4* remained unchanged in *Δcir1* mutants in either iron enrich media (IEM) or LIM. This confirmed the critical importance of the *Cryptococcus* iron regulator factor (Cir1p) in the cellular regulation and distribution of iron.

Like *Lys4*, *Leu1* (encoding 2-isopropylmalate synthase) expression was similarly regulated by Hap3 and HapX proteins in the LIM or IEM. Low iron supports the expression of *Leu1* and *Lys4* in *Δhap3* and *ΔhapX* mutants, but only a high level of iron favours the expression of *Leu1* in the *Δcir1* mutant [49]. This means that Cir1p positively regulates this cytoplasmic located Leu1p in *Δhap3* and *ΔhapX* mutants cultured in LIM but regulates negatively in IEM. There is speculation, however, that *Leu1* expression may be more under the regulation of Hap3 than HapX expression because while *ΔhapX* mutant produced a comparable *Leu1* transcript with the *wt*, *Δhap3* mutant had a significantly more transcript of *Leu1* under the same IEM conditions [49]. Like *Lys4* expression, *Leu1,* with a putative —CCAAT—HAP binding element at the promoter site for repression during the iron-limiting condition, is also very important in Leu biosynthesis; however, the uptake of these amino acids is generally under the influence of nitrogen catabolite repression (NCR).

3. Essential Enzymes of Metabolism and Membrane Transporters/Permeases are Controlled by Transcription Factors in cryptococcal cells for Virulence, Survival, Resistance, and Adaptation

3.1. Deleting phosphoglucose isomerase impairs the Hog1 pathway, capsule production, membrane integrity, and alternative pathways

To further appreciate the involvement of metabolic systems and their regulatory enzymes in the virulence, cell wall integrity, and stress-resistance phenotypic traits in *C. neoformans,* the lack of *Pgi1* expression (encoding phosphoglucose isomerase/glucose-6-phosphate isomerase), though showed no observable difference in the cell morphology but resulted in reduced capsule biosynthesis,

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 June 2023 doi:10.20944/preprints202306.1372.v1

impaired cell wall integrity, fragile cell membrane, osmotic stress hypersensitivity (due to impaired Hog1 pathway), and failure to utilise mannose and fructose [53]. Insertional T-DNA mutation at the promoter site of *Pgi1* reduces the activity of Pgip. In the presence of a 2% glucose supplement, the *Δpgi1* mutant could produce melanin due to the derepression of *Lac1*. Further work showed that an exogenous supply of cAMP could restore capsule deficiency in the *Δpgi1*, but this repressed the *Lac1* expression [53] **(Figure 2)**. Even in the presence of glucose, mutation of *Pgi1* expression may have limited glucose catabolism and subsequently altered the ATP homeostasis. Conversely, this condition favours Snf1p activation leading to the deactivation of Mig1p and subsequently derepresses *Lac1* for melanin production, **Figure 2**. Therefore, the involvement of the Snf1-Mig1 regulatory pathway through nutrient homeostasis cannot be ruled out in the pathogenesis and virulence of *C. neoformans;* however, this is tightly regulated by the Pgi activity under a considerable basal glucose level.

3.2. Low glucose level activates serine/threonine protein kinase 1 complex to induce genes with SRE-promoter sequence for stress response

The mammalian homologue of AMPK protein is Snf1p in the yeast, which is critical to cellular energy homeostasis through oxidative phosphorylation by facilitating sugar and fatty acid uptake. In a normal state, Snf1p (encoding serine/threonine protein kinase 1 complex) is turned off by Reg1-Glc7 type I protein phosphatase 1 (PP1) by dephosphorylation but activated by phosphorylation as the glucose level decreases [54-58]. Mutation in Reg1p or absence of Gpr1 sugar sensor caused increased activation of Snf1p with poor growth in the yeast; however, any alteration in the negative regulatory sub-units of cAMP/Pka (Ira1, Ira2, and Bcy1) will suppress Snf1 pathway activation [54], **Figure 2**.

High glucose level represses the Snf1 pathway in the yeast [54]. By speculation, the Snf1 pathway in *C. neoformans* may have been regulated similarly. Increased glucose level activates the Cac1→cAMP→Pka pathway to repress the C_2H_2 zinc-finger nuclear-translocated proteins, Msn2p and Msn4p, **Figure 2**. The Msn2 and Msn4 are programmable proteins that induce genes containing SRE-sequence promoters, such as *Hxt1, Hog1, Ras1* and other genes belonging to NSR, OSR, OMSR, ESR, GSR, HMSR, or CSR (see **Supplementary file** for definition), which Snf1 activation promotes under low glucose level, **Figure 2**. In addition, the cAMP/Pka inhibits the redundant upstream Snf1p-activation kinases, Sak1, Tos3, and Elm1, in the yeast to put Snf1 under repression at a high glucose level [54].

Relevant to *C. neoformans,* the high-glucose-deactivating Snf1 via the Pka leads to the activation of Mig1p by dephosphorylation to enhance the association of Mig1p with Suc2p (**Figure 2**). The Mig1p is a universal gene repressor in the yeasts [59], which binds the promoters of target genes, including the *Lac1* gene, to repress melanin biosynthesis as a response to glucose repression (**Figure 2**). In low glucose levels, however, the Cac1 activity is reduced to lower the cAMP level, which invariably elevates the Sak1 activity to activate Snf1p [60]. The activated Snf1p further inhibits the Cac1 to promote nuclear translocation of Msn2p to induce *Hxt7* and other similar stress response genes, including the *Lac1* gene, **Figure 2**. Unprecedentedly, the involvement of *Snf1* expression in antifungal, oxidative, and osmotic stress responses is less significant. Though Hu et al. claimed to have observed increasing sensitivity of *Δsnf1* mutants to amphotericin B relative to the *wt* response; however, the *Δsnf1* mutants showed no significant growth defect to AmpB and fluconazole but a very slight growth defect to rapamycin [61].

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 June 2023 doi:10.20944/preprints202306.1372.v1

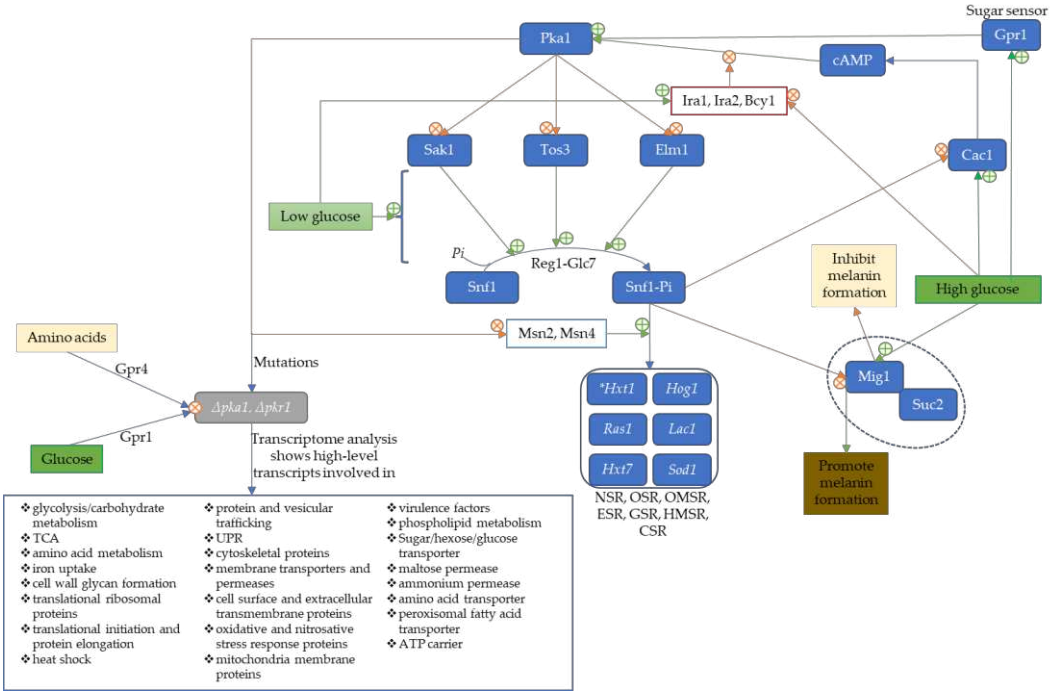


Figure 2. Glucose level dictates the conditional expression of *Snf1* and *Mig1* in the canonical regulation of genes with SRE-promoter sequence orchestrated by the nuclear translocation of Msn2p and Msn4p. The SRE genes are expressed for environmental adaptation, alternative sugar utilisation, virulence, osmotic, and oxidative responses. Ira1, Ira2, and Bcy1 are inherent negative regulatory factors of Pka activity. Low glucose level activates these intrinsic regulatory factors to prevent Pka-Snf1p inhibition. A high glucose level reverses this situation and eventually prevents the activation of Snf1p via the Pka-Msn2p inhibition. *gene solely depends on *Snf1* expression, especially in the non-glucose medium. Deletion of *Pka1* and *Pkr1* in the cAMP/Pka pathway induced several transcription factors for various metabolic pathways. Induced/activated (+); Repressed/inhibited (×).

3.3. Sugar/Hexose transporters (Hxt1 and Hxt7) are unexclusively dependent on the induction of serine/threonine protein kinase I complex (Snf1)

Generally, phenotypic defects of *Δsnf1* mutants become conspicuous with increasing temperature. For example, melanin formation decreases above 30°C and becomes a non-melanised mutant at 37°C [61]. Surprisingly, though the transcript level of *Snf1* is practically increased in a glucose-limited or acetate medium, there is no evidence of increased transcript in the cryptococcal cells recovered from the infected lungs [61]. Besides, *Δsnf1* mutants in a glucose-limited medium displayed an increased marginal level of *Ady2*, *Acs1*, *Ato2*, and *Pck1* transcripts; a marginal decrease in the expression levels of *Ctr4* and *Cgl1*; and a significant reduction in the *Hxt1* and *Cft1* transcripts [61]. So, the last two transcription factors depend solely on the *Snf1* expression, especially in the glucose-limited medium **(Figure 2)**. In the acetate medium, however, deletion of *Snf1* showed no effect on the expression of all these genes except the *Hxt1*, where an elevated transcript is observed [61]. Additionally, the report showed that *Δsnf1* mutants displayed a *wt Mls1* transcript irrespective of the media used [61]. The activation of Hxt1p in *Δsnf1* mutants cultivated in the acetate medium showed the characteristic of glucose-deprived cryptococcal cells and readiness to assimilate sugars as soon as it is available.

3.4. Low glucose level promotes melanin and antioxidative protein expression via serine/threonine protein kinase 1 complex

In addition to *Lac1* regulation, *Snf1* expression also affects the SOD activity. A drastic reduction of *Lac1* in *Δsnf1* mutants cultivated in the glucose-limited medium indicates the *Lac1* expression being regulated by Snf1p [61]. Similarly, the *Sod1* transcript is reduced in *Δsnf1* mutants in a glucose-limited medium, even in the acetate medium [61]. Irrespective of the medium, deletion of *Snf1* failed to affect

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 June 2023 doi:10.20944/preprints202306.1372.v1

the expression of *Fhb1, Tps1, Glr1,* and *Skn7* significantly, but *Lac2, Ssa1,* and *Tsa1* transcripts are increased considerably in this mutant when cultured in 2% acetate media [61]. This means that in the glucose-limiting medium, Snf1p orchestrates the expression of *Lac1* (by preventing the Mig1-Suc2 association, **Figure 2**) and *Lac2* (in alternative carbon source) to produce melanin and the expression of *Sod1* against oxidative stress in the *wt* [61].

Strategically, *Lac2* expression may be preferable for melanin synthesis under alternative carbon sources coupled with oxidative and nitrosative stress, which might have been addressed by the concomitant expression of *Tsa1* under the same nutrient-limiting medium. Though the *Δlac1* and *Δlac1Δlac2* mutants are *albino* in nutrient-limiting conditions, alternative carbon sources appeared to elevate *Lac2* expression in the *Δlac1* mutants background to cause infection in a murine model [61,62]. In contrast, *Δlac2* mutants are melanised in catecholamine-enriched media [63]. Therefore, an elevated *Lac2* transcript more than *Lac1* in the presence of alternative carbon sources such as acetate [61] is a reminiscence of *Lac2* possibly involvement in melanin production when unusual carbon sources weaken *Lac1* involvement.

3.5. Deleting acetyl-CoA forming enzymes in C. neoformans impairs alternative use of carbon sources and attenuates the virulence

The roles of *Acs1* (acetyl-CoA synthetase) have been investigated in the cryptococcal cells recovered from the lungs of infected animals. Evidence showed an elevated expression of the *Acs1* gene in *C. neoformans* during infection, as well as Kbc1, coupled with concurrent elevated expression of different membrane transporters and stress response genes, indicating a nutrient-limited environment and the route for alternative carbon sources as facilitated by the expression of *Snf1* [61]. Including Acl1 (ATP-citrate lyase) and Kbc1 (2-ketobutyryl-CoA/acetoacetyl-CoA synthetase), any singly mutated of these genes impairs phagocytic resistance of *C. neoformans,* and doubly mutated genes (*Δacs1Δkbc1)* reduced CNS fungal burden further [64]. Glucose is equally consumed in the *Δkbc1, Δacl1,* and *Δacs1Δkbc1* mutants compared to the *wt;* however, *Δacs1* and *Δkbc1Δacl1* mutants showed a slight reduction in glucose consumption. In addition, *Δacs1* and *Δacs1Δkbc1* mutants failed to utilise acetate and are significantly susceptible to FCZ [64]. Deleting *Acs1* yielded moderately attenuated mutants that could not use alternative carbon sources such as acetate, and deleting *Acs1* and *Acl1* generated sterile mutants. This shows that Acs1 and Kbc1 may be necessary enzymes needed for an alternative carbon source utilisation in *C. neoformans*.

3.6. Phospholipases B is a crucial enzyme to cellular tight-junction penetration by C. neoformans

The secretion of extracellular phospholipases (PLs) by *C. neoformans* occurs as a cluster of PLs comprised of PLB, lysoPL hydrolase, and lysoPL transacylase/acyltransferase (all together referred to as PLB). These three oligomeric proteins are borne on the same coding gene, the *Plb* gene, just like in other yeasts such as *Penicillium notatum* [65], *S. cerevisiae* [66], and *C. albicans* [67]. PLs, best secreted at 37°C [68], are a highly diverse group of hydrolytic enzymes targeting the ester linkages of glycerophospholipids carbonyl linkages and can also transfer acyl-chain to the lysophospholipid to form diacylglycerophospholipid. Chen et al. showed that PLs are well secreted between 6 – 10 hours of cell culture at 30°C, with optimal activity between pH 3.5 – 4.5 and stability at pH 3.8. The enzyme activity was not affected by exogenous serine protease inhibitors such as leupeptin, phenylmethylsulphonyl fluoride (PMSF), divalent cations (Ca^{2+}, Mg^{2+}, and Zn^{2+}), and EDTA [69]. The stability of this enzyme at acidic pH reveals one of the *C. neoformans* tolerance phenotypes in the phagosome micro-acidic environment. In so doing, the PLs may break down the phagosomal membrane leading to tissue dissemination.

PLs degrade membrane phospholipids in response to signal transduction. It is a complex extracellularly released enzyme that facilitates fungi-host cell attachment, tissue invasion, and hydrolysis of the epithelial plasma membrane for penetration. The activity of the PLs at the cell wall promotes survival against temperature, antifungals, oxidative stress, and cell wall destabilisers [68]. Moreover, the involvement of PLC as a component of PLs in the virulence expression, antifungal resistance,

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 June 2023 doi:10.20944/preprints202306.1372.v1

homeostasis and IP3 kinase activity, and cytokinesis displays the significant functions of this membrane-associated enzyme [70,71].

Every *Δplb1* mutant is usually marked with a drastic reduction in the activity of each enzyme compared to the *wt* [69,72]. By correlation, PL activity corroborated the mortality rate and mucosal invasion by *Candida albicans* in animal models [73]. Similarly, *C. neoformans* may use these hydrolytic enzymes to invade tissue and cross the blood-brain barrier (BBB). The production of PL appeared similar in environmental and clinical strains of *C. neoformans* var. *gattii*, but the environmental isolates of *C. neoformans* var. *neoformans* produced more PL than the clinical isolates [69]. Various isolates of *C. neoformans* possess different degrees of PL secretion. This was shown to correlate with the fungal virulence and tissue burden in the lungs and brains of mice inoculated intravenously [69,72].

The *Ste12α* gene has been implicated in the regulation of extracellular PLs. Deletion of the *Ste12α* gene significantly reduced the production of PL in the egg yolk agar media [74]. As crucial as PLs are to the virulence of *C. neoformans*, the absence of these hydrolytic enzymes may not affect the virulence phenotypes, such as growth at 37°C, urease activity, capsule, and melanin formation. Paradoxically, such a mutant is less virulent in the *in vivo* mouse inhalation and rabbit meningitis infection models when compared to the *wt* in so much that the mutant growth is defective in a macrophage-like cell line [72]. The attenuated virulence observed in the *Δplb1* mutant must have come from the lower density of the capsule; notwithstanding, the capsule and the cell wall dimensions are relatively retained compared to the *wt* [68].

The attenuated virulence observed in the *Δplb1* mutant is likely comparable to *Δure1* and *Δlac1*. The PLB and the accessory enzymes are generally associated with *C. neoformans* virulence, yet an infection can still progress in their absence but at a slower rate [68,72]. Increasing the temperature from 30 – 37°C promotes transmigration of the PLB from the cell membrane to the cell wall with a concomitant increase in the cytoplasmic translation, perhaps due to constitutive secretion from the Golgi apparatus. However, secretion of PLB under this heat stress is highly minimised to maintain cell wall integrity and promote membrane homeostasis [68].

3.7. Deleting membrane-associated antioxidative enzymes severely reduced PLB, urease, and laccase expression in C. neoformans

Efficient antioxidant control has been linked to PLB production. Mutation of plasma membrane-associated Cu/Zn SOD encoded by the *Sod1* gene has been characterised by a severe reduction in the phospholipase B, urease, and laccase expressions, leading to attenuated virulence manifested in the low brain colonisation and persistence in a mouse model of cryptococcosis [75]. Though the *Δsod1* mutants appeared normal in their growth, capsule production, mating, and sporulation, the mutants are susceptible to menadione (a redox cycling agent) and PMN (neutrophils) killing [76]. The mutant culture was also characterised by high mannitol production that possibly acted as an antioxidant to complement the loss of Cu/Zn SOD activity [76,77].

Similarly, mutation of mitochondrial SOD (Mn-SOD) encoded by *Sod2* produced thermosensitive and growth defective mutants in normoxic conditions coupled with the accumulation of reactive oxidants in the presence of stress-induced agents like antimycin A, menadione, high salt contents, and nutrient limitations [78]. Furthermore, the poor growth of the mutant at 37°C ensued in either avirulent or severely attenuated infectious conditions in mouse models of cryptococcosis. An exogenous supply of antioxidants like ascorbate could restore poor mutant growth [78].

Sequential deletion of *Sod1* and *Sod2* genes produced summative disadvantages in *C. neoformans* infections and tissue invasion. Further virulent attenuation, PMN killing, poor growth at 37°C, osmotic stress, and susceptibility to Paraquat™ and oxytetracycline but not to menadione are the general features of *Δsod1Δsod2* mutants [78].

3.8. Phospholipase C orchestrates the release of PLB, DAG, and IP_3 during C. neoformans infection

PLB covalently attaches to the cell wall chitin protruding *β*-1,3-linked glucan via its *β*-1,6-linked glucan. This arrangement is anchored by the glycosylphosphatidylinositol (GPI) within the

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 June 2023 doi:10.20944/preprints202306.1372.v1

membrane lipid raft proteins. The release of PLB from the GPI-anchor is possibly orchestrated by the phosphatidylinositol-specific phospholipase C (PI-PLC, putatively encoded by *Plc1* and *Plc2*), and the inclusion of *β*-1,6-linked glucan to this release confirms the cell wall localisation of the PLB [68,70]. This shows that PLB constitutes part of the proteins involved in the cell wall integrity because *Δplb1* mutant exhibited morphological cell wall defect, which is sensitive to SDS and Congo red disruptions but not caffeine [68]. The *Δplc1* mutant of *C. neoformans* var. *grubii* strain H99 failed to produce melanin, refused to grow at 37°C and exhibited impaired PLB secretion with concomitant accumulation of cytoplasmic PLB [70,71]. Furthermore, this mutant displayed high antifungal sensitivity, poor replication, and a defective cell wall with irregular morphology due to impaired activation of the Pkc/MAPK pathway [38].

Because the *Δplc1* mutant had poor growth at 37°C, its virulence was attenuated in the mice study. Also, even at 25°C, this mutant failed to kill a significant number of *Caenorhabditis elegans* compared to the *wt* or reconstituted mutant [70,71]. The actual killing of the worms at 25°C by the *wt* or reconstituted mutant indicates that the virulence involvement of Plc1 may be temperature independent. Contrarily, the *Δplc2* mutant failed to exhibit any significant defect and is as virulent as the *wt* [70].

Activated PLC breaks membrane-associated phosphatidylinositol-4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). The IP_3 produced by Plc1 can bind its ER receptors to release Ca^{2+} intracellularly or serve as a substrate for IP_3 kinase (encoded by *Arg1*) to produce inositol polyphosphate. Expectedly, the *Δplc1* mutant generally accumulates PIP_2 but a reduced level of IP_3, whereas in the *Δarg1* mutant, IP_3 was accumulated [71]. Invariably, these two mutants shared similar phenotypic defects, including impaired thermotolerance, defective cell walls, impaired virulence factors, improper cell division, and defective mating and filamentation [70,71]. Further analysis showed that *Δarg1* accumulated larger intracellular vacuoles (excessive vacuolar fusion) than the *wt* and *Δplc1* mutant [71]. Therefore, Arg1p inositol polyphosphate anabolism (IP_{4-8}) is as important as the Plc1p catabolism for *C. neoformans* virulence, and IP_3 may be acting as a biological relay molecule for functional Plc1p.

Being a eukaryote, *C. neoformans* compartmentalise and adopt differential regulation of transcription factors to facilitate catabolism (in the mitochondria, glyoxysome, and peroxisome) or anabolism (in the cytoplasm) depending on the inducing factor and the available nutrients. For example, IEM elevates enzymes (including fatty acid synthase and acetyl-CoA carboxylase) in the cytoplasmic lipogenesis and promotes extracellular digestion of lipids (lipolysis) which is usually commenced with the release of phospholipase B (encoded by *Plb1*) [43,47,79,80]. Analysis showed that *C. neoformans* possessed genes for lipase (encoded by *Tgl2* among at least three encoding genes) and enoyl-CoA hydratase [43,80]. However, there is no specific evidence of the functional extracellular lipase expressed in this fungus due to the lack of a signal peptide in these proteins, unlike the lipase secreted by *C. albicans* [79,80].

3.9. Glycerol-3-phosphate phosphatase controls S-containing amino acid synthesis via cystathionine-γ-lyase and calcineurin pathways

In a DNA microarray analysis, nearly 20% of total gene expression is dedicated to carbohydrates and amino acid metabolism [81]. The expression of *Gpp2* encoding glycerol-3-phosphate phosphatase controls various metabolic systems as well as osmotic and cold shock responses. Deletion of Gpp2 induced various permeases/transporters (like Aap, Mup1, Mup3, Ena1, Nha1, Plb1, Hxt1, Stl1, Uga4, sulphite, myo-inositol, and pantothenate transporters), major facilitator superfamily genes (MFS) (encoding secondary transporters like uniporter, symporter, and antiporter), anti-oxidative enzymes (like Cat2, Ccp1, and Gst1), and enzymes for sulphur-containing amino acid biosynthesis (such as Cys3, Sul1, Soa1, Bds1, Met17, and Jlp1) [82]. However, various genes involved in the redox process, the glycolytic pathway, and the TCA cycle are downregulated in *Δgpp2* mutant [82,83].

Among the sulfur-containing amino acid biosynthesis regulated by Gpp2p are Met and Cys. The deletion of *Gpp2* alters the biosynthetic regulation of these amino acids due to the perpetual activation

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 June 2023 doi:10.20944/preprints202306.1372.v1

of amino acid permease (Aap), including Aap4 and Aap5, which most likely explains the increased sensitivity of the *Δgpp2* mutants to eugenol because the deletion of *Aap4* and *Aap5* is hitherto shown to allow eugenol resistance in *C. neoformans* [82]. The functional absence of Gpp2 has not been shown to be restored by exogenous glycerol or other types of amino acids [82]. Comparatively, *Δhog1* mutants are also sensitive to eugenol because Aaps are induced in *Δhog1* mutants [84]. Thus, the osmotic-related stress response in *C. neoformans* is controlled by the release of glycerol in this pathogenic yeast. In the absence of glycerol-coordinated osmotic resistance (*Δgpp2* or *Δhog1*), a reductive glutathione cycle is induced in *C. neoformans*, such that Cys feeds the glutathione cycle with -SH (sulfhydryl) to regenerate GSH (reduced glutathione), which is channelled against osmotic stress and reactive oxidants [82].

Just as in *A. nidulans* and *N. crasssa*, deletion of *Cys3* (encoding cystathionine-γ-lyase) in *C. neoformans* has been shown to reduce the expression of *Sul1*, and in the synthetic dextrose (SD) media, the two genes are grossly repressed in the presence of *S*-containing amino acids (Met or Cys) more than in the YPD [83]. Again, Cys appears better as a sulphur source than Met because methionine could be formed from cysteine in a transulphurylation reaction [83]. Further investigation on *Δcys3* mutants showed a *wt* level of urease activity and capsule size yet attenuated for virulence in *Galleria mellonella* [83]. Bad still, the experimental conditioning with Cys or Met in the culture media failed to support the melanin and phospholipase assay of this mutant [83]. This means the full virulence of *C. neoformans* is not just an individual and independent involvement of transcription factors but a concerted mechanism of many functional factors.

The proteomic analysis that showed a concurrent induction of *Met3*, *Tef1*, *Gpd1*, *Ccp1*, *Rps0*, *Rps1*, *Tif1*, *Sub2*, *Tm8*, *Gpp2*, *Cna1*, and *Cnb1* established a metabolic relationship among the *S*-containing amino acid biosynthesis, glycerol phosphatase, and calcineurin pathway (Cna1/Cnb1) [82,83]. Under the influence of Ca^{2+}, the expression of Cna1/Cnb1 induces and maintains Cys3p protein levels for nuclear localisation and subsequently increases the Cys3p-target genes such as *Sul1*, *Met2*, *Met3*, *Met10*, *Str1*, and *Sam1* to promote sulphur, purine, Gly, Ser, Thr, Asp, Asn, Cys, and Met metabolism. However, Gpp2p orchestrates the inactivation of Cys3p by proteolytic degradation to maintain amino acid homeostasis. A proposed mechanism is that inorganic sulphur induces Cna1/Cnb1 activation to deactivate Gpp2p. The Gpp2p deactivation promotes Cys3p and activates downstream genes involved in *S*-containing amino acid biosynthesis [83]. However, organic sulphur in Met and Cys deactivates Cna1/Cnb1, which activates Gpp2p to deactivate Cys3p and repress the downstream genes involved in *S*-containing amino acid biosynthesis [83], **Figure 3**.

Among the genes induced in *Δcys3* mutant are putative *Aro3* and *Aro4* involved in aromatic amino acids biosynthesis; *Jlp1* in sulfonate metabolism to provide SO_4^{2-} for Met5/Met10p; sulphiredoxin in oxidative stress response; and *Clr6* that encodes class I histone deacetylase [85]. However, it is interesting that repressing *Clr6* in the *C. neoformans wt* promotes capsule formation and biosynthesis of various amino acids [86] (**Figure 3**).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 June 2023 doi:10.20944/preprints202306.1372.v1

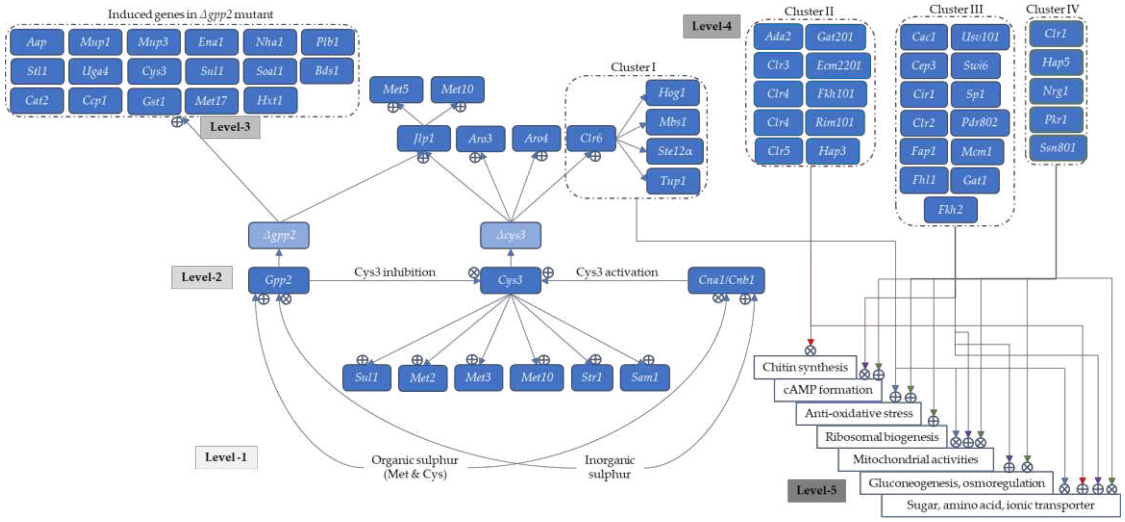


Figure 3. Gpp2, Cys3, and Cna1/Cnb1 interplay control *C. neoformans* response to inorganic or organic sulphur (Level 1). At (Level 2), Cys3p is activated in the presence of inorganic sulphur via Cna1/Cnb1 cascade to induce various enzymes involved in synthesising *S*-containing amino acids. Met or Cys represses these pathways but promotes Gpp2 activation that inhibits Cys3. Numerous metabolic-involving enzymes are induced in *Gpp2* and *Cys3* mutants (*Δgpp2* and *Δcys3*) (Level 3), and these include amino acid permeases, ionic transporters, PLB, antioxidant enzymes, and hexose transporters in *Δgpp2* while *Δcys3* induced aromatic amino acid metabolic enzymes. Jlp1p is an important sulphonate enzyme induced in *Δgpp2 and Δcys3* and channels sulphonate for Met synthesising enzymes. Certain clusters of genes (Level 4) could also affect the general metabolisms at different levels, as shown in Level 5. From the mapping work of Maier et al. [86] on gene clusters and regulation of virulence in *C. neoformans*, *Clr6*, along with *Hog1*, *Mbs1*, *Ste12α*, and *Tup1*, is classified as a cluster of genes induced against reactive oxidants but repressor of mitochondrial activities and also a repressor of sugar, amino acid, and ionic transporters that are needed for growth. The cluster of *Ada2*, *Clr3*, *Clr4*, *Clr5*, *Ecm2201*, *Fkh101*, *Gat201*, *Hap3*, and *Rim101* induced ionic transporters but repressed chitin synthesis. The cluster of *Cac1*, *Cep3*, *Cir1*, *Clr2*, *Fap1*, *Fhl1*, *Fkh2*, *Gat1*, *Mcm1*, *Pdr802*, *Sp1*, *Swi6*, and *Usv101* are involved in the mitochondrial respiratory process and the activation of proteins/enzymes involved in gluconeogenesis and ionic transporters for osmoregulation; however, cAMP signalling is repressed, and the cluster of *Clr1*, *Hap5*, *Nrg1*, *Pkr1*, and *Ssn801* enhanced ribosomal biogenesis, response to oxidative stress, amino acid biosynthesis, cAMP signalling but repressed mitochondrial activities, ionic transporters, gluconeogenesis, and osmoregulatory process. Induced/activated (⁺); Repressed/inhibited (ˣ).

3.10. Transcriptional activation factor controls the lipid and sterol biosynthesis in cryptococcal cells for membrane integrity and thermotolerance

Lipid biosynthesis is an essential biochemical pathway leading to cell membrane integrity. The Mga2 is an orthologue transcription activation factor for the component of fatty acid synthesis and regulates *in vitro* normal growth at a range of temperatures [87]. Therefore, mutation of *Mga2* makes *C. neoformans* thermosensitive and hypersensitive to FCZ with a concomitant reduction in the expression of Fas1, Acc1, Rpl11, Rps7, Leu1, Pho, Pdh, eEF3, and other orthologue proteins. However, expression of Lys2, Hxt, Cat, Pak1, Chs, Mdr1, ABC-family, Vps, Hsp78, Hsp104, calnexin, Rho-GAP domain protein, exo-1,3-*β*-glucanase, and other relevant proteins are induced in this mutant [87]. Ambiguously, it is unclear how the presence of Mga2 regulates polarisation and filamentation growth in *C. neoformans* from the work of Kraus et al. because *Δmga2* mutants are characterised with elevated expression of the homologues of small GTPase effectors, which control the cell polarisation, and the Pak1 that controls the filamentation [87]. Notwithstanding, it is possible that during this morphogenesis, Mga2 expression is repressed at ≤30°C for GTPases and Pak1 expressions that will promote polarisation during growth, but this, however, will contradict the involvement of Mga2 in

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 June 2023 doi:10.20944/preprints202306.1372.v1

thermotolerance and morphogenesis as reported by Kraus et al. [87]. Besides, the dependency of Fas1 (fatty acid synthase β-subunit) and Acc1 (acetyl-CoA decarboxylase) expressions on Mga1 for lipid and sterol biosynthesis through the elevated acyl-CoA desaturase I, Ole1 (an ER membrane enzyme for the production of unsaturated fatty acids), will promote polarisation growth, mating, and haploid fruiting [88,89]. Thus, the background expression of Mga1 may have promoted polarisation and cell growth in *Δmga2* mutant.

3.11. Cryptococcus virulence is under nitrogen catabolite repression regulated by GATA-sequence binding factors

With NCR, certain nitrogen sources are not assimilated, perhaps because of the energy cost of catabolism. Deletion of *Gat1*, among other GATA transcription factors, promotes poor to no growth of *C. neoformans* in the majority of different nitrogen sources except in the presence of Pro and, to some extent, Arg, while single deletion of other GATA family genes (*Δgat201, Δgat204, Δbwc2* or *Δcir1*) failed to shut down totally the fungal growth in all the popular nitrogen sources [90]. This means that various amino acid permeases (Aaps) may still be activated to assimilate other nitrogen sources under later conditions, contrary to *Δgat1* mutants where ammonium transporter (*Amt*) and most *Aap* gene expressions are impaired. However, Pro and Arg may not be under NCR control.

To affirm this, the Pro medium induces *Put1* gene expression for proline oxidase in the *wt* under the influence of NCR as mediated by *Gat1* expression in the presence of NH_4^+ [90]. In addition, the expression of *Put5* (a paralogue of *Put1*) and *Put2* genes, which are involved in the downstream catabolism of Pro to Glu, remained unchanged in *Δgat1* mutants and *wt* [90]. This means that though Gat1 mediates NCR, Pro metabolism is Gat1-independent. The ability to form Glu from Arg may also explain the moderate growth of *Δgat1* mutants in Arg-containing media. Gln and His can also form Glu directly. However, because the cytoplasmic content of Gln is always high as a form of nitrogen donor in most metabolic reactions and His metabolism is also very complex, this may explain why *Δgat1* mutants had poor growth in a medium with each of these two amino acids as nitrogen sources.

The growth defect of *Δgat1* strain H99 mutant in urea, urate, or creatinine-containing media with reduced expression of genes involved in nitrogen metabolism (such as *Gdh1, Amt1,* and *Amt2*) is a serendipitous observation that contradicts the ecological niche of *C. neoformans.* A similar survival growth observed with the *wt* and *Δgat1* mutants cultivated in pigeon guano media is an indication that though this ecological niche is rich in urea, urate, and creatine, which may probably be selective by the NCR, at the same time, the survival of the *Δgat1* mutant in the same environment poses an investigation into the unique nitrogen sources suitable for such a mutant. Perhaps, such media are rich in Pro or Arg, as inferred from Lee et al. observations [90]. Not only this, but Kmetzsch et al. also observed the growth of *Δgat1* mutant in Pro, Arg, urea, and NH_4^+-containing media [6,91]. Notwithstanding, limited nitrogen sources in the environmental pigeon guano may be suspected to have repressed the *Gat1* mutant and prevented the mating process that promotes propagule/basidiospore formation in the filamentous stage of this opportunistic human infectious fungus. Lee et al., instead, discovered that mating is greatly enhanced through the robust filament and basidia formation in the unilateral or bilateral crossing of *Δgat1* mutants [90].

In the same way, deletion of *Gat1* drastically reduces capsule formation, enhances melanin formation at physiological temperature, and promotes extreme thermotolerance but shows no significant effect on urease activity. Unexpectedly, these conditions seem to reduce the pathogenic efficiency of *Δgat1* mutants moderately compared to the *wt* [90]. This shows that the critical virulence factor of *C. neoformans* is firmly under the influence of NCR [91].

Keeping NCR under repression by Gat1p is necessary to facilitate the utilisation of other nitrogen sources. Lee et al. discovered that capsule formation increased considerably in the strain H99 when cultivated in a medium containing Asn, urea, urate, or creatinine but not Gln, Pro or Ala-containing medium. This capsule formation is prosaically affected by the presence of NH_4^+ in each media [90]. This shows that NH_4^+ represses capsule formation by activating NCR even in capsule-induced nitrogen sources, but, on the contrary, the presence of NH_4^+ induces melanin formation. To buttress

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 June 2023 doi:10.20944/preprints202306.1372.v1

this, Lee et al. observed melanin formation in each media mentioned above when prepared with NH_4^+ except in the Pro medium, where a slight melanisation was observed even when NH_4^+ was present [90]. Without NH_4^+, creatinine and urate failed to support melanin formation, and only minimal melanisation was observed in the Ala medium. However, melanin was significantly induced in urea, Asn, or Gln-containing media [90]. This shows that NCR differentially regulates virulence accessory factors and that certain amino acids can override the NCR effect in the dire need for virulence. So, while NCR induces NH_4^+ to repress capsule formation in the presence of different nitrogen sources, melanin is significantly induced.

3.12. A limited number of amino acid permeases/transporters are crucial for Cryptococcus virulence

It was shown that among the numerous amino acid permeases, *Aap2, Aap4, Aap5, Aap6,* and *Aap8* are more differentially expressed in the amino acid-fortified synthetic dextrose (SD) minimal media than the rich media (YPD) [92,93], which means that NCR is highly induced in YPD compared to the SD. Without NH_4^+ (a preferred nitrogen source), global amino acid control is triggered by nitrogen starvation leading to preponderant production of *t*-RNA for amino acid biosynthesis in the yeast. For example, nitrogen starvation induces Trp biosynthetic enzymes encoded by *Trp2, Trp3, Trp4,* and *Trp5* [92]; also, under repressive NCR, *Aap2* and *Aap3* are induced in the presence of Lys-containing media [52]. However, amino acid supplements in SD do repress the general amino acid control (GAAC) but promote the APC-superfamily membrane transporters/permeases, such as APC, AAAP, AGCS, CCC, HAAAP, BCCT, SSS, NSS, NCS1, NCS2, and SulP families [94-96].

The transcription analysis further showed that NCR could repress *Aap2, Aap5,* and *Aap8* in the presence of NH_4^+ but not *Aap4*, which means *Aap4* is sensitive to any nitrogen sources; however, *Aap1* and *Aap7* remained minimally expressed [92]. Though the NCR selectively represses Aap, carbon catabolite repression (CCR) appears to repress all the *Aap* expression in the presence of glucose (preferred carbon source) compared to galactose (alternative carbon source) [92].

The *Δaap1Δaap2* mutants are thermosensitive (at 37°C in synthetic media fortified with amino acids), hypocapsulated, and hypovirulent in the *G. mellonella* infection model [97]. Mutants such as *Δaap1, Δaap2, Δaap6, Δaap8, and Δaap1Δaap2* failed to display any significant difference in growth when compared to the *wt* at 30 or 37°C in SD fortified with amino acids/NH_4^+ or in YPD; however, growth reduction was observed in liquid culture of *Δaap1* containing Met or Pro at 30 or 37°C [97]. Also, a liquid culture of *Δaap1Δaap2* mutant containing Gln or Arg displayed growth reduction at 30°C, and *Δaap8* mutant showed growth reduction in media containing Met, Glu, and Trp [97].

Generally, *Δaap1Δaap2* mutants appeared to show defective growth in 60% of all culturable amino acid media (AAM), which means these amino acid permeases are essential for growth at 37°C on fortified SD media, just like Aap4 and Aap5. However, *Aap1, Aap2, Aap6,* and *Aap8* appeared non-essential for virulence because mutants of these genes, including *Δaap1Δaap2* mutant, failed to show any significant difference in the mating, filamentation, melanin expression, PLB and urease activities when compared to the *wt* [97]. Apart from being non-essential for virulence, none of the mutants seemed affected by oxidative stress agents, alkaline conditions, osmotic and salt stress agents, and cell wall stress agents at 30 or 37°C. Surprisingly, unlike the single mutants *(Δaap1, Δaap2, Δaap4, Δaap5, Δmup1,* and *Δmup3)*, the double mutants *Δaap1Δaap2, Δaap4Δaap5,* and *Δmup1Δmup3* displayed reduced capsule size at 37°C when compared to the *wt*. However, the capsules are similar at 30°C, and the virulence of *Δaap1Δaap2* and *Δaap8* mutants only is attenuated in *G. mellonella* [97].

From Martho et al., *Δaap2, Δaap4, Δaap5, Δmup1, Δmup3,* and *Δmup1Δmup3* showed no sign of attenuated virulence in *G. mellonella,* but *Δaap4Δaap5* mutants are hypovirulent at 30 or 37°C [98]. Furthermore, virulence investigation in the murine model showed that mice inoculated with the *wt, Δaap4,* and *Δaap5* died within a month, while the mice inoculated with *Δaap4Δaap5* mutants and PBS-treated strain survived with viable recovery colonies from the lungs and livers [98]. In addition, *Δaap2, Δaap4, Δaap5,* and *Δaap4Δaap5* mutants displayed *wt* phenotypic sensitivity to AmpB but are hypersensitive to FCZ, and each of the mutants is hyper-resistant to eugenol due to Aap inactivation. This shows that Aap4 and Aap5 are important permeases for thermotolerance, antifungal resistance,

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 June 2023 doi:10.20944/preprints202306.1372.v1

and oxidative stress response for tissue invasion, survival, and virulence in the animal model. By speculation, they are a potential target for therapeutic application [99]; notwithstanding, the compensatory essence of permeases/transporters cannot be ruled out. By harmonising several works on amino acid permeases (Aap, Mup, and Dao), all mutants of *Aap* and *Mup*:

- are not affected in rich media (YPD) at high temperatures except *Δaap4Δaap5*,
- are not affected in AAM at 37°C and in capsule formation except *Δaap1Δaap2*, *Δaap4Δaap5*, and *Δmup1Δmup3*,
- are not sensitive to any stress agent except *Δaap4Δaap5*,
- are virulent in *G. mellonella* except for *Δaap1Δaap2* and *Δaap8* (hypovirulent), *Δaap4Δaap5* (avirulent as well as in murine model),
- shared appreciable level of sequence homology (*Aap1* vs *Aap2*, 80.9%; *Aap1* vs *Aap2* vs *Aap3*, 49%; *Aap4* vs *Aap5*, 89.5%; and *Aap6* vs *Aap7*, 41.4%), and each of these *Aaps* in the doubly mutated yeast appeared redundant in their functions,
- the *Aap3* and *Aap7* expressions are below detection in YPD or SD; however, Lys-containing medium induced *Aap2* and *Aap3* expressions under alleviated NCR,
- irrespective of the nitrogen sources, the expression of *Aap6* remained relatively unchanged,
- the *Aap8* responds to amino acid-supplemented media only, but the highest expression is usually found in *Aap2*, *Aap4*, and *Aap5*,
- *Mup1* and *Mup3* expressions are under the NCR regulation as *Aap2* and *Aap5* but can be induced by His, Trp, and Met when NRC is shut down,
- only *Mup1* can be induced by *S*-containing amino acids,
- galactose induces the expression of all the *Aap* and *Mup* genes,
- expression of *Aap6* and *Aap8* is temperature-independent,
- Aap4, Aap5, and Mup1 induction increased from 30 to 37°C in SD medium,
- *Aap2*, *Aap4*, *Aap5*, and *Mup3* expressions are further repressed from 30 to 37°C except for *Mup1*, which is further induced,
- irrespective of the growing media, there is no significant change in the growth of *Δaap2*, *Δaap4*, *Δaap5*, *Δmup1*, *Δmup3* and *Δmup1Δmup3* mutants at 30 or 37°C,
- the significant growth defect of *Δaap4Δaap5* mutants at especially 37°C in YPD or SD showed that the two permeases (or at least one of them) are important for thermotolerance,
- the use of amino acids as nitrogen sources impaired the growth in *Δaap4Δaap5* mutants,
- relative to NH_4^+ at 30°C, Val, Ile, and Met-containing SD media poorly support the growth of *C. neoformans*, but Leu, Ser, Lys, and Phe are better nitrogen sources,
- Gly, Asp, Asn, Glu, Gln, Arg, Trp, and Pro are highly competitive with NH_4^+ in culturable AAM,
- at 37°C, Val and Met poorly support the growth of *C. neoformans* in SD media, but Gly, Leu, Ile, Ser, Trp, and Phe are good nitrogen sources, while Asp, Asn, Glu, Gln, Arg, Lys, and Pro are better nitrogen sources,
- stereospecifically, var. *gattii* metabolise *D*-amino acids because of the more active expression of *Dao1*, *Dao2*, and *Dao3* genes (encoding *D*-amino acid oxidase) but less metabolisable for var. *neoformans*, which prefers *L*-amino acids as nitrogen sources due to the inefficient evolutionary expression of *Dao* genes,
- in all, growth is denser in *L*-amino acids containing media than the corresponding *D*-amino acids media,
- pathologically, *Δdao* mutants of *C. neoformans* are virulent, but *Δdao* mutants of *C. gattii* are attenuated,
- positive correlation of *Gat1* expression that represses NCR is confirmed to aid the expression of *Aap* when the preferred nitrogen source is absent/limited but uncertain with *Dao* expression,

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 June 2023 doi:10.20944/preprints202306.1372.v1

- *L*-Tyr failed to dissolve at permissive pH for culturing *C. neoformans*, hence not suitable to be tested,
- Aap2, Aap3, Mup1, and Mup3 may be global amino acid permeases/transporters because of the most significant growth defect of their corresponding mutants, especially in their double mutant states, from 30 to 37°C and their ability to be induced by various amino acids,
- in addition to being a global permease and redundant transcription factors, Aap4 and Aap5 promote thermotolerance and response to oxidative stress, and the growth of the double mutant is significantly impacted from 30 to 37°C in a single amino acid medium or the presence of ≥5 mM H_2O_2,
- unlike the *Δaap4* and *Δaap5* mutants, the growth defect of *Δaap4Δaap5* mutants at 37°C appeared to be restored as pH increased gradually into the alkaline state or when supplemented with 0.75 M NaCl (this condition generates H^+ via Na^+/H^+ antiporter that drives other amino acid permeases to compensate for the deletion of *Aap4* and *Aap5*),
- functionally, Aap2, Aap3, Mup1, and Mup5 are regarded as minor permeases while Aap4 and Aap5 are major permeases [97,98,100].

3.13. Mutating the Ras1 gene attenuates Cryptococcus thermotolerance, capsule formation, and a more significant proportion of amino acid permeases/transporters

The thermotolerance in cryptococcal cells is majorly controlled by Ras1. Surprisingly, it has been shown that the Ras1 pathway also controls amino acid permeases leading to thermotolerance [97]. The *Δras1* mutants had shown growth reduction compared to the *wt* in various amino acid-fortified cultures at 30 or 37°C except for media with Ala, Cys, His, Tyr, and Thr that failed to support the growth of *Δras1* mutants [97]. The involvement of Ras1 in the assimilation of amino acids is a unique feature of this transcription factor to facilitate thermotolerance [101-103]; however, Aap4, Aap5, and Mup1 are among the few selected expressible permeases in this non-permissive temperature. Nutritionally, all *Aap* are generally more induced in fortified SD media (without NH_4^+) than YPD at 30°C [104].

The controlling effect of Ras1p on the expression of *Aap* was further investigated by quantifying the expression level of each *Aap* transcript in *Δras1* mutants. All *Aap* and *Mup* transcripts were repressed in *Δras1* mutants in SD fortified with Trp, His, and Met at 30 and 37°C except *Aap5*, which showed a *wt* transcript level at 37°C [97]. The exception of *Aap5* may suggest a different regulatory mechanism in addition to or different from Ras1 expression. This shows the importance of the Ras1/MAPK signalling pathway in the nutritional balance of *C. neoformans* against thermal stress, alternative nutrient source, morphogenesis, and virulence. Just as Aap4 and Aap5 were identified as potential drug targets against cryptococcal infection, the unique region of sequence homology within the regulatory domain of Ras1 protein across a few numbers of pathogenic yeast has also been speculated as a drug target [105].

4. Transcriptional Factors Regulate Virulence-Associated Membrane-Anchored Proteins, Extracellular Enzymes, and Vesicular Secretions in Cryptococcal Cells for Morphology, Growth, and Virulence

Several extracellular hydrolytic enzymes from *C. neoformans* have been studied with diverse functions. They are freely secreted under the regulatory *N*-terminal signal peptide or cleavage from the cell wall located GPI-anchored proteins or remain covalently attached to the cell wall glycans. Though the specific functional proteins, such as those designated for antioxidative functions, may lack *N*-terminal signal peptide and GPI-anchor [106], most of the proteins involved in adhesion may require GPI-anchorage.

Cryptococcal secretomes are controlled by several *Sec* expressions, which encode factors that orchestrate the secretory pathways of extracellular proteins, especially the glycosylated proteins. *S.*

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 June 2023 doi:10.20944/preprints202306.1372.v1

cerevisiae has been well-studied regarding the mechanism of exocytic secretion of proteins. Several haploid double *Δsec* mutants of *S. cerevisiae* on invertase secretion showed that extracellular secretion by yeast required several forms of *Sec* factors, ATP, temperature, and a moderate level of glucose (<1%) [107]. The endoplasmic reticulum-Golgi Body migration of secretory protein required energy for posttranslational modification. The final vesicular packaging and plasma membrane fusion for budding also required energy, permissive temperature, and several other *Sec* factors.

Secretions are generally poor at non-permissive temperatures but are reversible when transferred to permissive temperature in the presence of glucose, as found in intracellular accumulation of invertase by *Δsec7* and *Δsec16* mutants [107]. Mutation of *Sec7* impairs secretome activity, followed by intracellular accumulation of different organelles involved in the secretion. Furthermore, *Sec* expression has a way of regulating each other. For example, *Sec18* was epistatic to *Sec1* and *Sec17*, which was shown by intracellular accumulation of invertase in *Δsec18*, *Δsec1Δsec18*, and *Δsec7Δsec18* mutants but not in *Δsec1* and *Δsec7* [107].

Extracellularly released proteins could be inducible/constitutive depending on the signalling factors. Proteases are released to degrade proteins, lipases degrade triglycerides, phospholipases degrade phospholipids and sphingolipids, urease degrades urea to cytotoxic ammonia and CO_2, laccase is involved in pigmentation, and chitin synthase is involved in cell wall chitin formation. Each of these enzymes is examined further as follows:

4.1. Proteases (Proteinases/Peptidases)

Various hydrolytic enzymes, including endoproteinases and exoproteinases, have been implicated in the cryptococcal invasion and colonisation of the CNS (neurotropism). Vu et al. discovered that extracellular secreted metalloproteinase, which is an M36 protease class of fungalysins, is highly required for fungal colonisation of the CNS and brain endothelium but not dissemination and the deletion of the *Mpr1* gene incapacitated the fungal penetration of the BBB endothelial layer to establish infection [108]. This prolongs the survival of the infected host due to reduced brain pathology.

The nature of the media determines the type of secreted protease. Animal tissue culture induces metalloproteinase and serine endoprotease as major peptidases, but aspartyl endoprotease activity dominates in minimal media. Remarkably, the deletion of *Qsp1* reduced aspartyl endoprotease in the minimal media but elevated the secretion of metalloproteinase and serine endoprotease in animal tissue culture more than the *wt* [109]. Likewise, Brueske discovered that media supplemented with NH_4^+ and glucose could inhibit the total secreted proteolytic enzymes of *C. neoformans*, while growth in essential salts medium supplemented with bovine serum albumin only enhanced the extracellular protease profile [110].

The involvement of *C. neoformans* extracellular proteinases in the degradation of host immunological proteins has been demonstrated, and this is responsible for fungal invasion and dissemination. Such proteinases could degrade *β*-casein, murine IgG_1, bovine IgG, human C_5, haemoglobin, and *γ*-globulin in media containing carbon and nitrogen sources [111,112]. Regarding the potential to cause infection, a relatively low proteinase profile of *C. neoformans* var. *gattii* may have reduced tissue invasion, dissemination, and systemic spread of this serotype compared to the *C. neoformans* var. *neoformans*. However, there is no difference in the total proteolytic activities of the environmental isolates compared to the clinical isolates of *C. neoformans* [113]. Proteases are released at permissive and non-permissive temperatures, irrespective of their sources. A positive correlation exists between the capsule size and the protease activity in the clinical isolates, which was not found in the environmental/bird-dropping isolates [114]. The majority of these proteinases are serine proteases having optimal activities between pH 7 – 8 at 37°C [111]

In another related study, aspartyl endopeptidase (putatively encoded by *May1*) and carboxypeptidase D1 (putatively encoded by *Cxd1*, *Cxd2* or *Cxd3*) have been identified as the major extracellular endoprotease and carboxypeptidase, respectively, in *C. neoformans* that help cope with the acidic environment and significantly enhance virulence [115]. Specifically, the total proteolytic activity in the mutants lacking metalloendopeptidase or metalloproteinase *(*encoded by *Mpr1)*, serine

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 June 2023 doi:10.20944/preprints202306.1372.v1

carboxypeptidase (encoded by *Scx1)*, carboxypeptidase C (encoded by *Prc1)*, serine endopeptidase (encoded by *Prb1*), carboxypeptidase D (encoded by *Cxd2, Cxd3)*, and aspartyl endopeptidase (encoded by *Pep4)* was not significantly different from the *wt* in an enriched medium. However, *Δmay1* and *Δcxd1* mutants had significantly reduced activity of the extracellular proteases because they encoded major secreted protease tagged major pepsin-like aspartyl endopeptidase with optimal expression in an acidic medium.

This aspartyl endopeptidase, by mechanisms, can be inhibited by pepstatin A and antiviral protease inhibitors [115-117]. Mutation of these extracellular protease-encoding genes might not affect melanin production. Still, since melanin synthesis is also a cell wall localised process enriched with mixed GPI-anchored proteins, chitin, chitosan, and glycan, the deletion of *Prb1* has shown hypomelanisation in such a mutant [115].

Targeting the proteolytic activities of pathogens is a significant way of therapeutic approach against infections. A few works have shown that antiretroviral drugs in AIDS patients could antagonise some key extracellularly released hydrolytic enzymes and virulence factors in pathogenic fungi such as *C. albicans* and *C. neoformans,* and this invariably inhibits fungal growth. Unlike Saquinavir, Nelfinavir, Lopinavir, Darunavir, Tipranavir, Atazanavir, Amprenavir, Brecanavir, Darunavir, Indinavir, Ritonavir, and Saquinavir as well as macrocycles have been shown to inhibit fungal growth, attenuate capsule production, and inhibit urease and proteases but not phospholipase and melanin production in *C. neoformans* [115-117].

The use of specific and effective antiretroviral drugs in a co-morbidity patient with cryptococcosis may be responsible for the higher clearance of the cryptococcal cells because of the increased susceptibility of the weakened cryptococcal cells to the killing effect of innate effector cells, such as monocytes, neutrophils, and macrophages [116]. Unlike *C. albicans,* which are easily cleared by antiretroviral drugs, *C. neoformans* may be relatively difficult. *C. neoformans* aspartyl protease is one of the proteases not inhibited by highly active antiretroviral therapy, such as Indinavir, Lopinavir, and Ritonavir. Apparently, the transcriptional level of aspartyl protease is strategically independent of the media used [118].

4.2. Urease

Urease (encoded by *Ure1*) is another hydrolytic metalloenzyme that hydrolyses the urea to ammonia and carbamate. Apart from Ure1, other transcription factors like Ure4, Ure6, and Ure7 have been associated with the production of accessory proteins required for the nickel-dependent functional protein complex [119]. Via the nickel/cobalt transporter (encoded by *Nic1*), Ni^{2+} is shuttled into the cytoplasm to combine with the apo-urease (encoded by *Ure1*) via Ure7p to form an active metallocentric urease. The urease reaction may increase the media pH to create an alkali environment. While this may be deleterious to the infected host cells, *C. neoformans* deploy the pH-sensing signalling factors, such as Rim21-Rim8-Rim20-Rim13-complex, to counter the pH surge via the activation of Rim101 signalling pathway [29].

The *Δure1* mutants displayed comparable phenotypic and virulence features with the *wt;* however, evidence existed that deleting this gene may attenuate the progression of infection caused by *C. neoformans*. This is corroborated by the outcome of Cox et al. that murine infected with *Δure1* mutant, whether intranasally/intravenously, failed to show pulmonary distress, displayed delayed hydrocephalus, and wasting, and significantly lived longer than the *wt*-infected mice [120]. Hydrocephalus and wasting are delayed manifestations of meningoencephalitis caused by *Δure1* mutants after dissemination into the brain, but the infection caused by urease-positive strains proceeds rapidly in the lungs to result in acute pneumonitis capable of killing the mice before the brain encephalitis sets in [120]. Notwithstanding, wholistic urease activation is essential for the microcapillary sequestration, dissemination, and brain invasion of *C. neoformans* because *Δure1, Δure7,* and *Δnic1* mutants, which lacked urease activities, significantly displayed defective growth in the presence of urea with poor blood-to-brain invasion in an animal model [119,121]. Though urease seems to be essential to the dissemination, invasion, and survival of *C. neoformans in vivo* yet, clinically infectious urease-negative

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 June 2023 doi:10.20944/preprints202306.1372.v1

isolates capable of dissemination and invasion have been reported in AIDs patients [122,123], which means this enzyme may be playing second fiddle in the pathogenesis of *C. neoformans* [124].

4.3. Laccase

Laccase is a cell-wall-associated pigment-forming enzyme in *C. neoformans*. This iron/copper-dependent metalloenzyme is encoded by *Lac1* (major) and *Lac2* (minor) genes [62,125]. Four major transcription factors, Bzp4, Usv101, Mbs1, and Hob1, are involved in the induction and regulation of the *Lac1* gene [126]. The enzyme can act on several neurotransmitters and other amino compounds to synthesise melanin that confers camouflaging [127,128], resistance (against fungicides, photolysis, thermolysis, acidolysis, oxidants, denaturants) [129-133],[134,135], and elongating propagule lifespan [136] in *C. neoformans*. Several mutants of cryptococcal cells displayed impaired melanin formation. Mutating the *Snf7* gene in cryptococcal cells impaired polysaccharide secretion, capsule, and melanin formation, especially at physiological temperature, with a complete loss of virulence in an intranasal model of murine cryptococcosis [137].

Low glucose level facilitates the nuclear translocation of Bzp4 and Usv101; and, together with the residential nuclear transcription factor (Mbs1), initiates the transcription of the *Lac1* gene [126]. Conversely, Hog1 provides a repressive regulation of *Bzp4* and *Lac1* to coordinate melanin production. The *Lac1* expression was particularly shown to be upregulated in *Δssk1*, *Δhog1*, and *Δskn7* mutants [84]. In the early investigation, *Snf5* and *Mbf1* have been implicated in regulating laccase transcription, poor growth observed in non-glucose media, bilateral sterility, and mating defect in *C. neoformans* [138]. Also, because melanin production is anchored by the cell wall chitin and governed by the cytoplasmic ionic homeostasis, Chs3, Ccc2, Atx1, Cuf1, and Sit1 have been further identified as important transcription factors for melanisation [138].

Two oppositely regulated signalling pathways govern melanin production – cAMP/Pka and Hog1 [139,140] (**Figure 4**). Low glucose concentration activates cAMP/Pka-repressing pathways while repressing the Hog1 signalling pathways. This condition induces the *Lac1* gene for melanin production. Hog1 expression could hinder the Pka downstream activity leading to melanin production in serotype A, *Hog1* deletion effectively restored the melanin production in hitherto non-melanized *Δgpa1*, *Δcac1*, or *Δpka1* mutant cultured in 0.1% glucose media (**Figure 4**); however, *Hog1* mutation failed to restore melanin production in serotype D *Δpka2* mutant [140]. From the two-component system of the Hog1 pathway, deletion of *Tco* genes did not contribute significantly to melanisation, but *Δtco1* or *Δtco1Δtoc2* mutants showed enhanced melanisation at 37°C even in up to 2% glucose media, and this melanin content was considerably higher than the *Δhog1* mutant [141]. This shows that Tco1 kinase is a key repressor of *Lac1* expression, just like Ssk1, Pbs2, Hog1, and Skn7 proteins. However, complementing the *Δtco1* mutant (*Δtco1::Tco1*) reduced the melanin to the *wt* level.

The Ypd1 is also a component of the Hog1 pathway, which regulates melanin and capsule formation. At 0.1% glucose, *Δypd1Δhog1* mutant produced a highly significant level of melanin just like *Δhog1*, *Δssk1* and *Δskn7* mutants at 30°C, and at 37°C, only the *Δypd1Δhog1* and *Δskn7* could retain the melanin [142]. Similarly, Bahn et al. reported comparable melanin formation in the *wt*, *Δhog1*, *Δpbs2*, *Δssk1*, and *Δskn7* mutants at 37°C [141]. In 1.0% glucose medium, *Δssk1*, *Δhog1*, and *Δskn7* mutants melanised as *wt*, but all mutants, including the *wt*, failed to retain this melanin at 37°C. This shows that the efficiency of melanin formation is more negatively affected by increasing glucose levels than by increasing temperature.

Surprisingly, the deletion of *Ypd1* represses melanin formation in the *Δhog1* background mutant irrespective of the temperature [142] (**Figure 4**). Contrary to this, *Δhog1*, *Δpbs2*, *Δssk1*, and *Δskn7* mutants still retained pigmentation in 1.0% glucose media at 37°C while melanin formation had already been lost in the *wt* and reconstituted *Δssk1*. Yet, in 2% glucose media, pigmentations are still found in the same set of mutants with significant melanisation in the *Δskn7* than the rest of the mutants [141]. Because of the regulatory effect of Hog1p on the expression of *Mbs1* (encoding DNA-binding basic helix-loop-helix protein 1), then Song et al. showed significant melanin and capsule defect in

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 June 2023 doi:10.20944/preprints202306.1372.v1

Δcac1 compared to *Δmbs1* mutants at 37°C, notwithstanding *Δmbs1* mutant still showed a reduced virulence with low degree tissue fungal burden and titanisation compared to the *wt* or complemented *Δmbs1* mutant (*Δmbs1::Mbs1*) [143].

While it is reasonable to summate that increasing cAMP production would elevate melanin synthesis, D'Souza et al. showed that high levels of cAMP led to overexpression of Pka1 and the repression of melanin production as compared to the *wt* [144] (**Figure 4**). However, capsule production appeared highly induced [144]. This means that capsule production may be more critical to virulence than melanin. Though the same upstream regulatory factors may control melanin and capsule productions, different transcription factors are involved in the terminal responses. Evidence exists that melanin is produced in the infected tissue isolates, but not as much is produced *in vitro*. Again, the fact that larger capsules have been produced in the infected tissue isolates than culture media means that capsules are more involved in the *C. neoformans in vivo* virulence and pathogenesis than melanin. Notwithstanding, an appreciable level of melanin is necessary for effective virulence, tissue invasion, antifungal resistance, and macrophage survival within the infected tissues [145-148].

Ironically, the presence of melanin in the infected tissues has been proposed with uncertainty. Liu et al. discovered that laccase-dependent catecholamine oxidative products such as pyrrole-2,3,5-tricarboxylic and pyrrole-2,3-dicarboxylic acids rather than melanin might be produced in the mouse brain during infection to induce oxidative cytotoxic effects within the infected tissue [149]. The accrued evidence came from the work of Ito et al. that dopamine-*o*-quinone is one of the reactive catecholamine oxidative intermediates, which are formed during melanin synthesis, and this intermediate strongly attacks the sulfhydryl groups of protein cysteine [150]. This can be further oxidised to pyrrole acids in alkaline peroxide [151,152]. Therefore, this contrary discovery means that if melanin is actually produced during infection, then it may be stringently controlled for moderate pigmentation against oxidative damage caused by heterologous quinone-like derivatives, which are cytotoxic catecholamine oxidative intermediate products channelled to destroy the host cells.

Mutation of the *Pka1* gene in *C. neoformans* usually leads to sterility and avirulent because the strain failed to produce melanin and capsules; however, overexpression of Ste12*α* in the *Δpka1* mutant restored the mating but was unable to restore the virulence of *Δpka1* mutant [144]. Contrarily, the mutation of the *Pkr1* gene, encoding the regulatory Pka subunit protein, failed significantly to affect melanin production in serotype A *MATα*, unlike the mutation of *Gpa1*. Interestingly, it was shown that the *Δpkr1Δgpa1* double mutant produced a comparable level of melanin to the *wt* better than the *Δgpa1* mutant [144]. This indicates that melanin and capsule production is under the multifactorial transcription factors downstream of the Pka protein. Furthermore, mutation of the *Crg1* gene has been shown to enhance melanin production in the *MATα* strain, which depends on the *Ste12α* expression. This mutation directly increased the virulence in the animal study in a Cpk1-independent manner [153].

Deleting the *Sit1* gene (encoding siderochrome-iron uptake transporter) in the serotype D background enhances melanisation better than the serotype A *Δsit1* mutant. The melanin production seemed better with increasing DOPA concentration in the medium—however, 1.0% glucose repressed melanin formation in the *Δsit1* mutants of the two serotypes. Additionally, the *Sit1* expression appears to control copper availability to Cu^{2+}-binding pocket of laccase. So, loss of *Sit1* gene apparently attenuates ionic homeostasis and increases laccase activity more significantly in the mutants than the *wt*. This means laccase in the mutants has more access to copper because of the absence of Sit1p, which ordinarily could have distributed the copper among other copper-dependent proteins. In this case, Tangen et al. exogenously supplied copper and discovered an elevated laccase activity in the wt and reconstituted Δsit1 strain, comparable to the *Δsit1* mutant [125]. Notwithstanding, an exogenous supply of copper or iron in the absence of glucose generally seemed to favour melanisation in serotype D *ΔSit1* mutant.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 June 2023 doi:10.20944/preprints202306.1372.v1

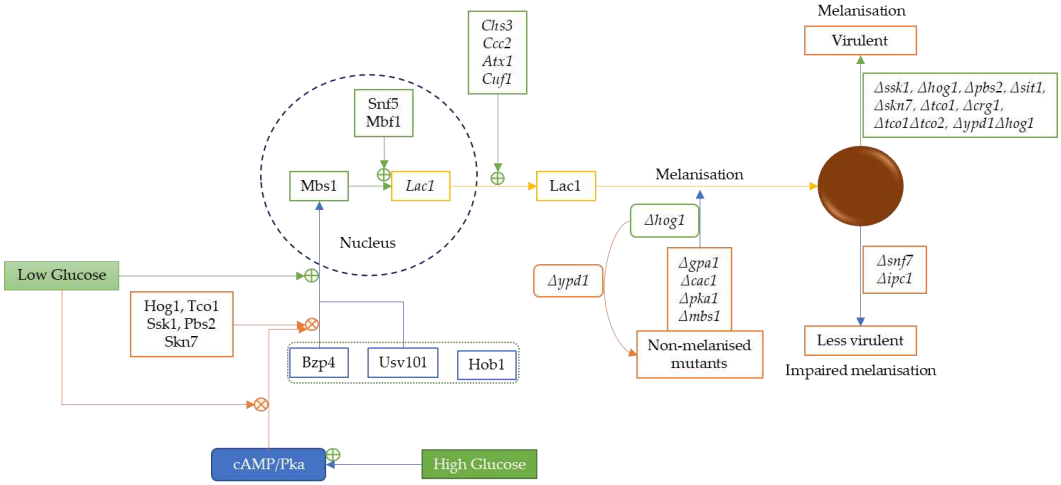


Figure 4. Melanisation in *C. neoformans*. The level of glucose modulates melanin synthesis. Generally, low glucose suppresses cAMP/Pka activity but promotes the nuclear transition of inducing factors – Bzp4, Usv101, and Hob1, to induce *Lac1* transcription via the association with Mbs1. High glucose levels increase cAMP/Pka activity and prevent Bzp4 transition to the nucleus. As shown above, mutants of Hog1-associated factors melanised and are virulent; however, cAMP/Pka mutants are non-melanised. However, deleting Hog1 in the cAMP/Pka background mutants will promote melanisation. Hence Hog1-associated transcription factors are melanin suppressors. Induced/activated (+); Repressed/inhibited (×).

4.4. Inositol-phosphorylceramide synthase

Luberto et al. exposed the vital role of the *Ipc1*/*Aur1* gene encoding inositol-phosphorylceramide synthase 1, which catalyses the formation of membrane-associated sphingolipids complex called inositol-phosphorylceramide (IPC) and diacylglycerol (DAG) from phytoceramides and phosphatidylinositol (PI) in fungi [154]. The DAG is a second messenger of mitogen and protein kinase *c* (Pkc) activation. Based on this function, inositol-phosphorylceramide synthase 1 is critical in membrane integrity during growth.

Suppressing the *Ipc1* expression under the P_{gal7}-regulated inducible promoter, it was shown that while the glucose-repressing condition reduced melanin production by 60%, the galactose-inducing condition increased melanin production by 80% [154]. Furthermore, this repressive condition impaired the virulence, survival rate, *in vivo* growth, replication, and cellular diffusion of infectious strain H99 in immunocompromised animal and macrophage-like cell line models [154]. Again, because of the increased melanin production in the *Hog1* deleted background mutant, Ko et al. discovered that upregulation of *Ipc1* in the *Δssk1* and *Δhog1* mutants might synergistically enhance melanin production in *C. neoformans* [84]. This condition may be adopted by cryptococcal cells *in vivo* during infection to enhance resistance, stability, adaptation, growth, and development.

4.5. Chitin synthase

Though the presence of chitin and other unique cell wall components predisposes the cryptococcal cells to the host PAMPs-PRRs (pathogen-associated molecular patterns-pattern recognition receptors) recognition to induce anti-cryptococcal defence strategies [155] but with the capsule formation and vesicular secretion of capsular components, *C. neoformans* could shield the chitin and circumvent the host's PAMPs effect [28,156]. Not only this, but *Cryptococcus* also has intrinsic chitin deacetylase encoded by seemingly redundant *Cda1*, *Cda2*, and *Cda3* functional genes (the fourth, *Fdp1*, remained undetermined), which can convert cell wall chitin produced by chitin synthase (encoded by *Chs3*), in the presence of its regulator (encoded by *Csr2*), to chitosan (a non-rigid soluble polymer) [157,158]. Interestingly, *C. neoformans* prefers the Cda1 gene, which is highly upregulated in the recovery strain from mammalian lungs and primarily selected for fungal proliferation [159].

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 June 2023 doi:10.20944/preprints202306.1372.v1

Eight genes encode chitin synthase, *viz Chs1* to *Chs8*, but only three chitin synthase regulators, *Csr1 – Csr3*, are functionally active. Unexplainably, as many of these encoding genes are in cryptococcal cells, a compensatory expression for each mutant rarely occurs except for *Δchs3*, where a slight increase in *Chs5* and *Chs7* was detected [157]. Furthermore, the Chs3-Csr2-Cda1 complex is essential for chitosan production for the cryptococcal cells to grow, disseminate, and invade the host at physiological conditions [160,161]. Under vegetative growth, *Chs7* expression is entirely repressed, while *Chs6* is remarkably and minimally detected in contrast to *Chs1* and *Chs4* expressions. However, *Chs2*, *Chs3*, *Chs5*, and *Chs8* are highly induced during vegetative growth [157]. Surprisingly, none of these chitin-associated genes or regulators is essential for cryptococcal viability at 30°C, but *Δchs3* and *Δcsr2* mutants are particularly hypersensitive to non-permissive temperatures and cell wall stressors such as Calcofluor White (CFW), Congo red, SDS, and caffeine.

4.6. Dnases

Cryptococcal cells can assimilate nitrogenous bases and nucleosides to produce endogenous nucleotides via the salvage pathway. By producing extracellular deoxyribonucleases (DNases), most infectious and parasitic fungi degrade the host and surrounding nucleic acids to salvage intermediates molecules needed to build nucleotides. The release of DNases is generally correlated with the increased activity of ureases that degrade excessive nitrogenous compounds in most fungi except in a few isolates of *Endomycopsis fibuligera* and *Lipomyces starkeyi* [162]. Though not too many studies are available on the cryptococcal extracellular nucleases [162]; however, available few studies have predicted the inclusion of secreted nucleases as virulence factors [163,164] because it may play a role in innate immune evasion as found with Group A *Streptococcus* infection characterised with polymorphonuclear neutrophil infiltration at the inflammatory region caused by bacterial infection [165,166]. A wide range of cryptococcal strain assessments for the production of DNase showed that *neoformans* and *gattii* species could secrete DNase. Based on their natural environment, the clinical isolates usually produce higher levels of DNase than environmental isolates [163,164].

4.7. Phosphatase

Ectophosphatase is a membrane-surface hydrolytic enzyme that often releases phosphate preferentially from phosphothreonine, phosphotyrosine, and phosphoserine substrates and is liable to be inhibited irreversibly by sodium orthovanadate (Na_3VO_4) [167]. Irreversible inhibition of this enzyme in *C. neoformans* has been characterised by reduced adhesion to animal epithelial cells – an important step towards fungal pathogenesis and tissue invasion [167].

The removal of phosphate groups (dephosphorylation) by phosphatase from transcription factors that are usually activated/deactivated by serine/threonine/tyrosine phosphorylation catalysed by protein kinases is one of the core regulatory pathways of MAPK. This controls the *on*/*off* regulation of signalling pathways orchestrated by transcription factors such as Hog1 MAPK, Ste20/Pak kinase, Pkc1 MAPK, and cAMP/Pka. Such phosphatase could operate optimally in an acidic medium (acid phosphatase) or alkali medium (alkaline phosphatase). For example, *C. neoformans* can release extracellular acid phosphatase encoded by *Aph1* [168], which facilitates fungal adhesion to monocytes, but deletion of this gene attenuates the fungal virulence [167,169].

In a relevant study, an ecto-ATPase has been identified in *C. neoformans* with totally different activities compared to phosphatase and other types of ATPases. Cryptococcal ecto-ATPase, classified as E-type ATPase [170], functioned optimally in the presence of Mg^{2+}, Mn^{2+}, Zn^{2+}, and Ca^{2+} in that others with a variety of nucleoside triphosphates as substrates (preferentially from ATP>GTP>UTP>ITP>CTP) [171]. Surprisingly, the secretion of this enzyme induced moderate antifungal resistance to FCZ in the presence of ATP [171].

4.8. Multifunctional hydrolytic enzymes in C. neoformans

The lack of trypsin, chymotrypsin, *β*-galactosidase, *β*-glucuronidase, and *α*-mannosidase in the species *Cryptococcus* showed that certain proteins and *β-D*-sugars might not be suitable for culturing

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 19 June 2023 doi:10.20944/preprints202306.1372.v1

this fungus [79]. However, many species of cryptococcal cells do possess esterase lipase (active against triacylglycerol with C4 – C8 acyl chain), α-galactosidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-fucosidase, galactokinase, *D*-lactate dehydrogenase [43,79], which means that various α-*D*-sugars, amino sugars, and disaccharides are suitable carbon sources for cryptococcal cells. Special attention is needed on some extracellular lipases capable of inducing antibodies in infected animal studies. Chen et al. discovered that 92% of the tested cryptococcal strains secreted butyrate and caprylate esterase lipases [172]. At the same time, some also produced acid phosphatase, naphthol-AS-BI-phosphohydrolase, and β-glucosidase, and the sera of infected rodents could precipitate several of these secreted proteins [172]. This shows that extracellular vesicles from *C. neoformans* contain proteins of immunological functions, which can be modified as vaccine candidates against systemic cryptococcosis.

4.9. Extracellular vesicles

Extracellular vesicles (EVs) described as "virulence bags" are exosomal secretory vesicles containing capsular components (GXM, GalXM, and MP88) and enzymes like laccase *(Lac1)*, urease, chitin deacetylase *(Cda1, Cda2,* and *Cda3)*, glyoxal oxidase *(Gox1, Gox2,* and *Gox3)*, multicopper oxidase *(Cfo1* and *Cfo2)*, ricin-type lectin-domain containing proteins *(Ril1, Ril2,* and *Ril3)*, Sul7/PalI family motif protein (*Tsh1, Tsh2,* and *Tsh3)*, Pr4/Barwin domain protein family *(Blp2* and *Blp4)*, and phosphatase [28,173-176]. Studies showed that the key components of EVs are virulent and antigenic, capable of activating macrophages to produce pro-inflammatory cytokines and inducing protection in animal studies [174,177-179]. As a standalone component, it could enhance cryptococcal cell infectivity and BBB penetration [180]. The production, packaging, and secretion of these vesicles are controlled by *Sec* genes, such as *Sec6* [181].

The proteomic analysis of the *C. neoformans* secretory vesicles showed diverse functional proteins and enzymes classified as antioxidative and heat-shock proteins (Hsp70 and Hsp90), signal transduction and nucleotide salvage enzymes, ribosomal proteins, metabolic enzymes (for sugar, lipid, protein, and amino acid), nucleoproteins, membrane proteins (transporters, carriers, pumps, channels, adhesins, inositol-3-phosphate synthase, Rab proteins), cytoskeleton proteins (actin and actin-binding proteins, tubulin, and annexin), and a few mitochondria membrane proteins [28]. In addition, the presence of UDP-glucose dehydrogenase (encoded by *Ugd1*) and UDP-glucuronate decarboxylase (encoded by *Uxs1*) in the extracellular vesicles of *C. neoformans* showed the presence of functional enzymes that not only promote capsule formation but also enhance cell wall integrity and thermotolerance [182]. Cryptococcal EVs witnessed a considerably wide range of studies recently and have opened a new way of presenting the component of these vesicles as potential vaccine candidates against cryptococcal infections [183]. Many more studies will be needed to characterise the strain specificity in the antigenic properties of these vesicles.

5. Conclusions and Perspectives

The significant involvement of different hydrolytic enzymes, secreted proteins, and membrane-associated proteins like pumps, permeases, transporters, laccase, redox proteins, and ion-affinity proteins related to the pathogenesis of *C. neoformans* was discussed in this article. While each of these proteins plays a vital role in the survival, adaptation, and pathogenesis of *C. neoformans* in animals and humans, some are redundant with barely known functions. These attributes are common in other invasive fungi that cause candidiasis and aspergillosis. The multiplicity and redundancy of these proteins may be critical in catabolite repression to rescue the fungi when in an environment with alternative carbon sources, low oxygen, fluctuating pH, high salt contents, high oxidant level, low phosphate content, low ammonium content, and low micronutrient elements. The ability to secrete extracellular enzymes and virulence proteins is an alternative way of inducing cellular morphology, biofilm formation, cellular communication, filamentation, fruiting, mating, and nutrient assimilation. We described these proteins as weapons that facilitate tissue invasion, persistence, and infection in animal models of cryptococcosis. With the increasingly unfriendly environment and incessant use of

antibiotics, pathogenic fungi are developing resistance to classical antifungals coupled with the emergence of sporadic strains that pose a threat to humans. Therefore, studies that target virulence-induced proteins and the membrane proteins necessary for fungi nutrient assimilation and survival should be targeted for new antifungal drug development; however, a careful consideration that will preclude the consequences in humans is highly recommended.

Supplementary Materials: Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org., File: Genes, Transcription Factors, Protein Kinases, Intracellular Proteins, and Other Abbreviations as Used in the Article.

Author Contributions: "Conceptualisation, O.S. and O.M.; methodology, O.S.; software, O.S.; validation, O.S. and O.M.; formal analysis, O.S.; investigation, O.S.; resources, O.S. and O.M.; data curation, O.S.; writing—original draft preparation, O.S.; writing—review and editing, O.S. and O.M.; visualization, O.S. and O.M.; supervision, O.M.; project administration, O.M.; funding acquisition, O.M. All authors have read and agreed to the published version of the manuscript.

Funding: "The authors declared that no funding was obtained for this article."

Institutional Review Board Statement: "Not applicable."

Informed Consent Statement: "Not applicable."

Data Availability Statement: "Not applicable."

Acknowledgments: The authors appreciated the conducive research environment and administrative procedures established by the Research Office of the University of the Free State.

Conflicts of Interest: "The authors declare no conflict of interest."

References

1. Elhariri, M.; Hamza, D.; Elhelw, R.; Refai, M. Eucalyptus Tree: A Potential Source of *Cryptococcus neoformans* in Egyptian Environment. *Int J Microbiol* **2016**, *2016*, 4080725, doi:10.1155/2016/4080725.
2. Cogliati, M.; D'Amicis, R.; Zani, A.; Montagna, M.T.; Caggiano, G.; De Giglio, O.; Balbino, S.; De Donno, A.; Serio, F.; Susever, S. Environmental distribution of *Cryptococcus neoformans* and *C. gattii* around the Mediterranean basin. *FEMS yeast research* **2016**, *16*.
3. Ergin, C.; Sengul, M.; Aksoy, L.; Dogen, A.; Sun, S.; Averette, A.F.; Cuomo, C.A.; Seyedmousavi, S.; Heitman, J.; Ilkit, M. *Cryptococcus neoformans* Recovered From Olive Trees (*Olea europaea*) in Turkey Reveal Allopatry With African and South American Lineages. *Front Cell Infect Microbiol* **2019**, *9*, 384, doi:10.3389/fcimb.2019.00384.
4. Feldmesser, M.; Kress, Y.; Novikoff, P.; Casadevall, A. *Cryptococcus neoformans* is a facultative intracellular pathogen in murine pulmonary infection. *Infect Immun* **2000**, *68*, 4225-4237, doi:10.1128/IAI.68.7.4225-4237.2000.
5. Kmetzsch, L.; Staats, C.C.; Cupertino, J.B.; Fonseca, F.L.; Rodrigues, M.L.; Schrank, A.; Vainstein, M.H. The calcium transporter Pmc1 provides Ca^{2+} tolerance and influences the progression of murine cryptococcal infection. *FEBS J* **2013**, *280*, 4853-4864, doi:10.1111/febs.12458.
6. Kmetzsch, L.; Staats, C.C.; Simon, E.; Fonseca, F.L.; Oliveira, D.L.; Joffe, L.S.; Rodrigues, J.; Lourenco, R.F.; Gomes, S.L.; Nimrichter, L.; et al. The GATA-type transcriptional activator Gat1 regulates nitrogen uptake and metabolism in the human pathogen *Cryptococcus neoformans*. *Fungal Genet Biol* **2011**, *48*, 192-199, doi:10.1016/j.fgb.2010.07.011.
7. Aslanyan, L.; Sanchez, D.A.; Valdebenito, S.; Eugenin, E.A.; Ramos, R.L.; Martinez, L.R. The Crucial Role of Biofilms in *Cryptococcus neoformans* Survival within Macrophages and Colonization of the Central Nervous System. *Journal of Fungi* **2017**, *3*, 10.
8. Costerton, J.W.; Stewart, P.S.; Greenberg, E.P. Bacterial biofilms: a common cause of persistent infections. *Science* **1999**, *284*, 1318-1322.
9. Ramage, G.; Mowat, E.; Jones, B.; Williams, C.; Lopez-Ribot, J. Our current understanding of fungal biofilms. *Critical reviews in microbiology* **2009**, *35*, 340-355.
10. Costerton, J.W.; Lewandowski, Z.; Caldwell, D.E.; Korber, D.R.; Lappin-Scott, H.M. Microbial biofilms. *Annual review of microbiology* **1995**, *49*, 711-745.

11. Martinez, L.R.; Casadevall, A. *Cryptococcus neoformans* cells in biofilms are less susceptible than planktonic cells to antimicrobial molecules produced by the innate immune system. *Infection and immunity* **2006**, *74*, 6118-6123.
12. Martinez, L.R.; Casadevall, A. Susceptibility of Cryptococcus neoformans biofilms to antifungal agents in vitro. *Antimicrobial agents and chemotherapy* **2006**, *50*, 1021-1033.
13. Martinez, L.R.; Casadevall, A. Cryptococcus neoformans biofilm formation depends on surface support and carbon source and reduces fungal cell susceptibility to heat, cold, and UV light. *Applied and environmental microbiology* **2007**, *73*, 4592-4601.
14. Martinez, L.R.; Casadevall, A. Biofilm Formation by Cryptococcus neoformans. *Microbiology Spectrum* **2015**, *3*, 3.3.05, doi:doi:10.1128/microbiolspec.MB-0006-2014.
15. Benaducci, T.; Sardi Jde, C.; Lourencetti, N.M.; Scorzoni, L.; Gullo, F.P.; Rossi, S.A.; Derissi, J.B.; de Azevedo Prata, M.C.; Fusco-Almeida, A.M.; Mendes-Giannini, M.J. Virulence of *Cryptococcus* sp. Biofilms *In Vitro* and *In Vivo* using *Galleria mellonella* as an Alternative Model. *Front Microbiol* **2016**, *7*, 290, doi:10.3389/fmicb.2016.00290.
16. Walsh, T.J.; Schlegel, R.; Moody, M.M.; Costerton, J.W.; Salcman, M. Ventriculoatrial shunt infection due to *Cryptococcus neoformans*: an ultrastructural and quantitative microbiological study. *Neurosurgery* **1986**, *18*, 373-375, doi:10.1227/00006123-198603000-00025.
17. Bach, M.C.; Tally, P.W.; Godofsky, E.W. Use of cerebrospinal fluid shunts in patients having acquired immunodeficiency syndrome with cryptococcal meningitis and uncontrollable intracranial hypertension. *Neurosurgery* **1997**, *41*, 1280-1283.
18. Braun, D.K.; Janssen, D.A.; Marcus, J.R.; Kauffman, C.A. Cryptococcal infection of a prosthetic dialysis fistula. *American journal of kidney diseases* **1994**, *24*, 864-867.
19. Banerjee, U.; Gupta, K.; Venugopal, P. A case of prosthetic valve endocarditis caused by *Cryptococcus neoformans* var. neoformans. *J Med Vet Mycol* **1997**, *35*, 139-141.
20. Santi, L.; Beys-da-Silva, W.O.; Berger, M.; Calzolari, D.; Guimaraes, J.A.; Moresco, J.J.; Yates, J.R., 3rd. Proteomic profile of *Cryptococcus neoformans* biofilm reveals changes in metabolic processes. *J Proteome Res* **2014**, *13*, 1545-1559, doi:10.1021/pr401075f.
21. Martinez, L.R.; Casadevall, A. Specific Antibody Can Prevent Fungal Biofilm Formation and This Effect Correlates with Protective Efficacy. *Infection and Immunity* **2005**, *73*, 6350-6362, doi:doi:10.1128/IAI.73.10.6350-6362.2005.
22. Garcia-Rodas, R.; Zaragoza, O. Catch me if you can: phagocytosis and killing avoidance by *Cryptococcus neoformans*. *FEMS Immunol Med Microbiol* **2012**, *64*, 147-161, doi:10.1111/j.1574-695X.2011.00871.x.
23. Steenbergen, J.N.; Shuman, H.A.; Casadevall, A. *Cryptococcus neoformans* interactions with amoebae suggest an explanation for its virulence and intracellular pathogenic strategy in macrophages. *Proceedings of the National Academy of Sciences* **2001**, *98*, 15245-15250, doi:doi:10.1073/pnas.261418798.
24. Steenbergen, J.N.; Nosanchuk, J.D.; Malliaris, S.D.; Casadevall, A. *Cryptococcus neoformans* virulence is enhanced after growth in the genetically malleable host *Dictyostelium discoideum*. *Infection and immunity* **2003**, *71*, 4862-4872.
25. Fu, M.S.; Liporagi-Lopes, L.C.; Dos Santos, S.R.J.; Tenor, J.L.; Perfect, J.R.; Cuomo, C.A.; Casadevall, A. Amoeba Predation of *Cryptococcus neoformans* Results in Pleiotropic Changes to Traits Associated with Virulence. *mBio* **2021**, *12*, doi:10.1128/mBio.00567-21.
26. Neilson, J.; Ivey, M.; Bulmer, G. *Cryptococcus neoformans*: pseudohyphal forms surviving culture with *Acanthamoeba polyphaga*. *Infection and Immunity* **1978**, *20*, 262-266.
27. Paul, C.; Emeka, N. Pathogenicity of Cryptococcus neoformans VNI (ST 32) recovered from environmental and clinical isolates in Nigeria. *Comparative Clinical Pathology* **2019**, *28*, 1013-1024.
28. Rodrigues, M.L.; Nakayasu, E.S.; Oliveira, D.L.; Nimrichter, L.; Nosanchuk, J.D.; Almeida, I.C.; Casadevall, A. Extracellular vesicles produced by *Cryptococcus neoformans* contain protein components associated with virulence. *Eukaryot Cell* **2008**, *7*, 58-67, doi:10.1128/EC.00370-07.
29. Maliehe, M.; Ntoi, M.A.; Lahiri, S.; Folorunso, O.S.; Ogundeji, A.O.; Pohl, C.H.; Sebolai, O.M. Environmental Factors That Contribute to the Maintenance of *Cryptococcus neoformans* Pathogenesis. *Microorganisms* **2020**, *8*, 180, doi:10.3390/microorganisms8020180.
30. Derengowski Lda, S.; Paes, H.C.; Albuquerque, P.; Tavares, A.H.; Fernandes, L.; Silva-Pereira, I.; Casadevall, A. The transcriptional response of *Cryptococcus neoformans* to ingestion by Acanthamoeba castellanii and macrophages provides insights into the evolutionary adaptation to the mammalian host. *Eukaryot Cell* **2013**, *12*, 761-774, doi:10.1128/EC.00073-13.

31. Wang, Z.A.; Li, L.X.; Doering, T.L. Unraveling synthesis of the cryptococcal cell wall and capsule. *Glycobiology* **2018**, *28*, 719-730, doi:10.1093/glycob/cwy030.
32. Panepinto, J.; Liu, L.; Ramos, J.; Zhu, X.; Valyi-Nagy, T.; Eksi, S.; Fu, J.; Jaffe, H.A.; Wickes, B.; Williamson, P.R. The DEAD-box RNA helicase Vad1 regulates multiple virulence-associated genes in *Cryptococcus neoformans*. *J Clin Invest* **2005**, *115*, 632-641, doi:10.1172/JCI23048.
33. Fan, W.; Kraus, P.R.; Boily, M.J.; Heitman, J. *Cryptococcus neoformans* gene expression during murine macrophage infection. *Eukaryot Cell* **2005**, *4*, 1420-1433, doi:10.1128/EC.4.8.1420-1433.2005.
34. Price, M.S.; Betancourt-Quiroz, M.; Price, J.L.; Toffaletti, D.L.; Vora, H.; Hu, G.; Kronstad, J.W.; Perfect, J.R. *Cryptococcus neoformans* requires a functional glycolytic pathway for disease but not persistence in the host. *mBio* **2011**, *2*, e00103-00111, doi:10.1128/mBio.00103-11.
35. Griffiths, E.J.; Hu, G.; Fries, B.; Caza, M.; Wang, J.; Gsponer, J.; Gates‐Hollingsworth, M.A.; Kozel, T.R.; De Repentigny, L.; Kronstad, J.W. A defect in ATP‐citrate lyase links acetyl‐CoA production, virulence factor elaboration and virulence in *Cryptococcus neoformans*. *Molecular microbiology* **2012**, *86*, 1404-1423.
36. Fan, W.; Kraus, P.R.; Boily, M.-J.; Heitman, J. *Cryptococcus neoformans* Gene Expression during Murine Macrophage Infection. *Eukaryotic Cell* **2005**, *4*, 1420-1433, doi:doi:10.1128/EC.4.8.1420-1433.2005.
37. Xue, C.; Bahn, Y.S.; Cox, G.M.; Heitman, J. G protein-coupled receptor Gpr4 senses amino acids and activates the cAMP-PKA pathway in *Cryptococcus neoformans*. *Mol Biol Cell* **2006**, *17*, 667-679, doi:10.1091/mbc.e05-07-0699.
38. Bahn, Y.S.; Hicks, J.K.; Giles, S.S.; Cox, G.M.; Heitman, J. Adenylyl cyclase-associated protein Aca1 regulates virulence and differentiation of *Cryptococcus neoformans* via the cyclic AMP-protein kinase A cascade. *Eukaryot Cell* **2004**, *3*, 1476-1491, doi:10.1128/EC.3.6.1476-1491.2004.
39. Hu, G.; Steen, B.R.; Lian, T.; Sham, A.P.; Tam, N.; Tangen, K.L.; Kronstad, J.W. Transcriptional regulation by protein kinase A in *Cryptococcus neoformans*. *PLoS Pathog* **2007**, *3*, e42, doi:10.1371/journal.ppat.0030042.
40. Lorenz, M.C.; Bender, J.A.; Fink, G.R. Transcriptional response of *Candida albicans* upon internalization by macrophages. *Eukaryot Cell* **2004**, *3*, 1076-1087, doi:10.1128/EC.3.5.1076-1087.2004.
41. Rude, T.H.; Toffaletti, D.L.; Cox, G.M.; Perfect, J.R. Relationship of the glyoxylate pathway to the pathogenesis of *Cryptococcus neoformans*. *Infect Immun* **2002**, *70*, 5684-5694, doi:10.1128/IAI.70.10.5684-5694.2002.
42. McClelland, E.E.; Ramagopal, U.A.; Rivera, J.; Cox, J.; Nakouzi, A.; Prabu, M.M.; Almo, S.C.; Casadevall, A. A Small Protein Associated with Fungal Energy Metabolism Affects the Virulence of *Cryptococcus neoformans* in Mammals. *PLoS Pathog* **2016**, *12*, e1005849, doi:10.1371/journal.ppat.1005849.
43. Lian, T.; Simmer, M.I.; D'Souza, C.A.; Steen, B.R.; Zuyderduyn, S.D.; Jones, S.J.; Marra, M.A.; Kronstad, J.W. Iron-regulated transcription and capsule formation in the fungal pathogen *Cryptococcus neoformans*. *Mol Microbiol* **2005**, *55*, 1452-1472, doi:10.1111/j.1365-2958.2004.04474.x.
44. Jung, W.H.; Saikia, S.; Hu, G.; Wang, J.; Fung, C.K.; D'Souza, C.; White, R.; Kronstad, J.W. HapX positively and negatively regulates the transcriptional response to iron deprivation in *Cryptococcus neoformans*. *PLoS Pathog* **2010**, *6*, e1001209, doi:10.1371/journal.ppat.1001209.
45. Eisenstein, R.S. Iron regulatory proteins and the molecular control of mammalian iron metabolism. *Annu Rev Nutr* **2000**, *20*, 627-662, doi:10.1146/annurev.nutr.20.1.627.
46. Ihrig, J.; Hausmann, A.; Hain, A.; Richter, N.; Hamza, I.; Lill, R.; Mühlenhoff, U. Iron regulation through the back door: iron-dependent metabolite levels contribute to transcriptional adaptation to iron deprivation in *Saccharomyces cerevisiae*. *Eukaryotic cell* **2010**, *9*, 460-471.
47. Ramos-Alonso, L.; Romero, A.M.; Martinez-Pastor, M.T.; Puig, S. Iron Regulatory Mechanisms in *Saccharomyces cerevisiae*. *Front Microbiol* **2020**, *11*, 582830, doi:10.3389/fmicb.2020.582830.
48. Garcia-Santamarina, S.; Uzarska, M.A.; Festa, R.A.; Lill, R.; Thiele, D.J. *Cryptococcus neoformans* Iron-Sulfur Protein Biogenesis Machinery Is a Novel Layer of Protection against Cu Stress. *mBio* **2017**, *8*, e01742-01717, doi:10.1128/mBio.01742-17.
49. Do, E.; Hu, G.; Caza, M.; Oliveira, D.; Kronstad, J.W.; Jung, W.H. Leu1 plays a role in iron metabolism and is required for virulence in *Cryptococcus neoformans*. *Fungal Genet Biol* **2015**, *75*, 11-19, doi:10.1016/j.fgb.2014.12.006.
50. Nazi, I.; Scott, A.; Sham, A.; Rossi, L.; Williamson, P.R.; Kronstad, J.W.; Wright, G.D. Role of homoserine transacetylase as a new target for antifungal agents. *Antimicrob Agents Chemother* **2007**, *51*, 1731-1736, doi:10.1128/AAC.01400-06.

51. Pascon, R.C.; Ganous, T.M.; Kingsbury, J.M.; Cox, G.M.; McCusker, J.H. *Cryptococcus neoformans* methionine synthase: expression analysis and requirement for virulence. *Microbiology (Reading)* **2004**, *150*, 3013-3023, doi:10.1099/mic.0.27235-0.
52. Do, E.; Park, M.; Hu, G.; Caza, M.; Kronstad, J.W.; Jung, W.H. The lysine biosynthetic enzyme Lys4 influences iron metabolism, mitochondrial function and virulence in *Cryptococcus neoformans*. *Biochem Biophys Res Commun* **2016**, *477*, 706-711, doi:10.1016/j.bbrc.2016.06.123.
53. Zhang, P.; Wei, D.; Li, Z.; Sun, Z.; Pan, J.; Zhu, X. Cryptococcal phosphoglucose isomerase is required for virulence factor production, cell wall integrity and stress resistance. *FEMS Yeast Res* **2015**, *15*, doi:10.1093/femsyr/fov072.
54. Barrett, L.; Orlova, M.; Maziarz, M.; Kuchin, S. Protein kinase A contributes to the negative control of Snf1 protein kinase in *Saccharomyces cerevisiae*. *Eukaryot Cell* **2012**, *11*, 119-128, doi:10.1128/EC.05061-11.
55. Hong, S.-P.; Leiper, F.C.; Woods, A.; Carling, D.; Carlson, M. Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases. *Proceedings of the National Academy of Sciences* **2003**, *100*, 8839-8843.
56. McCartney, R.R.; Rubenstein, E.M.; Schmidt, M.C. Snf1 kinase complexes with different beta subunits display stress-dependent preferences for the three Snf1-activating kinases. *Current genetics* **2005**, *47*, 335-344.
57. McCartney, R.R.; Schmidt, M.C. Regulation of Snf1 kinase: activation requires phosphorylation of threonine 210 by an upstream kinase as well as a distinct step mediated by the Snf4 subunit. *Journal of Biological Chemistry* **2001**, *276*, 36460-36466.
58. Tu, J.; Carlson, M. REG1 binds to protein phosphatase type 1 and regulates glucose repression in Saccharomyces cerevisiae. *The EMBO Journal* **1995**, *14*, 5939-5946.
59. Nehlin, J.O.; Ronne, H. Yeast MIG1 repressor is related to the mammalian early growth response and Wilms' tumour finger proteins. *EMBO J* **1990**, *9*, 2891-2898, doi:10.1002/j.1460-2075.1990.tb07479.x.
60. Nicastro, R.; Tripodi, F.; Gaggini, M.; Castoldi, A.; Reghellin, V.; Nonnis, S.; Tedeschi, G.; Coccetti, P. Snf1 Phosphorylates Adenylate Cyclase and Negatively Regulates Protein Kinase A-dependent Transcription in *Saccharomyces cerevisiae*. *J Biol Chem* **2015**, *290*, 24715-24726, doi:10.1074/jbc.M115.658005.
61. Hu, G.; Cheng, P.Y.; Sham, A.; Perfect, J.R.; Kronstad, J.W. Metabolic adaptation in *Cryptococcus neoformans* during early murine pulmonary infection. *Mol Microbiol* **2008**, *69*, 1456-1475, doi:10.1111/j.1365-2958.2008.06374.x.
62. Pukkila-Worley, R.; Gerrald, Q.D.; Kraus, P.R.; Boily, M.J.; Davis, M.J.; Giles, S.S.; Cox, G.M.; Heitman, J.; Alspaugh, J.A. Transcriptional network of multiple capsule and melanin genes governed by the *Cryptococcus neoformans* cyclic AMP cascade. *Eukaryot Cell* **2005**, *4*, 190-201, doi:10.1128/EC.4.1.190-201.2005.
63. Zhu, X.; Williamson, P.R. Role of laccase in the biology and virulence of *Cryptococcus neoformans*. *FEMS Yeast Res* **2004**, *5*, 1-10, doi:10.1016/j.femsyr.2004.04.004.
64. Alden, K.M.; Jezewski, A.J.; Beattie, S.R.; Fox, D., 3rd; Krysan, D.J. Genetic Interaction Analysis Reveals that *Cryptococcus neoformans* Utilizes Multiple Acetyl-CoA-Generating Pathways during Infection. *mBio* **2022**, *13*, e0127922, doi:10.1128/mbio.01279-22.
65. Saito, K.; Sugatani, J.; Okumura, T. Phospholipase B from *Penicillium notatum*. In *Methods in enzymology*; Elsevier: 1991; Volume 197, pp. 446-456.
66. Lee, K.S.; Patton, J.L.; Fido, M.; Hines, L.K.; Kohlwein, S.D.; Paltauf, F.; Henry, S.A.; Levin, D.E. The *Saccharomyces cerevisiae* PLB1 gene encodes a protein required for lysophospholipase and phospholipase B activity. *J Biol Chem* **1994**, *269*, 19725-19730.
67. Leidich, S.D.; Ibrahim, A.S.; Fu, Y.; Koul, A.; Jessup, C.; Vitullo, J.; Fonzi, W.; Mirbod, F.; Nakashima, S.; Nozawa, Y.; et al. Cloning and disruption of caPLB1, a phospholipase B gene involved in the pathogenicity of *Candida albicans*. *J Biol Chem* **1998**, *273*, 26078-26086, doi:10.1074/jbc.273.40.26078.
68. Siafakas, A.R.; Sorrell, T.C.; Wright, L.C.; Wilson, C.; Larsen, M.; Boadle, R.; Williamson, P.R.; Djordjevic, J.T. Cell wall-linked cryptococcal phospholipase B1 is a source of secreted enzyme and a determinant of cell wall integrity. *J Biol Chem* **2007**, *282*, 37508-37514, doi:10.1074/jbc.M707913200.
69. Chen, S.C.; Muller, M.; Zhou, J.Z.; Wright, L.C.; Sorrell, T.C. Phospholipase activity in *Cryptococcus neoformans*: a new virulence factor? *J Infect Dis* **1997**, *175*, 414-420, doi:10.1093/infdis/175.2.414.
70. Chayakulkeeree, M.; Sorrell, T.C.; Siafakas, A.R.; Wilson, C.F.; Pantarat, N.; Gerik, K.J.; Boadle, R.; Djordjevic, J.T. Role and mechanism of phosphatidylinositol-specific phospholipase C in survival and virulence of *Cryptococcus neoformans*. *Mol Microbiol* **2008**, *69*, 809-826, doi:10.1111/j.1365-2958.2008.06310.x.
71. Lev, S.; Desmarini, D.; Li, C.; Chayakulkeeree, M.; Traven, A.; Sorrell, T.C.; Djordjevic, J.T. Phospholipase C of *Cryptococcus neoformans* regulates homeostasis and virulence by providing inositol trisphosphate as a substrate for Arg1 kinase. *Infect Immun* **2013**, *81*, 1245-1255, doi:10.1128/IAI.01421-12.

72. Cox, G.M.; McDade, H.C.; Chen, S.C.; Tucker, S.C.; Gottfredsson, M.; Wright, L.C.; Sorrell, T.C.; Leidich, S.D.; Casadevall, A.; Ghannoum, M.A.; et al. Extracellular phospholipase activity is a virulence factor for *Cryptococcus neoformans*. *Mol Microbiol* **2001**, *39*, 166-175, doi:10.1046/j.1365-2958.2001.02236.x.
73. Ibrahim, A.S.; Mirbod, F.; Filler, S.G.; Banno, Y.; Cole, G.T.; Kitajima, Y.; Edwards, J.E., Jr.; Nozawa, Y.; Ghannoum, M.A. Evidence implicating phospholipase as a virulence factor of *Candida albicans*. *Infect Immun* **1995**, *63*, 1993-1998, doi:10.1128/iai.63.5.1993-1998.1995.
74. Chang, Y.C.; Wickes, B.L.; Miller, G.F.; Penoyer, L.A.; Kwon-Chung, K.J. *Cryptococcus neoformans* STE12α regulates virulence but is not essential for mating. *J Exp Med* **2000**, *191*, 871-882, doi:10.1084/jem.191.5.871.
75. Narasipura, S.D.; Chaturvedi, V.; Chaturvedi, S. Characterisation of *Cryptococcus neoformans* variety *gattii* SOD2 reveals distinct roles of the two superoxide dismutases in fungal biology and virulence. *Mol Microbiol* **2005**, *55*, 1782-1800, doi:10.1111/j.1365-2958.2005.04503.x.
76. Narasipura, S.D.; Ault, J.G.; Behr, M.J.; Chaturvedi, V.; Chaturvedi, S. Characterisation of Cu,Zn superoxide dismutase (SOD1) gene knock-out mutant of *Cryptococcus neoformans* var. *gattii*: role in biology and virulence. *Mol Microbiol* **2003**, *47*, 1681-1694, doi:10.1046/j.1365-2958.2003.03393.x.
77. Chaturvedi, V.; Wong, B.; Newman, S.L. Oxidative killing of Cryptococcus neoformans by human neutrophils - Evidence that fungal mannitol protects by scavenging reactive oxygen intermediates. *Journal of Immunology* **1996**, *156*, 3836-3840.
78. Narasipura, S.D.; Chaturvedi, V.; Chaturvedi, S. Characterization of *Cryptococcus neoformans* variety *gattii* SOD2 reveals distinct roles of the two superoxide dismutases in fungal biology and virulence. *Molecular microbiology* **2005**, *55*, 1782-1800.
79. Garcia-Martos, P.; Marin, P.; Hernandez-Molina, J.M.; Garcia-Agudo, L.; Aoufi, S.; Mira, J. Extracellular enzymatic activity in 11 *Cryptococcus* species. *Mycopathologia* **2001**, *150*, 1-4, doi:10.1023/a:1010868223582.
80. Park, M.; Do, E.; Jung, W.H. Lipolytic enzymes involved in the virulence of human pathogenic fungi. *Mycobiology* **2013**, *41*, 67-72, doi:10.5941/MYCO.2013.41.2.67.
81. Maeng, S.; Ko, Y.J.; Kim, G.B.; Jung, K.W.; Floyd, A.; Heitman, J.; Bahn, Y.S. Comparative transcriptome analysis reveals novel roles of the Ras and cyclic AMP signaling pathways in environmental stress response and antifungal drug sensitivity in *Cryptococcus neoformans*. *Eukaryot Cell* **2010**, *9*, 360-378, doi:10.1128/EC.00309-09.
82. Martho, K.F.; Brustolini, O.J.B.; Vasconcelos, A.T.; Vallim, M.A.; Pascon, R.C. The Glycerol Phosphatase Gpp2: A Link to Osmotic Stress, Sulfur Assimilation and Virulence in *Cryptococcus neoformans*. *Front Microbiol* **2019**, *10*, 2728, doi:10.3389/fmicb.2019.02728.
83. de Melo, A.T.; Martho, K.F.; Roberto, T.N.; Nishiduka, E.S.; Machado, J., Jr.; Brustolini, O.J.B.; Tashima, A.K.; Vasconcelos, A.T.; Vallim, M.A.; Pascon, R.C. The regulation of the sulfur amino acid biosynthetic pathway in *Cryptococcus neoformans*: the relationship of Cys3, Calcineurin, and Gpp2 phosphatases. *Sci Rep* **2019**, *9*, 11923, doi:10.1038/s41598-019-48433-5.
84. Ko, Y.J.; Yu, Y.M.; Kim, G.B.; Lee, G.W.; Maeng, P.J.; Kim, S.; Floyd, A.; Heitman, J.; Bahn, Y.S. Remodeling of global transcription patterns of *Cryptococcus neoformans* genes mediated by the stress-activated HOG signaling pathways. *Eukaryot Cell* **2009**, *8*, 1197-1217, doi:10.1128/EC.00120-09.
85. Grewal, S.I.; Bonaduce, M.J.; Klar, A.J. Histone deacetylase homologs regulate the epigenetic inheritance of transcriptional silencing and chromosome segregation in fission yeast. *Genetics* **1998**, *150*, 563-576, doi:10.1093/genetics/150.2.563.
86. Maier, E.J.; Haynes, B.C.; Gish, S.R.; Wang, Z.A.; Skowyra, M.L.; Marulli, A.L.; Doering, T.L.; Brent, M.R. Model-driven mapping of transcriptional networks reveals the circuitry and dynamics of virulence regulation. *Genome Res* **2015**, *25*, 690-700, doi:10.1101/gr.184101.114.
87. Kraus, P.R.; Boily, M.J.; Giles, S.S.; Stajich, J.E.; Allen, A.; Cox, G.M.; Dietrich, F.S.; Perfect, J.R.; Heitman, J. Identification of *Cryptococcus neoformans* temperature-regulated genes with a genomic-DNA microarray. *Eukaryot Cell* **2004**, *3*, 1249-1260, doi:10.1128/EC.3.5.1249-1260.2004.
88. Chellappa, R.; Kandasamy, P.; Oh, C.S.; Jiang, Y.; Vemula, M.; Martin, C.E. The membrane proteins, Spt23p and Mga2p, play distinct roles in the activation of *Saccharomyces cerevisiae* OLE1 gene expression. Fatty acid-mediated regulation of Mga2p activity is independent of its proteolytic processing into a soluble transcription activator. *J Biol Chem* **2001**, *276*, 43548-43556, doi:10.1074/jbc.M107845200.
89. Martin, C.E.; Oh, C.S.; Jiang, Y. Regulation of long chain unsaturated fatty acid synthesis in yeast. *Biochim Biophys Acta* **2007**, *1771*, 271-285, doi:10.1016/j.bbalip.2006.06.010.
90. Lee, I.R.; Chow, E.W.; Morrow, C.A.; Djordjevic, J.T.; Fraser, J.A. Nitrogen metabolite repression of metabolism and virulence in the human fungal pathogen *Cryptococcus neoformans*. *Genetics* **2011**, *188*, 309-323, doi:10.1534/genetics.111.128538.

91. Ries, L.N.A.; Beattie, S.; Cramer, R.A.; Goldman, G.H. Overview of carbon and nitrogen catabolite metabolism in the virulence of human pathogenic fungi. *Mol Microbiol* **2018**, *107*, 277-297, doi:10.1111/mmi.13887.
92. Fernandes, J.D.; Martho, K.; Tofik, V.; Vallim, M.A.; Pascon, R.C. The Role of Amino Acid Permeases and Tryptophan Biosynthesis in *Cryptococcus neoformans* Survival. *PLoS One* **2015**, *10*, e0132369, doi:10.1371/journal.pone.0132369.
93. Kingsbury, J.M.; McCusker, J.H. Threonine biosynthetic genes are essential in *Cryptococcus neoformans*. *Microbiology (Reading)* **2008**, *154*, 2767-2775, doi:10.1099/mic.0.2008/019729-0.
94. Wong, F.H.; Chen, J.S.; Reddy, V.; Day, J.L.; Shlykov, M.A.; Wakabayashi, S.T.; Saier, M.H., Jr. The amino acid-polyamine-organocation superfamily. *J Mol Microbiol Biotechnol* **2012**, *22*, 105-113, doi:10.1159/000338542.
95. Mikros, E.; Diallinas, G. Tales of tails in transporters. *Open Biol* **2019**, *9*, 190083, doi:10.1098/rsob.190083.
96. Wyman, C.L. *Analysis Of Membrane Transporter Systems Expressed During Symbiotic Nitrogen Fixation In The Model Legume Medicago Truncatula*; West Virginia University: 2018.
97. Calvete, C.L.; Martho, K.F.; Felizardo, G.; Paes, A.; Nunes, J.M.; Ferreira, C.O.; Vallim, M.A.; Pascon, R.C. Amino acid permeases in *Cryptococcus neoformans* are required for high temperature growth and virulence; and are regulated by Ras signalling. *PLoS One* **2019**, *14*, e0211393, doi:10.1371/journal.pone.0211393.
98. Martho, K.F.; de Melo, A.T.; Takahashi, J.P.; Guerra, J.M.; Santos, D.C.; Purisco, S.U.; Melhem, M.S.; Fazioli, R.D.; Phanord, C.; Sartorelli, P.; et al. Amino Acid Permeases and Virulence in *Cryptococcus neoformans*. *PLoS One* **2016**, *11*, e0163919, doi:10.1371/journal.pone.0163919.
99. Garbe, E.; Vylkova, S. Role of Amino Acid Metabolism in the Virulence of Human Pathogenic Fungi. *Current Clinical Microbiology Reports* **2019**, *6*, 108-119, doi:10.1007/s40588-019-00124-5.
100. Chang, Y.C.; Khanal Lamichhane, A.; Bradley, J.; Rodgers, L.; Ngamskulrungroj, P.; Kwon-Chung, K.J. Differences between *Cryptococcus neoformans* and *Cryptococcus gattii* in the Molecular Mechanisms Governing Utilization of *D*-Amino Acids as the Sole Nitrogen Source. *PLoS One* **2015**, *10*, e0131865, doi:10.1371/journal.pone.0131865.
101. Alspaugh, J.A.; Cavallo, L.M.; Perfect, J.R.; Heitman, J. RAS1 regulates filamentation, mating and growth at high temperature of *Cryptococcus neoformans*. *Mol Microbiol* **2000**, *36*, 352-365, doi:10.1046/j.1365-2958.2000.01852.x.
102. Nichols, C.B.; Perfect, Z.H.; Alspaugh, J.A. A Ras1-Cdc24 signal transduction pathway mediates thermotolerance in the fungal pathogen *Cryptococcus neoformans*. *Mol Microbiol* **2007**, *63*, 1118-1130, doi:10.1111/j.1365-2958.2006.05566.x.
103. Vallim, M.A.; Nichols, C.B.; Fernandes, L.; Cramer, K.L.; Alspaugh, J.A. A Rac homolog functions downstream of Ras1 to control hyphal differentiation and high-temperature growth in the pathogenic fungus *Cryptococcus neoformans*. *Eukaryot Cell* **2005**, *4*, 1066-1078, doi:10.1128/EC.4.6.1066-1078.2005.
104. Fernandes, J.D.S.; Martho, K.; Tofik, V.; Vallim, M.A.; Pascon, R.C. The Role of Amino Acid Permeases and Tryptophan Biosynthesis in *Cryptococcus neoformans* Survival. *PLOS ONE* **2015**, *10*, e0132369, doi:10.1371/journal.pone.0132369.
105. Pentland, D.R.; Piper-Brown, E.; Muhlschlegel, F.A.; Gourlay, C.W. Ras signalling in pathogenic yeasts. *Microb Cell* **2017**, *5*, 63-73, doi:10.15698/mic2018.02.612.
106. Eigenheer, R.A.; Jin Lee, Y.; Blumwald, E.; Phinney, B.S.; Gelli, A. Extracellular glycosylphosphatidylinositol-anchored mannoproteins and proteases of *Cryptococcus neoformans*. *FEMS Yeast Res* **2007**, *7*, 499-510, doi:10.1111/j.1567-1364.2006.00198.x.
107. Novick, P.; Ferro, S.; Schekman, R. Order of events in the yeast secretory pathway. *Cell* **1981**, *25*, 461-469.
108. Vu, K.; Tham, R.; Uhrig, J.P.; Thompson, G.R., 3rd; Na Pombejra, S.; Jamklang, M.; Bautos, J.M.; Gelli, A. Invasion of the central nervous system by *Cryptococcus neoformans* requires a secreted fungal metalloprotease. *mBio* **2014**, *5*, e01101-01114, doi:10.1128/mBio.01101-14.
109. Homer, C.M.; Summers, D.K.; Goranov, A.I.; Clarke, S.C.; Wiesner, D.L.; Diedrich, J.K.; Moresco, J.J.; Toffaletti, D.; Upadhya, R.; Caradonna, I.; et al. Intracellular Action of a Secreted Peptide Required for Fungal Virulence. *Cell Host Microbe* **2016**, *19*, 849-864, doi:10.1016/j.chom.2016.05.001.
110. Brueske, C.H. Proteolytic activity of a clinical isolate of *Cryptococcus neoformans*. *J Clin Microbiol* **1986**, *23*, 631-633, doi:10.1128/jcm.23.3.631-633.1986.
111. Yoo Ji, J.; Lee, Y.S.; Song, C.Y.; Kim, B.S. Purification and characterisation of a 43-kilodalton extracellular serine proteinase from *Cryptococcus neoformans*. *J Clin Microbiol* **2004**, *42*, 722-726, doi:10.1128/JCM.42.2.722-726.2004.
112. Chen, L.C.; Blank, E.S.; Casadevall, A. Extracellular proteinase activity of *Cryptococcus neoformans*. *Clin Diagn Lab Immunol* **1996**, *3*, 570-574, doi:10.1128/cdli.3.5.570-574.1996.

113. Ruma-Haynes, P.; Brownlee, A.G.; Sorrell, T.C. A rapid method for detecting extracellular proteinase activity in *Cryptococcus neoformans* and a survey of 63 isolates. *J Med Microbiol* **2000**, *49*, 733-737, doi:10.1099/0022-1317-49-8-733.
114. Leone, R.; Cabeli, P.; Sinicco, A.; Ito-Kuwa, S.; Aoki, S.; Vidotto, V. Relationship between protease production and capsule size in Cryptococcus neoformans. *Journal De Mycologie Medicale* **1999**, *9*, 42-44.
115. Clarke, S.C.; Dumesic, P.A.; Homer, C.M.; O'Donoghue, A.J.; La Greca, F.; Pallova, L.; Majer, P.; Madhani, H.D.; Craik, C.S. Integrated Activity and Genetic Profiling of Secreted Peptidases in *Cryptococcus neoformans* Reveals an Aspartyl Peptidase Required for Low pH Survival and Virulence. *PLoS Pathog* **2016**, *12*, e1006051, doi:10.1371/journal.ppat.1006051.
116. Monari, C.; Pericolini, E.; Bistoni, G.; Cenci, E.; Bistoni, F.; Vecchiarelli, A. Influence of indinavir on virulence and growth of *Cryptococcus neoformans*. *J Infect Dis* **2005**, *191*, 307-311, doi:10.1086/426828.
117. Sidrim, J.J.; Perdigao-Neto, L.V.; Cordeiro, R.A.; Brilhante, R.S.; Leite, J.J.; Teixeira, C.E.; Monteiro, A.J.; Freitas, R.M.; Ribeiro, J.F.; Mesquita, J.R.; et al. Viral protease inhibitors affect the production of virulence factors in *Cryptococcus neoformans*. *Can J Microbiol* **2012**, *58*, 932-936, doi:10.1139/w2012-075.
118. Pinti, M.; Orsi, C.F.; Gibellini, L.; Esposito, R.; Cossarizza, A.; Blasi, E.; Peppoloni, S.; Mussini, C. Identification and characterisation of an aspartyl protease from *Cryptococcus neoformans*. *FEBS Lett* **2007**, *581*, 3882-3886, doi:10.1016/j.febslet.2007.07.006.
119. Singh, A.; Panting, R.J.; Varma, A.; Saijo, T.; Waldron, K.J.; Jong, A.; Ngamskulrungroj, P.; Chang, Y.C.; Rutherford, J.C.; Kwon-Chung, K.J. Factors required for activation of urease as a virulence determinant in *Cryptococcus neoformans*. *mBio* **2013**, *4*, e00220-00213, doi:10.1128/mBio.00220-13.
120. Cox, G.M.; Mukherjee, J.; Cole, G.T.; Casadevall, A.; Perfect, J.R. Urease as a virulence factor in experimental cryptococcosis. *Infect Immun* **2000**, *68*, 443-448, doi:10.1128/IAI.68.2.443-448.2000.
121. Olszewski, M.A.; Noverr, M.C.; Chen, G.H.; Toews, G.B.; Cox, G.M.; Perfect, J.R.; Huffnagle, G.B. Urease expression by *Cryptococcus neoformans* promotes microvascular sequestration, thereby enhancing central nervous system invasion. *Am J Pathol* **2004**, *164*, 1761-1771, doi:10.1016/S0002-9440(10)63734-0.
122. Bava, A.J.; Negroni, R.; Bianchi, M. Cryptococcosis produced by a urease negative strain of *Cryptococcus neoformans*. *J Med Vet Mycol* **1993**, *31*, 87-89.
123. Ruane, P.J.; Walker, L.J.; George, W.L. Disseminated infection caused by urease-negative *Cryptococcus neoformans*. *J Clin Microbiol* **1988**, *26*, 2224-2225, doi:10.1128/jcm.26.10.2224-2225.1988.
124. Almeida, F.; Wolf, J.M.; Casadevall, A. Virulence-Associated Enzymes of *Cryptococcus neoformans*. *Eukaryot Cell* **2015**, *14*, 1173-1185, doi:10.1128/EC.00103-15.
125. Tangen, K.L.; Jung, W.H.; Sham, A.P.; Lian, T.; Kronstad, J.W. The iron- and cAMP-regulated gene SIT1 influences ferrioxamine B utilisation, melanisation and cell wall structure in *Cryptococcus neoformans*. *Microbiology (Reading)* **2007**, *153*, 29-41, doi:10.1099/mic.0.2006/000927-0.
126. Lee, D.; Jang, E.H.; Lee, M.; Kim, S.W.; Lee, Y.; Lee, K.T.; Bahn, Y.S. Unraveling Melanin Biosynthesis and Signalling Networks in *Cryptococcus neoformans*. *mBio* **2019**, *10*, e02267-02219, doi:10.1128/mBio.02267-19.
127. Wang, Y.; Aisen, P.; Casadevall, A. *Cryptococcus neoformans* melanin and virulence: mechanism of action. *Infect Immun* **1995**, *63*, 3131-3136, doi:10.1128/iai.63.8.3131-3136.1995.
128. Durrell, L.W. The Composition and Structure of Walls of Dark Fungus Spores. *Mycopathol Mycol Appl* **1964**, *23*, 337-345, doi:10.1007/BF02049005.
129. Rosas, A.L.; Casadevall, A. Melanization affects susceptibility of *Cryptococcus neoformans* to heat and cold. *FEMS Microbiol Lett* **1997**, *153*, 265-272, doi:10.1111/j.1574-6968.1997.tb12584.x.
130. Wang, Y.; Aisen, P.; Casadevall, A. Melanin, melanin "ghosts," and melanin composition in *Cryptococcus neoformans*. *Infect Immun* **1996**, *64*, 2420-2424, doi:10.1128/iai.64.7.2420-2424.1996.
131. Garcia-Rivera, J.; Eisenman, H.C.; Nosanchuk, J.D.; Aisen, P.; Zaragoza, O.; Moadel, T.; Dadachova, E.; Casadevall, A. Comparative analysis of *Cryptococcus neoformans* acid-resistant particles generated from pigmented cells grown in different laccase substrates. *Fungal Genet Biol* **2005**, *42*, 989-998, doi:10.1016/j.fgb.2005.09.003.
132. Garcia-Rivera, J.; Casadevall, A. Melanization of *Cryptococcus neoformans* reduces its susceptibility to the antimicrobial effects of silver nitrate. *Med Mycol* **2001**, *39*, 353-357, doi:10.1080/mmy.39.4.353.357.
133. Wang, Y.; Casadevall, A. Growth of *Cryptococcus neoformans* in the presence of *L*-dopa decreases its susceptibility to amphotericin B. *Antimicrob Agents Chemother* **1994**, *38*, 2648-2650, doi:10.1128/AAC.38.11.2648.
134. Wang, Y.; Casadevall, A. Susceptibility of melanised and nonmelanised *Cryptococcus neoformans* to nitrogen- and oxygen-derived oxidants. *Infect Immun* **1994**, *62*, 3004-3007, doi:10.1128/iai.62.7.3004-3007.1994.

135. Rosas, A.L.; Nosanchuk, J.D.; Gomez, B.L.; Edens, W.A.; Henson, J.M.; Casadevall, A. Isolation and serological analyses of fungal melanins. *J Immunol Methods* **2000**, *244*, 69-80, doi:10.1016/s0022-1759(00)00255-6.
136. Sussman, A.S. *The Fungal Population: An Advanced Treatise*; Elsevier Science: 2013; pp. 447-486.
137. Godinho, R.M.; Crestani, J.; Kmetzsch, L.; Araujo Gde, S.; Frases, S.; Staats, C.C.; Schrank, A.; Vainstein, M.H.; Rodrigues, M.L. The vacuolar-sorting protein Snf7 is required for the export of virulence determinants in members of the *Cryptococcus neoformans* complex. *Sci Rep* **2014**, *4*, 6198, doi:10.1038/srep06198.
138. Walton, F.J.; Idnurm, A.; Heitman, J. Novel gene functions required for melanisation of the human pathogen *Cryptococcus neoformans*. *Mol Microbiol* **2005**, *57*, 1381-1396, doi:10.1111/j.1365-2958.2005.04779.x.
139. Alspaugh, J.A.; Perfect, J.R.; Heitman, J. *Cryptococcus neoformans* mating and virulence are regulated by the G-protein alpha subunit GPA1 and cAMP. *Genes Dev* **1997**, *11*, 3206-3217, doi:10.1101/gad.11.23.3206.
140. Bahn, Y.S.; Kojima, K.; Cox, G.M.; Heitman, J. Specialization of the HOG pathway and its impact on differentiation and virulence of *Cryptococcus neoformans*. *Mol Biol Cell* **2005**, *16*, 2285-2300, doi:10.1091/mbc.e04-11-0987.
141. Bahn, Y.S.; Kojima, K.; Cox, G.M.; Heitman, J. A unique fungal two-component system regulates stress responses, drug sensitivity, sexual development, and virulence of *Cryptococcus neoformans*. *Mol Biol Cell* **2006**, *17*, 3122-3135, doi:10.1091/mbc.e06-02-0113.
142. Lee, J.W.; Ko, Y.J.; Kim, S.Y.; Bahn, Y.S. Multiple roles of Ypd1 phosphotransfer protein in viability, stress response, and virulence factor regulation in *Cryptococcus neoformans*. *Eukaryot Cell* **2011**, *10*, 998-1002, doi:10.1128/EC.05124-11.
143. Song, M.H.; Lee, J.W.; Kim, M.S.; Yoon, J.K.; White, T.C.; Floyd, A.; Heitman, J.; Strain, A.K.; Nielsen, J.N.; Nielsen, K.; et al. A flucytosine-responsive Mbp1/Swi4-like protein, Mbs1, plays pleiotropic roles in antifungal drug resistance, stress response, and virulence of *Cryptococcus neoformans*. *Eukaryot Cell* **2012**, *11*, 53-67, doi:10.1128/EC.05236-11.
144. D'Souza, C.A.; Alspaugh, J.A.; Yue, C.; Harashima, T.; Cox, G.M.; Perfect, J.R.; Heitman, J. Cyclic AMP-dependent protein kinase controls virulence of the fungal pathogen *Cryptococcus neoformans*. *Mol Cell Biol* **2001**, *21*, 3179-3191, doi:10.1128/MCB.21.9.3179-3191.2001.
145. Salas, S.D.; Bennett, J.E.; Kwon-Chung, K.J.; Perfect, J.R.; Williamson, P.R. Effect of the laccase gene CNLAC1, on the virulence of *Cryptococcus neoformans*. *J Exp Med* **1996**, *184*, 377-386, doi:10.1084/jem.184.2.377.
146. Nosanchuk, J.D.; Rosas, A.L.; Lee, S.C.; Casadevall, A. Melanisation of *Cryptococcus neoformans* in human brain tissue. *Lancet* **2000**, *355*, 2049-2050, doi:10.1016/S0140-6736(00)02356-4.
147. Rosas, A.L.; Nosanchuk, J.D.; Feldmesser, M.; Cox, G.M.; McDade, H.C.; Casadevall, A. Synthesis of polymerised melanin by *Cryptococcus neoformans* in infected rodents. *Infect Immun* **2000**, *68*, 2845-2853, doi:10.1128/IAI.68.5.2845-2853.2000.
148. Rhodes, J.C.; Polacheck, I.; Kwon-Chung, K.J. Phenoloxidase activity and virulence in isogenic strains of *Cryptococcus neoformans*. *Infect Immun* **1982**, *36*, 1175-1184, doi:10.1128/iai.36.3.1175-1184.1982.
149. Liu, L.; Wakamatsu, K.; Ito, S.; Williamson, P.R. Catecholamine oxidative products, but not melanin, are produced by *Cryptococcus neoformans* during neuropathogenesis in mice. *Infect Immun* **1999**, *67*, 108-112, doi:10.1128/IAI.67.1.108-112.1999.
150. Ito, S.; Wakamatsu, K. Chemical degradation of melanins: application to identification of dopamine-melanin. *Pigment Cell Res* **1998**, *11*, 120-126, doi:10.1111/j.1600-0749.1998.tb00721.x.
151. Napolitano, A.; Pezzella, A.; d'Ischia, M.; Prota, G. New pyrrole acids by oxidative degradation of eumelanins with hydrogen peroxide. Further hints to the mechanism of pigment breakdown. *Tetrahedron* **1996**, *52*, 8775-8780, doi:10.1016/0040-4020(96)00418-8.
152. Napolitano, A.; Pezzella, A.; Vincensi, M.R.; Prota, G. Oxidative degradation of melanins to pyrrole acids: A model study. *Tetrahedron* **1995**, *51*, 5913-5920, doi:10.1016/0040-4020(95)00259-b.
153. Wang, P.; Cutler, J.; King, J.; Palmer, D. Mutation of the regulator of G protein signalling Crg1 increases virulence in *Cryptococcus neoformans*. *Eukaryot Cell* **2004**, *3*, 1028-1035, doi:10.1128/EC.3.4.1028-1035.2004.
154. Luberto, C.; Toffaletti, D.L.; Wills, E.A.; Tucker, S.C.; Casadevall, A.; Perfect, J.R.; Hannun, Y.A.; Del Poeta, M. Roles for inositol-phosphoryl ceramide synthase 1 (IPC1) in the pathogenesis of *C. neoformans*. *Genes Dev* **2001**, *15*, 201-212, doi:10.1101/gad.856001.
155. Lee, C.G.; Da Silva, C.A.; Dela Cruz, C.S.; Ahangari, F.; Ma, B.; Kang, M.J.; He, C.H.; Takyar, S.; Elias, J.A. Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodelling, and injury. *Annu Rev Physiol* **2011**, *73*, 479-501, doi:10.1146/annurev-physiol-012110-142250.

156. Fries, B.C.; Goldman, D.L.; Cherniak, R.; Ju, R.; Casadevall, A. Phenotypic switching in *Cryptococcus neoformans* results in changes in cellular morphology and glucuronoxylomannan structure. *Infect Immun* **1999**, *67*, 6076-6083, doi:10.1128/IAI.67.11.6076-6083.1999.
157. Banks, I.R.; Specht, C.A.; Donlin, M.J.; Gerik, K.J.; Levitz, S.M.; Lodge, J.K. A chitin synthase and its regulator protein are critical for chitosan production and growth of the fungal pathogen *Cryptococcus neoformans*. *Eukaryot Cell* **2005**, *4*, 1902-1912, doi:10.1128/EC.4.11.1902-1912.2005.
158. Baker, L.G.; Specht, C.A.; Donlin, M.J.; Lodge, J.K. Chitosan, the deacetylated form of chitin, is necessary for cell wall integrity in *Cryptococcus neoformans*. *Eukaryot Cell* **2007**, *6*, 855-867, doi:10.1128/EC.00399-06.
159. Upadhya, R.; Baker, L.G.; Lam, W.C.; Specht, C.A.; Donlin, M.J.; Lodge, J.K. *Cryptococcus neoformans* Cda1 and Its Chitin Deacetylase Activity Are Required for Fungal Pathogenesis. *mBio* **2018**, *9*, e02087-02018, doi:10.1128/mBio.02087-18.
160. Baker, L.G.; Specht, C.A.; Lodge, J.K. Cell wall chitosan is necessary for virulence in the opportunistic pathogen *Cryptococcus neoformans*. *Eukaryot Cell* **2011**, *10*, 1264-1268, doi:10.1128/EC.05138-11.
161. Upadhya, R.; Lam, W.C.; Maybruck, B.; Specht, C.A.; Levitz, S.M.; Lodge, J.K. Induction of Protective Immunity to Cryptococcal Infection in Mice by a Heat-Killed, Chitosan-Deficient Strain of *Cryptococcus neoformans*. *mBio* **2016**, *7*, e00547-00516, doi:10.1128/mBio.00547-16.
162. Cazin, J., Jr.; Kozel, T.R.; Lupan, D.M.; Burt, W.R. Extracellular deoxyribonuclease production by yeasts. *J Bacteriol* **1969**, *100*, 760-762, doi:10.1128/jb.100.2.760-762.1969.
163. Sanchez, M.; Colom, F. Extracellular DNase activity of *Cryptococcus neoformans* and *Cryptococcus gattii*. *Rev Iberoam Micol* **2010**, *27*, 10-13, doi:10.1016/j.riam.2009.11.004.
164. Gumasta, R.; Nawange, S.R.; Singh, S.M.; Garg, A.; Sethi, R.; Yadu, R. Extracellular Protease and DNase Activities in Clinical and Environmental Isolates of *Cryptococcus neoformans* Species Complex from Central India. *Journal of Drug Delivery and Therapeutics* **2019**, *9*, 328-333.
165. Sumby, P.; Barbian, K.D.; Gardner, D.J.; Whitney, A.R.; Welty, D.M.; Long, R.D.; Bailey, J.R.; Parnell, M.J.; Hoe, N.P.; Adams, G.G. Extracellular deoxyribonuclease made by group A *Streptococcus* assists pathogenesis by enhancing evasion of the innate immune response. *Proceedings of the National Academy of Sciences* **2005**, *102*, 1679-1684.
166. Fazekas, G.; Schwarz, J. Histology of experimental murine cryptococcosis. *The American Journal of Pathology* **1958**, *34*, 517.
167. Collopy-Junior, I.; Esteves, F.F.; Nimrichter, L.; Rodrigues, M.L.; Alviano, C.S.; Meyer-Fernandes, J.R. An ectophosphatase activity in *Cryptococcus neoformans*. *FEMS Yeast Res* **2006**, *6*, 1010-1017, doi:10.1111/j.1567-1364.2006.00105.x.
168. Mahvi, T.A.; Spicer, S.S.; Wright, N.J. Cytochemistry of acid mucosubstance and acid phosphatase in *Cryptococcus neoformans*. *Canadian Journal of Microbiology* **1974**, *20*, 833-838.
169. Lev, S.; Crossett, B.; Cha, S.Y.; Desmarini, D.; Li, C.; Chayakulkeeree, M.; Wilson, C.F.; Williamson, P.R.; Sorrell, T.C.; Djordjevic, J.T. Identification of Aph1, a phosphate-regulated, secreted, and vacuolar acid phosphatase in *Cryptococcus neoformans*. *mBio* **2014**, *5*, e01649-01614, doi:10.1128/mBio.01649-14.
170. Plesner, L. Ecto-ATPases: identities and functions. *International review of cytology* **1995**, *158*, 141-214.
171. Junior, I.C.; Rodrigues, M.L.; Alviano, C.S.; Travassos, L.R.; Meyer-Fernandes, J.R. Characterization of an ecto-ATPase activity in *Cryptococcus neoformans*. *FEMS yeast research* **2005**, *5*, 899-907.
172. Chen, L.C.; Pirofski, L.A.; Casadevall, A. Extracellular proteins of *Cryptococcus neoformans* and host antibody response. *Infect Immun* **1997**, *65*, 2599-2605, doi:10.1128/iai.65.7.2599-2605.1997.
173. Nicola, A.M.; Frases, S.; Casadevall, A. Lipophilic dye staining of *Cryptococcus neoformans* extracellular vesicles and capsule. *Eukaryot Cell* **2009**, *8*, 1373-1380, doi:10.1128/EC.00044-09.
174. Rizzo, J.; Wong, S.S.W.; Gazi, A.D.; Moyrand, F.; Chaze, T.; Commere, P.H.; Novault, S.; Matondo, M.; Pehau-Arnaudet, G.; Reis, F.C.G.; et al. *Cryptococcus* extracellular vesicle properties and their use as vaccine platforms. *J Extracell Vesicles* **2021**, *10*, e12129, doi:10.1002/jev2.12129.
175. Reis, R.S.; Bonna, I.C.F.; Antonio, I.; Pereira, S.A.; Nascimento, C.; Ferraris, F.K.; Brito-Santos, F.; Ferreira Gremiao, I.D.; Trilles, L. *Cryptococcus neoformans* VNII as the Main Cause of Cryptococcosis in Domestic Cats from Rio de Janeiro, Brazil. *J Fungi (Basel)* **2021**, *7*, e00125-00121, doi:10.3390/jof7110980.
176. Rizzo, J.; Taheraly, A.; Janbon, G. Structure, composition and biological properties of fungal extracellular vesicles. *microLife* **2021**, *2*, doi:10.1093/femsml/uqab009.
177. Oliveira, D.L.; Freire-de-Lima, C.G.; Nosanchuk, J.D.; Casadevall, A.; Rodrigues, M.L.; Nimrichter, L. Extracellular vesicles from *Cryptococcus neoformans* modulate macrophage functions. *Infect Immun* **2010**, *78*, 1601-1609, doi:10.1128/IAI.01171-09.

178. Bielska, E.; Sisquella, M.A.; Aldeieg, M.; Birch, C.; O'Donoghue, E.J.; May, R.C. Pathogen-derived extracellular vesicles mediate virulence in the fatal human pathogen *Cryptococcus gattii*. *Nat Commun* **2018**, *9*, 1556, doi:10.1038/s41467-018-03991-6.
179. Marina, C.L.; Bürgel, P.H.; Agostinho, D.P.; Zamith-Miranda, D.; Las-Casas, L.d.O.; Tavares, A.H.; Nosanchuk, J.D.; Bocca, A.L. Nutritional Conditions Modulate *C. neoformans* Extracellular Vesicles' Capacity to Elicit Host Immune Response. *Microorganisms* **2020**, *8*, 1815.
180. Huang, S.H.; Wu, C.H.; Chang, Y.C.; Kwon-Chung, K.J.; Brown, R.J.; Jong, A. *Cryptococcus neoformans*-derived microvesicles enhance the pathogenesis of fungal brain infection. *PLoS One* **2012**, *7*, e48570, doi:10.1371/journal.pone.0048570.
181. Panepinto, J.; Komperda, K.; Frases, S.; Park, Y.D.; Djordjevic, J.T.; Casadevall, A.; Williamson, P.R. Sec6-dependent sorting of fungal extracellular exosomes and laccase of *Cryptococcus neoformans*. *Mol Microbiol* **2009**, *71*, 1165-1176, doi:10.1111/j.1365-2958.2008.06588.x.
182. Moyrand, F.; Janbon, G. UGD1, encoding the *Cryptococcus neoformans* UDP-glucose dehydrogenase, is essential for growth at 37 degrees C and for capsule biosynthesis. *Eukaryot Cell* **2004**, *3*, 1601-1608, doi:10.1128/EC.3.6.1601-1608.2004.
183. Reis, F.C.G.; Costa, J.H.; Honorato, L.; Nimrichter, L.; Fill, T.P.; Rodrigues, M.L. Small Molecule Analysis of Extracellular Vesicles Produced by *Cryptococcus gattii*: Identification of a Tripeptide Controlling Cryptococcal Infection in an Invertebrate Host Model. *Front Immunol* **2021**, *12*, 654574, doi:10.3389/fimmu.2021.654574.