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Review

# Hydrolytic Enzymes, Proteases, Permeases, and Transporters as *Cryptococcus neoformans* Weapons in Human Infections

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**Abstract:** In this review, we present several extracellular proteases, enzymes, membrane permeases, and transporters as essential accessories proteins for nutrient assimilation, conservation, and transportation as determined by nutrient repletion or depletion. As an obligate aerobic pathogen, it is crucial for invading *Cryptococcus* (*C.*) *neoformans* to negotiate its adaptation to human internal organs like the brain and spinal cord, where the oxygen level is low compared to peripheral organs. Besides, essential metals like copper and iron are important cofactors to functional proteins; however, these metals are not usually freely available to invading human pathogens. Again, the phagolysosome low pH with glucose paucity, internal temperature, immune response, and complex extracellular matrixes are challenging environments that must be circumvented by *C. neoformans* in the systemic tissues for survival, adaptation, and infection in humans. We review extensive works on several extracellular proteases, enzymes, membrane permeases and transporters orchestrated by different transcription factors and present these proteins as weapons needed to outwit systemic resistance to invading pathogens. Lastly, we examine the extracellular secretory vesicles of *C. neoformans* as “an exosomal virulence bag” that harbours urease, laccase, phosphatase, and capsular components as additional secretory weapons for tissue invasion and persistence.

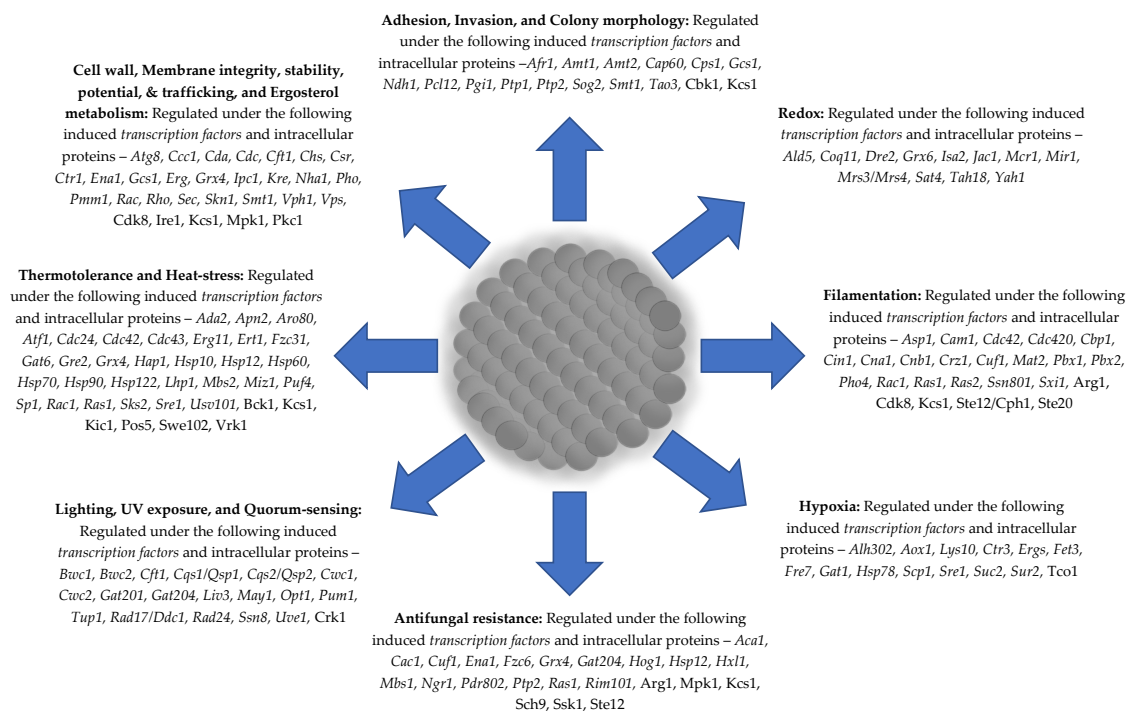
**Keywords:** *Cryptococcus*; extracellular protease; biofilm; secretory vesicles; hypoxia; hydrolytic enzyme; homeostasis; transcription factors; membrane permeases; membrane transporters

## 1. Introduction

*C. neoformans* is an opportunistic yeast associated with humous soil contaminated with Aves dropping and around trees. As an encapsulated and obligate aerobe, it becomes pathogenic and able to infect, invade, and colonise various animal and human organ systems. Being an environmental saprophytic fungus, the internalisation of extracellular nutrients is necessary for survival of this fungus. With the help of numerous membrane proteins (permeases and transporters), extracellular enzymes, and cytoplasmic enzymes, digested nutrients can be assimilated.

The assimilation of extracellularly digested nutrients and micro-nutrients is a complex metabolic system. Besides, other membrane proteins are strategically made active for adaptation, survival, drug resistance, and human infection. The redox process uses membrane-bound proteins to obtain ions such as  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ , and  $\text{Zn}^{2+}$  for membrane potentials. Pumps, such as  $\text{Na}^+/\text{K}^+$ -ATPase and P-type  $\text{Na}^+$ -ATPase (Ena1), are used to maintain membrane polarisation, while ABC (Afr1) and ABD-type multidrug homologue pumps (Pdr5, Yor1, and Snq1) are deployed for drug resistance. Several transporters and permeases like  $\text{Ca}^{2+}$  transporters (Cch1, Eca1, and Vcx1),  $\text{Na}^+/\text{H}^+$  antiporter (Nha1), aquaporin (Aqp1), phosphate affinity permeases (Pho) are implemented for cellular homeostasis, tolerance, resistance, antioxidation, and coping with environmental stress (oxidative and osmotic). Altogether, these have been studied to be important in cryptococcal cell survival, invasion, colonisation, and infection [1,2].

One of the attributes of cryptococcal cells like *C. albicans*, *A. fumigatus*, and *P. aeruginosa* is the ability to form biofilm. This is an important phenotypic display of invasive pathogenic fungi and bacteria that involves a conglomerate of cells working together with common goals – colonisation, extracellular matrix modelling, quorum sensing, resistance, protection, adhesion, survival, adaptation, communication, sharing, maturation, filamentation, and invasion [3-8], **Figure 1**. Biofilm formation in *C. neoformans* is metabolically regulated, induced by unfavourable conditions, and capsular components [9]. Either *in vivo* or *in vitro*, *C. neoformans* can form a biofilm, even on surfaces and invasive clinical instruments [9]. Studies have shown that the redox process, anaerobic metabolism, substrate-level phosphorylation, proteolysis, and anti-osmotic processes are upregulated during biofilm formation, but general metabolic processes, replication, transcription, translation, transportation, permeases, pump systems are generally repressed [10].



**Figure 1.** An aggregate of cryptococcal cells forms a matured biofilm unit inside which are numerous fungi enveloped with a thin film coat of extracellular protective sheath. The sheath consists of polymeric molecules of protein, chitins, nucleic acids,  $\beta$ -1,3-glycans, capsular components, and lipids derivatives. Check Supplementary 4 for as many details as possible for the transcription factor, kinase, protein, enzyme, and gene acronyms used in this review.

*C. neoformans*, an opportunistic environmental pathogen, encounters abiotic factors with adequate cellular taxis to survive these conditions. It also encounters predatory association with surrounding biotic soil organisms. Amoeboid protozoans can “catch” and “kill” internalised cryptococcal cells similarly to mammalian macrophages that phagocytose invading pathogens. Surprisingly, though cryptococcal cells can be engulfed, the fungi can survive and replicate intracellularly within the soil amoeba. These survival mechanisms are insurmountable, especially in immunocompromised patients when infected. This invariably promotes gradual tissue intrusion, leading to a life-threatening condition, meningoencephalitis, due to fungemia.

Based on their immediate interaction, cryptococcal cells could be environmental or clinical. Both showed similar pathogenicity in animal models [11] because of the similarity in the virulence factors – capsule, melanin, extracellular hydrolytic enzymes, and vesicular virulence bags (secretory vesicles containing microbodies, laccase, urease, and phosphatase) [12]. *C. neoformans* reprograms its gene expression profile to survive inadequate nutrient supply by upregulating the membrane transporters and enzymes involved in the shuttling of carbon intermediates from alternative pathways such as  $\beta$ -oxidation (mitochondria function) and glyoxylate cycle (peroxisome function) when phagocytosed

by amoeba/macrophage. These two pathways fetch cryptococcal cells acetyl-CoA [13], which is converted via gluconeogenesis into various sugars needed for the cell wall components [14]. In addition, *C. neoformans* resorts to anabolic pathways to generate resourceful intermediates for virulence expression, and 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 [13,15].

Besides these, 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 [16]. In extreme cases, the redox process, substrate-level phosphorylation, and anaerobic decarboxylation can be employed to generate energy that will maintain cellular function and growth [10]; however, the lack of glucose or other utilisable sugars generally impairs cryptococcal proliferation, virulence, persistence, and tissue invasion as displayed by  $\Delta pyk1$ ,  $\Delta pyk1\Delta mig1$ ,  $\Delta mig1$ ,  $\Delta pck1$ ,  $\Delta hxc1$ ,  $\Delta hxc2$ , and  $\Delta hxc1\Delta hxc2$  mutants in the presence of glucose, glycerol, and lactate [17]. These self-defence strategies are primarily enrooted in the signalling cascade events that interpret environmental factors into signalling sensors for effective metabolic and physiological responses [18]. For example, Fan et al. reported that genes in the Gpa1-cAMP-Pka pathway are upregulated during phagocytosis [16]. Contrarily, *C. neoformans* retrogressively limit the ribosome-related functional gene process and translation initiation or elongation [16], which tactically relates a central control response to phagocytosis and the initiation of spores for survival.

Though similarities occur in the responses of *C. neoformans* to soil amoeba and mammalian macrophages based on their intracellular attack, nevertheless, the ecological niches surrounding the amoeba are quite different from the extracellular environment. Interaction of *C. neoformans* with the soil amoeba is usually at the ambient temperature (28 – 30°C) in a slightly acidic environment, while the macrophage is at the physiological temperature of 37°C with 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*; however, only a few numbers of these proteins were differentially modulated [13].

So, the involvement of several membrane transporters, permeases, and extracellular proteins in the pathogenesis of *C. neoformans* is critical, as also their deployment in the nutrient inadequacy. Unfortunately, targeting many of these proteins for antifungal management may probably be a come by attempt as most of the proteins are common to human cells. We review several of these proteins and present a succinct analysis of their functional relevance to cryptococcal survival and adaptation in nutrient repletion or depletion, and in human infections. Careful consideration of their consequences to human cells is needed if any of these proteins is considered a target for antifungal formulation against invasive cryptococcal infections in humans.

#### **4. Regulatory Transcription Factors in Cryptococcal Cells Control Membrane Transporters, Permeases and Enzymes Activities in Response to Nutrient Repletion/Depletion**

The environmental transition of *C. neoformans* enables this fungus to cause infection in the susceptible hosts. The adaptation and survival of this pathogen in the nutrient-deprived macrophage milieu when phagocytosed are paramount to nutrient acquisition, assimilation, and metabolism. *C. neoformans* tactically switch on the important and inevitable transcription factors while some, considered unimportant and redundant, are temporarily switched off. When phagocytosed, the induced transcription factors include genes expressed for membrane transporters and secretion, cell wall formation, anti-oxidative and nitrosative stress, anti-autophagy and peroxisome functions, intracellular metabolism, mating, and genomic repair. However, the repressed genes are involved in ribosomal metabolisms and translational processes, most of which are repressed as the infection progresses [16]. This means that *C. neoformans* possesses a highly classical environmental sensor, fast enough to help decipher the micro-conditional changes and subsequently release the fort of



transcription factors for nutrient acquisition that translate into cellular morphology for immune evasion and induction of infections.

#### 4.1. Phospholipases

The secretion of extracellular phospholipases (PL) by *C. neoformans* was investigated by Chen et al., where a cluster of PL such as PLB, lysoPL hydrolase, and lysoPL transacylase/acyltransferase was detected and every  $\Delta plb1$  mutant usually marked with a drastic reduction in the activity of each enzyme compared to the *wt* [19,20]. By popular demand, PLB is generally used to represent the three enzymes. By correlation, PL activity corroborated the mortality rate and mucosal invasion by *Candida albicans* in animal models [21]. Similarly, *C. neoformans* may use these hydrolytic enzymes to invade tissue and cross the 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 [19]. Various isolates of *C. neoformans* possess a different degree of PL secretion, which correlates with the fungal virulence and tissue burden in the lungs and brains of mice inoculated intravenously [19,20].

PLs, best secreted at 37°C [22], are a highly diverse group of hydrolytic enzymes targeting the ester linkages of glycerophospholipids carbonyl linkage and can also transfer acyl chain to the lysophospholipid to form diacylglycerophospholipid. These three oligomeric proteins are borne on the same coding gene, the *Plb* gene, just like in other yeasts such as *Penicillium notatum* [23], *S. cerevisiae* [24], and *C. albicans* [25]. Chen et al. showed that PLs are well secreted at about 6 – 10 hours of cell culture at 30°C, with optimal activity at 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 ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{Zn}^{2+}$ ), and EDTA [19]. The stability of this enzyme at acidic pH vividly reveals one of the strategies put in place by *C. neoformans* to tolerate the micro-acidic environment of the phagosome, and, in so doing, the PLs may break down the phagosomal membrane leading to tissue dissemination.

The *Ste12 $\alpha$*  gene has been implicated in the regulation of extracellular PL. Deletion of the *Ste12 $\alpha$*  gene significantly reduced the production of PL in the egg yolk agar media [26]. As crucial as PLs are to the virulence of *C. neoformans*, the absence of these hydrolytic enzymes may not affect the masterminding virulence factors/phenotypes such as growth at 37°C, urease activity, capsule, and melanin formation. However, such a mutant is less virulent in the mouse inhalation and rabbit meningitis models when compared to the *wt* in so much that its growth is defective in a macrophage-like cell line [20]. The attenuated virulence observed in the  $\Delta plb1$  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* [22].

The attenuated virulence observed in the  $\Delta plb1$  mutant is likely comparable to  $\Delta ure1$  and  $\Delta lac1$ . PLB and their components are generally associated with *C. neoformans* virulence, yet an infection can still progress in their absence but at a slower rate. 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 [22].

The homologous *Ssn3/Ume5* gene was serendipitously discovered adjacent to the *Plb1* locus in *C. neoformans*. Thus, like *S. cerevisiae*, *C. neoformans* may be using the product of this low transcript to repress the transcription factor Hsp70 family. However, this repressing protein, known as Ume5p, is a cyclin-dependent protein kinase, which is sensitive to temperature- or oxidative-induced degradation via ubiquitin-mediated proteolysis [27], and may not be playing any significant role in the virulence factors [20].

Covalently, PLB is usually attached to the cell wall chitin protruding  $\beta$ -1,3-linked glucan via its  $\beta$ -1,6-linked glucan. This arrangement is anchored by the glycosylphosphatidylinositol (GPI) on the membrane lipid raft proteins. The release of PLB from the GPI-anchor is probably orchestrated by the phosphatidylinositol-specific phospholipase C (PI-PLC, putatively encoded by *Plc1* and *Plc2*), and

the inclusion of  $\beta$ -1,6-linked glucan to this release confirms the cell wall localisation of the PLB [22,28]. This shows that PLB constitutes part of the proteins involved in the cell wall integrity because  $\Delta plb1$  exhibited morphological cell wall defect, which is sensitive to disruption by SDS and Congo red; however, the growth is not affected by caffeine [22]. The  $\Delta plc1$  *C. neoformans* var. *grubii* strain H99 mutant, which failed to produce melanin and refused to grow at 37°C, exhibited less secretion of PLC with concomitant accumulation of cytoplasmic PLB because of the impaired secretion of PLB [28]. Furthermore, this mutant displayed high antifungal sensitivity (to 5-FC, azoles, and AmpB but not CpF), poor replication, and defective cell wall characterised by cell clumping and irregular morphology as confirmed by the diffusion of Calcofluor white staining and sensitivity to cell wall disruptors (Congo red, caffeine, and SDS – check **Supplementary 1** endnote for details) due to impaired activation of Pkc/MAPK pathway [28].

Because the  $\Delta plc1$  mutant had poor growth at 37°C, its virulence was attenuated in the mice study. Not only this, even at 25°C, this mutant failed to kill a significant number of *Caenorhabditis elegans* compared to the *wt* and reconstituted mutant [28]. Factually, the few numbers of *C. elegans* killed by this mutant means that attenuated virulence associated with this mutant is independent of temperature; after all, other virulence determinants are simultaneously attenuated as well. The actual killing of the worms at 25°C by the *wt* and reconstituted mutant indicates that the Plc1 virulence can be temperature-independent and that certain other factors may augment this virulence. In all, the  $\Delta plc2$  mutant failed to exhibit any other major defect, and it is as active as the *wt*.

The IP<sub>3</sub> produced by Plc1, besides the ER Ca<sup>2+</sup> release, serves as a substrate for IP<sub>3</sub> kinase (encoded by *Arg1*) to produce inositol polyphosphate. Expectedly, the  $\Delta plc1$  mutant generally accumulates PIP<sub>2</sub> but a reduced level of IP<sub>3</sub>, whereas in the  $\Delta arg1$  mutant, IP<sub>3</sub> will be accumulated [29]. 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. Further analysis showed that  $\Delta arg1$  accumulated larger intracellular vacuoles (excessive vacuolar fusion) than the *wt* and  $\Delta plc1$  mutant [29]. Therefore, Arg1p inositol polyphosphate anabolism (IP<sub>4-s</sub>) is as important as the Plc1p catabolism for *C. neoformans* virulence, and that IP<sub>3</sub> is a biological relay molecule for active Plc1p.

In another related work, efficient antioxidant control has been linked to PLB production. Mutation of Cu/Zn SOD encoded by the *Sod1* gene has been characterised by a severe reduction in the activities of phospholipase B, urease, and laccase leading to attenuated virulence manifested in the low brain colonisation and persistence. However, this failed to affect the capsule formation under LIM, and the mutant culture media were characterised by high mannitol production that probably acted as an antioxidant to complement the loss of Cu/Zn SOD activity [30,31] (for details on the phenotypic display of different cryptococcal cell mutants, check **Supplementary 1**).

#### 4.2. Phosphate Depletion

Ordinarily, *C. neoformans* prefers an acidic environment to neutral-alkaline or physiological pH, perhaps because of phosphate availability in the acidic environment. Intracellular inorganic phosphate (*P<sub>i</sub>*) is highly essential for kinases, activation of membrane transporter, membrane biogenesis, coupling reactions, and genomic stabilisation. Very importantly, the production of second messengers such as cAMP, IPC, and DAG, which are essential for virulence (melanin and capsule), mating, filamentation, membrane biogenesis and integrity, are essential phosphate-related metabolic products. It is likely possible that the IPC formed from inositol-phosphoryl ceramide synthase 1 activity is salvaged in the Golgi apparatus to produce membrane sphingolipids. Furthermore, the IPC can readily form a sterol pocket [32], which may take ergosterol to be fused at the membrane bilipid layer for fluidity and rafting. In *S. cerevisiae*, IPC-mannosyl transferase (encoded by redundant *Csg1* and *Csh1* genes) can take mannose instead of sterol to form mannose-IPC in the presence of intracellular Ca<sup>2+</sup>-binding protein (encoded by *Csg2* gene) [33,34]. With more IP, mannose diinositolphosphoryl ceramide can be produced [35]. It may be possible that *C. neoformans* employed the same phosphate-dependent glycosphingolipid intermediates as membrane components to

increase membrane thickness and thermal stability. Therefore, phosphate-containing intermediates are crucial to *C. neoformans* adaptation, survival, and pathogenesis.

*C. neoformans* deploys arrays of kinases, membrane transporters, and transcriptional regulatory factors to facilitate the phosphate-sensing and acquisition (PHO) pathway, a mechanism that seems to be absent in human cells. This enables the fungi to assimilate phosphate and perhaps store it as polyphosphate. These arrays of proteins and regulatory factors include:

- ❖ plasma membrane high-affinity phosphate transporters (including Cdk encoded by *Pho85*, Pho85-cyclin encoded by *Pho80*, Cdk inhibitor of phosphate signalling pathway encoded by *Pho81*, and transcriptional regulatory factor encoded by *Pho4*),
- ❖ low-affinity vacuolar phosphate transporter encoded by *Pho90*,
- ❖ polyphosphate storage and processing (including vacuolar transport chaperone encoded by *Vtc4*, exopolyphosphatase encoded by *Xpp1*, endopolyphosphatase encoded by *Epp1*, and diacylglycerol trimethylhomoserine (DGTS – betaine lipid) synthase encoded by *Bta1*),
- ❖ mitochondrial phosphate transporter encoded by putative *Mip1* and *Mip2* genes, and
- ❖ glycerophosphodiesterase encoded by *Gde2* [36,37].

Generally, *Pho4* gene expression is triggered whenever the level of  $P_i$  is critically low [36]. The high level of  $P_i$  increases the phosphorylation of Pho4p mediated by the Pho80-Pho85 complex because, under this condition, the *Pho81* remained repressed. Contrarily, a low level of  $P_i$  activates the *Pho81* expression to deactivate the Pho80-Pho85 complex and dephosphorylate the Pho4p regulatory factor, thereby increasing the induction and activation of Pho84, Pho89, and Pho840, which are high-affinity transmembrane  $P_i$  transporters and at the same time promoting the activities of *Pho2* and constitutive *Pho1* (both are orthologues of *S. cerevisiae* *Pho5* encoding acid phosphatase) and *Pho8* (encoding alkaline phosphatase).

This condition may probably promote the phosphate-scavenging activities of extracellular and vacuolar acid phosphatase (encoded by the *Aph1* gene) secreted by *C. neoformans*. The phosphate-released activity of this enzyme, under phosphate starvation, has been demonstrated against expendable metabolites such as  $\beta$ -glycerol phosphate,  $\alpha$ -D-glucose-1-phosphate, AMP, mannose-6-phosphate, O-phosphotyrosine. This enzyme, however, displayed less activity with ATP but no activity with phosphoserine and phosphothreonine [38]. *In vitro* assay with pNPP over an acidic pH range (5.0 – 6.9) showed that acidic phosphatase activity decreases as the pH approaches neutral value, which explains why *C. neoformans* survive phagolysosome (pH 4 – 5), cryptococcomas (pH 5.5), and inflammatory tissue (pH 6.5 – 6.9) [38]. The orthologues of *S. cerevisiae* *Pho5* phosphate-repressed acid phosphatase gene have been identified in cryptococcal cells. These putative genes include *Pho1* (constitutive acid phosphatase), *Pho2* (secreted and vacuolar acid phosphatase), and *Pho3* [36], while Lev et al. identified and designated these genes as *Aph1* (similar to *Pho2*), *Aph2*, *Aph3* (encoding phytases), and *Aph4* (perhaps similar to *Pho1*) [38,39]. Furthermore, *Aph2*, *Aph3*, and *Aph4* are suspected to be intracellular [39].

Experimentally, phosphate-starvation induces the expression of *Aph1* and secretion of acid phosphatase when co-cultured with activated THP-1 monocytes and such  $\Delta aph1$  mutants displayed attenuated virulence because infected mice survived longer than those infected with the *wt* serotype A H99 [38]. Mutation of *Pho84*, *Pho89*, and *Pho840* genes ( $\Delta\Delta\Delta pho$ ) in *C. neoformans* interfered with growth in low-phosphate media; such mutants further displayed reduced survival and attenuated the virulence. However, the  $\Delta vtc4$  mutant, which encoded the polyphosphate polymerase, behaved normally like the *wt* in terms of infection, but such a mutant lacked effective tissue invasion and dissemination from the lungs and failed to induce *in vitro* blood coagulation [37]. At 30 or 37°C, the impact of single/double mutations of *Pho* genes was not as conspicuous as the triple  $\Delta\Delta\Delta pho$  mutant when the growth in the acidic-to-alkaline pH and non-to-low phosphate supplement media was examined. However, a higher phosphate supplement (100 mM) was able to restore the poor growth [37].

Regulatory evidence of high-affinity phosphate transporters is glaring. The  $\Delta\Delta\Delta pho$  mutants inclined to accumulate intracellular sodium, iron, and zinc but failed to accumulate phosphate. This is tantamount to the poor and unrecovered formation of capsule and melanin compared to the

mutants with either single or double gene disruption [37]. Again, contrary to  $\Delta vtc4$  and  $\Delta epp-xpp1$  mutants, viable  $\Delta\Delta\Delta pho$  mutant failed to replicate in the macrophage [37].

The regulatory role of *Pho4* in facilitating the acquisition of *Pi* is inevitable. This helix-loop-helix (Hlh3) transcription factor senses *Pi* starvation, and the cytoplasmic-nucleus translocation is initiated to induce the expression of phosphate sensing and acquisition genes. Thus, the growth defect, hypovirulence, inefficient tissue invasion/systemic dissemination, and other phenotypic traits of  $\Delta pho4$  mutant can be restored with phosphate supplementation; however, these seem irreversible in an alkaline pH media and physiological pH [39]. Apparently, this alkaline pH intolerance creates phosphate starvation, which could have prevented the invasion and dissemination of cryptococcal infections, but the expression of the *pho4* gene enables this fungus to subtly acquire phosphate in this ostensive environment through the activation of the PHO pathway.

#### 4.3. Iron Depletion

Strategically, human physiological conditions never allow invading pathogens easy access to iron because iron is not usually found circulating without being locked in a storage protein like transferrin or transport protein like haemoglobin. Hence, invading pathogens must strive to obtain iron through the arsenal of high-affinity iron uptake and acquisition proteins. The arrays of membrane transporters (such as siderophores) and intracellularly expressed regulatory proteins (such as Cir1p) are released for iron acquisition and homeostasis (for details of cellular events that induced/repressed different membrane transporters and permeases in *C. neoformans*, check **Supplementary 2**). Interestingly, *C. neoformans* mainly released specific iron acquisition proteins based on the available iron sources *in vivo* or *in vitro*. Besides, *C. neoformans* can direct the expression of *HapX* or *Hap3* to shut down other iron-dependent functional pathways in LIM to maximise the utilisation of the scarce iron for more growth while capsule production is being initiated. Generally, capsule production enlarges as iron decreases with concomitant slower growth.

The expression of the GATA iron regulatory factor, *Cir1*, is majorly responsible for iron uptake for growth at 37°C and the formation of virulence factors in the host tissue [40]. This expression is enhanced by the *Hap* genes such as *HapX*. *Cryptococcus* ferroxidase (*Cfo1*) expression enables this fungus to survive LIM; however,  $\Delta cfo1$  mutant or as a background mutant to the deletion of *Hap3*, *Hap5*, or *HapX* cannot survive in the LIM unless feroxamine is supplied. Apart from the  $\Delta cfo1$  mutant that can survive in the media supplied with  $\text{FeCl}_3$  and hemin, none of the doubly mutated iron gene mutants can survive in these two iron sources [40].

It appears that *C. neoformans* employed multiple alternative routes of acquiring iron from its environment for growth and proliferation. Though  $\Delta cfo1$  mutant may have attenuated virulence notwithstanding deletion of *HapX* or *Hap3* gene failed to extenuate further the virulence of this mutant [40]. Comparatively, the deletion of *HapX* seemed to have a greater influence on various transcriptional genes than deleting *Hap3* or *Cir1* under iron-limiting or iron-repleting conditions [40].

Paradoxically, though *HapX* seems to influence a greater percentage of other transcription factors under iron-limiting conditions, including siderophore transporters, yet deletion of *Cir1* had a greater influence on the high-affinity iron transporters; however, this function is overlapping to some extent under low iron conditions. *HapX* and *Hap3* cooperatively repressed most iron-dependent mitochondrial functional pathways under low iron conditions, but this overlapping function is not found in the iron-repleting conditions. While *HapX* majorly controls the subset genes for siderophore transporters in different iron sources, *Hap3* mainly inhibits other iron-dependent regulatory pathways, especially in the mitochondria [40].

Though *Cir1* and *HapX* positively regulate *Sit1* expression for siderophore transporter, only *Cir1* appeared to regulate virulence genes such as *Lac1*. Also, a report has shown that *Cir1* positively affected the expression of Rim101 pH-responsive transcription factors, which further regulates the expression of various iron acquisition genes such as *Sit1* and *Cft1* [40]. Under low iron content, *HapX* influences the expression of *Cir1* and *Rim101*, but under iron-repleting conditions, *HapX* apparently loses its influence while *Cir1* influences the expression of *rim101* but withdraws its influence on *HapX* to minimise redundancy of *HapX* perhaps. In a related study, Grx4, a 2Fe-2S cluster sensing and



trafficking protein, was identified as an essential regulator of iron homeostasis capable of associating with Cir1p in the nucleus during iron depletion to induce multiple iron acquisition proteins [41,42]. This synergistic association may probably be under the influence of HapX during iron depletion. Reports showed that iron depletion promotes relocalisation of the Grx4p into the cytoplasm; besides, deletion of the *Grx4* gene alone is sufficient to dysregulate iron-dependent metabolisms leading to oxidative stress, mitochondria dysfunction, and impairment of DNA repair [42].

The ability to utilise different sources of iron is peculiar to *C. neoformans* and other invasive pathogenic fungi like *C. albicans*. Iron is usually acquired in a reduced form from inorganic (hemin, feroxamine B, and  $\text{FeCl}_3$ ) or organic/biological form (transferrin and heme), and the effective absorption is centred on the interplay of *Hap*, *Cfo1*, and *Cft1* genes. Acquisition of iron involves the reduction of  $\text{Fe}^{3+}$  by the cell surface reductases (Fre) to  $\text{Fe}^{2+}$  and then oxidised by the ferroxidase (*Cfo1*), which is coupled to *Cryptococcus* high-affinity iron permeases/transporters (*Cft1* and *Cft2*). Besides, *C. neoformans* can acquire the non-reduced  $\text{Fe}^{3+}$  through the ferric-bound siderophores transporter (*Sit1*) presented by other closely associated microorganisms. This attribute is also common in other fungi, such as *C. albicans* and *S. cerevisiae* [43].

There are two putative orthologues of ferroxidase, *Cfo1* and *Cfo2*, with the former being actively involved in the acquisition of iron while the latter plays a minor role. During infection, the austere iron withheld by the host tissues enables the fungus to express *Cft1*, which is trafficked to the plasma membrane under the influence of *Pka1* to utilise transferrin for growth and virulence expression. The  $\Delta cfo1$  and  $\Delta cfo1\Delta cfo2$  mutants, irrespective of the temperature, are characterised by growth defect, attenuated virulence, copper toxicity, increased susceptibility to FCZ and AmpB due to impaired heme formation as a cofactor needed for ergosterol biosynthesis [44]. However, an exogenous supply of heme and feroxamine (siderophore) but not  $\text{FeCl}_3$  can restore some of these phenotypic deficiencies meaning that  $\Delta cfo1$  mutant can still utilise iron through the low-affinity uptake proteins such as *Cfo2* and perhaps *Cft2*. Iron assimilation mediated by the *Cfo1* expression is independent of the *Sit1* (feroxamine) and heme; nevertheless, when supplied at 100  $\mu\text{g/mL}$ , these two iron sources can restore growth and relative resistance against FCZ in  $\Delta cfo1$  mutants [44].

The *Cft1-Cfo1* expression is needed to utilise iron from hemin and  $\text{FeCl}_3$  but not from heme or feroxamine, and any mutant with single/double deletion of these genes may not survive in the low-iron environment [44,45]. Thus, it seems the *Cft1* and *Cfo1* may be clustered on the same locus as observed in serotype D, and their transcripts are synergistically elevated with other iron regulatory genes in a low-iron condition [46]. Apart from these tandem iron-uptake-clustered genes, Lian et al. further suspected, in the LIM, other yeast orthologue genes involved in the intracellular iron transport and storage, and this includes:

- ❖ a low copy *Yfh1* transcript (encoding frataxin that mediates mitochondrial iron efflux cytosolic unbound iron),
- ❖ *Utr1p* (augmenting ferric reductase activity),
- ❖ *Atm1p/ABC* inner mitochondria iron transporter,
- ❖ *Mrs3p* and *Mrs4p* (inner membrane protein suppressing mitochondrial RNA (*mtRNA*) splicing defect in the yeast, and also function as inner mitochondrial iron transporter),
- ❖ *fet5* (encoding Fet3-related integral membrane multicopper oxidase required for high-affinity iron uptake across the vacuolar membrane in conjunction with *Cft1p* to mobilise iron from the storage), and
- ❖ *ccc1* (encoding vacuolar membrane transporter to facilitate cytosolic reduction of iron while storing the iron in the vacuole) [46-52].

The evidence that  $\Delta hap3\Delta cfo1$  and  $\Delta hapX\Delta cfo1$  failed to utilise hemin and yet are relatively virulence in mice experiment means that other additional iron acquisition transporters, apart from the *Cfo1*, may be orchestrating iron acquisition during infection [40].

The *Cft1* and *Cft2* are the two orthologues of high-affinity iron permease similar to the *Ftr1* of *S. cerevisiae*, and their expression is influenced by *Pka*. The *Cft1* expression is more effective and highly involved in the growth and iron uptake from  $\text{FeCl}_3$  and holo-transferrin than *Cft2*; however, the deletion of the two genes ( $\Delta cft1\Delta cft2$ ) attenuated the virulence accompanied by low tissue burden

(for details on the phenotypic display of different cryptococcal cell mutants, check **Supplementary 1**). In the presence of  $\leq 1.0$  mM  $\text{FeCl}_3$ , the  $\Delta pka1$  and  $\Delta pka2$  mutants significantly failed to raise the expression level of *cft1* compared to the *wt*, and even the  $\Delta pkr1$  mutant further displayed reduced expression of *cft1* by 2 – 3-fold. More significantly, the  $\Delta pka1$  mutant displayed a more increased expression of *cft2* compared to the  $\Delta pka2$  mutant. In contrast, the  $\Delta pkr1$  mutant failed to show any significant effect on the expression of *cft2* when compared to the *wt* [45]. Though no significant contribution has been traced to Cft2 high-affinity iron uptake, the upregulation of this gene by Pka, however, might suggest the importance of this seemingly insignificant iron transporter. The reduced growth observed by Lian et al. with  $\Delta cft1$  mutant in the low- and high-iron media [46] is redolent that Cft2 and perhaps Cfo expressions may be rescuing the mutant. This is like an SOS message received by the low-affinity iron uptake proteins to rescue the  $\Delta cft1$  mutant.

In the absence of ferrous iron, *C. neoformans* may be compelled to use a non-reductive ferric-bound siderophore complex *in vivo* or *in vitro* as the iron source. Several pathogenic fungi have evolved to possess various specific siderophore transporters (Sitp) through which ferric-bound siderophores can be utilised by their competitors. These competitors may be other microorganisms capable of producing siderophore as deferoxamine, which can subtly steal iron from transferrin or heme and present it as siderophore feroxamine bearing  $\text{Fe}^{3+}$ . A putative *sit1* transcript identified in *C. neoformans* encodes a siderophore transporter, which is highly expressed in cells cultured in the LIM alone or with a ferrous iron chelator, bathophenanthrolinedisulfonic acid (BPDA), supplemented with deferoxamine. Experimental observation from Tangen et al. showed that  $\Delta sit1$  mutants (either serotype A or D) could utilise ferrichrome but not feroxamine B as an iron source under iron-limiting conditions [53].

Furthermore, deletion of the *Sit1* gene in the serotype D background enhances melanisation better than the serotype A  $\Delta sit1$  mutant. The melanin production seemed better with increasing DOPA concentration in the medium. However, 1.0% glucose repressed melanin formation in the two serotype mutants. Additionally, the *Sit1* expression appears to control copper availability to  $\text{Cu}^{2+}$ -binding pocket of laccase. So, the loss of *Sit1* gene apparently attenuates ionic homeostasis and reduces laccase activity more significantly in the *wt* than the mutants. This means that laccase in the mutants has more access to copper because of the absence of Sitp, 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  $\Delta sit1$  strain, which is comparable to the  $\Delta sit1$  mutant [53]. Notwithstanding, an exogenous supply of copper or iron in the absence of glucose generally seems to favour melanisation in serotype D  $\Delta Sit1$  mutant.

In serotype A, the lack of the *Sit1* gene showed no significant effect on the *Lac1* transcript, even if copper or iron was supplied in a non-glucose medium. However, in serotype D, this mutant showed a significantly high level of *Lac1* transcript than the *wt* and reconstituted mutant – only when copper or iron is exogenously supplied in the absence of glucose [53]. With Calcofluor white, SDS, and Congo red in low-glucose or L-DOPA medium, serotype D  $\Delta sit1$  mutant only showed a slight growth sensitivity to these compounds compared to the *wt*, but no significant sensitivity was observed in the serotype A  $\Delta sit1$  mutant. Furthermore, serotype D  $\Delta sit1$  mutant showed increasing growth defect from 30 – 37°C, but such temperature sensitivity is not conspicuous in the serotype A  $\Delta sit1$  mutant [53]. This serotype differential phenotypic expression in response to copper and iron from *Sit* gene re-emphasises the existence of cellular morphotypes in *C. neoformans*.

To further emphasise the importance of *Sit1* expression in cell wall integrity,  $\Delta sit1$  mutants generally displayed a less dense and heterogenous cell wall thickness and polysaccharide fibril distribution. In addition, the serotype D  $\Delta sit1$  mutant cell wall is easily distorted compared to the serotype A  $\Delta sit1$  mutant, which reaffirms the phenotypical disadvantages earlier described in the serotype D  $\Delta sit1$  mutant. Therefore, it is not surprising to observe that serotype D  $\Delta pka1$  mutant could have a preponderant level of *Sit1* transcript and that the serotype A  $\Delta pka1$  and  $\Delta pka2$  mutants equally showed an elevated level of *sit1* transcript [53]. This observation is similar to the negative regulation of iron uptake by the cAMP-Pka event in *S. cerevisiae* [54]. To this effect, the  $\Delta sit1$  mutant failed to

improve defective capsule formation or induction in the  $\Delta pka$  mutant cultured in the low-iron or iron-supplemented medium. Also, the deletion of *Sit1* did not initiate unilateral mating defect in serotype A  $MAT\alpha \Delta sit1 \times MAT\alpha$  or serotype D  $MAT\alpha \Delta sit1 \times MAT\alpha$  [53].

Expectedly, the hypermelanised serotype D  $\Delta sit1$  mutant would have shown higher virulence than the *wt* or reconstituted mutant; however, Tangen et al. could not observe any significant difference in the virulence among these strains. Not only this, but the virulence from this serotype mutant is generally weak [53]. Still, serotype A  $\Delta sit1$  mutant, alongside the *wt* and the reconstituted mutant, actually induced infection almost equally with no significant difference in the survival rate of experimental animals after the infection, tissue colonisation/burden within the brain and lungs, and extrapulmonary dissemination [53]. Thus, this outcome generally presents the *Sit1* gene as not particularly required for virulence and infection in an animal model.

While *Cir1* positively influences the expression of *Cft1*, *Cft2* is negatively regulated. The transcription level of *Cft1* in  $\Delta cft2$  mutant decreases just like in the *wt* as  $FeCl_3$  concentration increases. However, the *Cft2* transcripts level in  $\Delta cft1$  mutant increases with a threshold at 10 mM  $FeCl_3$  but reduces in the *wt* as the iron level increases. The *Sit1* transcript, on the other hand, is generally reduced in the *wt* but becomes better responsive to  $FeCl_3$  level  $\leq 100 \mu M$  in  $\Delta cft1$  than  $\Delta cft2$ ; however, concentration beyond this makes *Sit1* entirely unresponsive and drastically reduced in the  $\Delta cft1$  [45]. This means that the loss of *Cft1* potentially reduces the intracellular iron content, and this condition makes *Sit1* transcription unresponsive even in the presence of 1.0 mM of  $FeCl_3$ . This, therefore, means that *Cft1* expression may come to rescue  $\Delta cfo1$  background mutant during infection. Not only this, but evidence also showed that *C. neoformans* could secrete reductant metabolites such as  $\alpha$ -ketoglutarate, 3-hydroxyanthranilate, and melanin to reduce  $Fe^{3+}$  to  $Fe^{2+}$  nonenzymatically for extracellular absorption [55]. This alternative reduction method might be responsible for late organ invasion observed with  $\Delta cft1$  mutant in experimental animals [45]. The possibility also exists that though  $\Delta cft1$  and  $\Delta cft1\Delta cft2$  mutants cannot easily utilise transferrin *in vitro*, heme might be unavoidably used to supply iron in the late systemic infection. Nevertheless, the brain fungal burden of these mutants is constantly lower compared to other systemic organ invasions, perhaps due to transferrin availability in the brain parenchyma.

Evidence showed that the pH of cryptococcomas (mass lesions within the brain parenchyma caused by the dissemination of cryptococcal cells) is between 5.4 – 5.6, and this favoured the accumulation of pro- and anti-inflammatory metabolites such as polyols, acetoin, dihydroxyacetone, and GABA [56]. Accumulation of superoxide in this neutrophil micro-acidic milieu may have subtly deceived the host tissue into deploying iron into this environment. This condition favours iron acquisition through low-affinity/ferrous iron-specific systems in the *C. neoformans*. Convincingly, the growth of  $\Delta hap3\Delta cfo1$ ,  $\Delta hap5\Delta cfo1$ , and  $\Delta hapX\Delta cfo1$  mutants are better rescued at pH 5.0 than 7.0 in media supplemented with  $FeCl_3$  or hemin. In addition, all the  $\Delta hap$  and  $\Delta cfo1$  mutants failed to survive low iron irrespective of the pH, but all can utilise feroxamine at pH 5.0 and 7.0. The better growth of  $\Delta cfo1$  mutants in the presence of  $FeCl_3$  and hemin, but the poorer growth of  $\Delta hap$  mutants, showed that Hap proteins function better to utilise different iron sources in a mutant lacking *Cfo1* for high-affinity and reductive iron uptake system [40].

There are many reports on the connection of iron level with major virulence factors and cell surface-associated proteins in *C. neoformans*. With SAGE, iron-enriched media elevate various transcription factors connected to iron storage, glycolysis, mitochondria oxidative function, lipid and amino acid metabolisms, and calmodulin-calcineurin signalling events. However, other important transcription factors are well elevated in the LIM. Lian et al. reported an increased level of *Cap60* gene, mannoprotein MP88, and GPI transamidase (which mediates cross-linking of cell wall mannoprotein to  $\beta$ -1,6-glucan) under iron-limiting conditions [46]. The transcript level of *Cig1* encoding an extracellular glycoprotein (like mannoprotein) needed for capsule attachment to the cell, together with  $\alpha$ -1,3-glucan (synthesised by  $\alpha$ -1,3-glucan synthase), is usually found upregulated under the iron-limiting condition as well [46,57]. Though this gene responded to low-iron conditions where  $\Delta cig1$  mutant is, the mutant is characterised by poor growth in a low-iron medium and failed to suppress capsule formation under an iron-supplemented medium. This means that *Cig1p* may be

involved in the iron-sensing relay process or probably participates in maintaining membrane iron retention through the siderophore transporter. Still, its role in capsule formation is dispensable.

#### 4.4. Copper Depletion

Copper is another micro-essential element needed for growth and virulence expression in *C. neoformans*. Copper acquisition and uptake are regulated by *Ctr1* and *Ctr4* (encoding copper transporters), copper transporter orthologue *ccc2*, *atx1* (encoding copper chaperone), and copper-responsive transcription factor (encoded by *cuf1*). Mutation in these genes impaired melanin formation because copper is highly needed for laccase and also impaired growth under iron-limiting media [58]. This means an interplay of homeostatic regulation between the copper uptake and iron acquisition and regulation in *C. neoformans* during pathogenesis. The  $\Delta cuf1$  mutants are characterised by impaired expression of *Lac1*, reduced dissemination, and tissue invasion coupled with growth defect in copper-limiting conditions because *Ctr4* is poorly upregulated [59]. Thus, *C. neoformans* invasion and dissemination within CNS seems to be centred on the requirement for copper and iron because the same poor tissue burden was reported for both  $\Delta cft1$  and  $\Delta cuf1$  mutants. Additionally,  $\Delta cir1$  mutant displayed positive upregulation of *Ctr4* transcript in LIM or negatively downregulated in high-iron media [60].

One anti-cryptococcal strategy of the host cells is to channel excessive copper into the cytoplasm to initiate copper toxicity that can kill cryptococcal cells. However, with the expression of the *Cmt* gene, excessive copper influx can be detoxified by this obligate aerobic fungus (for details on the phenotypic display of different cryptococcal cell mutants, check **Supplementary 1**). Cryptococcal infection is characterised by increased serum  $\text{Cu}^{2+}$  due to the efflux of excessive  $\text{Cu}^{2+}$  from cryptococcal cells and presumptive excessive  $\text{Cu}^{2+}$  initially channelled to toxify the fungus. Evidence showed an increase in the expression of alveolar macrophage *Ctr1* and a decreased expression of phagosomal  $\text{Cu}^{2+}$  transporter Atp7A during *C. neoformans* pulmonary infections [61]. Strategically, *C. neoformans* infections seem to promote nuclear factor- $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) that failed to coordinate macrophage growth, apoptosis, and inflammation. The preponderant level of NF- $\kappa\text{B}$  putatively binds the *Ctr1* promoter to maintain the expression of macrophage *Ctr1* while reducing the expression of phagosomal Atp7A ATPase to enhance fungal survival within the  $\text{Cu}^{2+}$  homeostatic phagocytes [61]. This, together with acidic phagocytic pH, probably keeps the infectious cryptococcal cell safe within the phagocyte until the condition is perturbed for non-lytic exocytosis, known as vomocytosis (phagosome extrusion/expulsion) [62].

Under  $\text{Cu}^{2+}$  limitation, Cuf1p acts as a transcriptional regulatory factor that enhances the expression of *Ctr1* and *Ctr4* as high-affinity  $\text{Cu}^{2+}$  transporters. Again, in the  $\text{Cu}^{2+}$ -enrich medium, Cuf1p instead stimulates the expression of *Cmt1* and *Cmt2* against  $\text{Cu}^{2+}$  detoxification – a situation usually encountered during infection [63]. Though individual deletion of *Cmt1* or *Cmt2* appeared not to affect the fungal survival in the lungs, double deletion of these redundant genes, however, yielded mutants with severely attenuated virulence despite insignificant differences in the observable virulence expressions and phagocytosis rate when compared to the *wt* [61]. Also, the expression of *Cmt1* and *Cmt2* is very specific and can only be induced in response to  $\text{Cu}^{2+}$  but not to any other ions. Compared to the *wt*, the  $\Delta cmt1\Delta cmt2$  mutant showed no growth defect in the presence of  $\text{Fe}^{2+}$ , bathophenanthroline disulfonate (BPS –  $\text{Fe}^{2+}$  chelator),  $\text{Cd}^{2+}$ , and menadione (MND), but the mutant was highly hypersensitive to 1 mM  $\text{Cu}^{2+}$  in the complete synthetic medium due to  $\text{Cu}^{2+}$  toxicity [61].

#### 4.5. Magnesium Homeostasis

Three  $\text{Mg}^{2+}/\text{H}^{+}$  antiporter encoding genes have been identified in *C. neoformans* (*Mgt1* – 3). Apart from *Mgt2*, none of these genes is induced by exogenous  $\text{Mg}^{2+}$ . Except for *Mgt2*, which seemed very important for intracellular  $\text{Mg}^{2+}$  homeostasis and proved difficult to be knocked out, *Mgt1* and *Mgt3* could not be associated with any phenotypical defect in this fungus [64]. Contrarily, different  $\text{Mg}^{2+}$  transporters have been identified in the yeasts, all of which are important to growth, mitochondrial functions, and spindle fibre formation during mitotic formation. Some of these transporters include plasma membrane  $\text{Mg}^{2+}$  transporters encoded by *Alr1* and *Alr2* [65], vacuolar  $\text{Mg}^{2+}$  transporter



encoded by *Mnr2* [66], and mitochondrial-associated  $Mg^{2+}$  importers encoded by *lpe10* and *Mrs2* [65] and exporter encoded by *Mme1* [67].

The *Mgt2* is a vacuolar membrane transporter and a homologue of *Mnr2* of *S. cerevisiae* that influences the regulation of several other transcription factors, a few of which appeared to be  $Mg^{2+}$ -independent. Suo et al. observed that  $\Delta mgt1$ ,  $P_{gal7-mgt2}$ ,  $\Delta mgt3$ , and  $\Delta mgt1\Delta mgt3$  mutants showed normal growth in YPGal. However, only the  $P_{gal7-mgt2}$  mutant exhibited a severe growth defect in YPD due to the significant downregulation of *Mgt2* in the presence of a catabolite repressor (for details on the phenotypic responses of different mutants of *Cryptococcus* against various quantitative external factors, check **Supplementary 3**). This growth defect could be rescued precisely by exogenous  $Mg^{2+}$  supplementation ( $\geq 50$  mmol/L) but not any other metallic salts. Among other downregulated genes in the transcriptome analysis of  $P_{gal7-mgt2}$  mutant cultivated in YPD alone or supplied with 50 mmol/L  $MgSO_4$  include *Aox1*, *Adh1*, *Tpo1*, *Fdh1*, *Hsp*, *Pdc1*, *Pcl12*, *Hxs1*, *Mup1*, *Mup3*, *Hxt1*, *Gal2*, *Aat*, *Pkp1*, and *Gal1* but *Mf1 $\alpha$* , *Agn1*, and many hypothetical proteins are upregulated mostly in the YPD alone [64]. The  $P_{gal7-mgt2}$  mutant is completely *albino* in L-DOPA without  $Mg^{2+}$ , but melanin is produced in the  $\Delta mgt1\Delta mgt3$ . Furthermore, adding  $Mg^{2+}$  restored melanin in the  $P_{gal7-mgt2}$  mutant; however, this pigmentation appeared less when compared to  $\Delta mgt1\Delta mgt3$  mutants [64]. On the other hand, capsule formation is highly induced in  $P_{gal7-mgt2}$  mutant only in the absence of  $Mg^{2+}$ , and this subsequently increased fungal proliferation and burden in the lungs of the infected animal model but with a *wt* tissue dissemination. Also, phagocytic inhibition was observed in murine macrophages infected with the  $P_{gal7-mgt2}$  mutant [64].

#### 4.6. Oxygen Depletion(Hypoxia)

*C. neoformans* is an encapsulated and environmental obligate aerobe (growing optimally under atmospheric oxygen at about 21% of the total air); therefore, adaptation to the relatively lower oxygen level of 1 – 5% in the human brain and lungs is inevitable to the survival of this fungus to cause infection. Oxygen availability is paramount to all the oxidative pathways, including mitochondria metabolism in this fungus and many other obligate aerobic basidiomycetes. These reactions are iron- and copper-dependent for electron transport leading to energy production. According to whole-genome microarray-based transcriptional profiling of *C. neoformans* under hypoxic conditions, about 347 transcripts are differentially regulated [68] and more than 100 expressed genes are statistically higher in the *wt* than the  $\Delta sre1$  mutant under 1%  $O_2$  level in 3 hours. However, more than 414 genes are significantly expressed in the  $\Delta sre1$  mutant than the *wt* under the same condition [69].

The major upregulated genes are involved in stress, iron and copper homeostasis, and the metabolism of sterol, heme, sphingolipid, sugar, and amino acids. In contrast, the downregulated genes are significantly involved in vesicular trafficking, cell wall formation, capsule synthesis, and ribosomal metabolism. Surprisingly, stress and antioxidant-related genes are highly upregulated in  $\Delta sre1$  mutant via the cAMP/Pka or Hog1 pathway than the *wt* under the hypoxic condition. These genes include *Tco2*, *Cat1*, *Cat3*, *Cas34*, *Hsp12*, *Hsp31*, *Gst1*, *Ecm4*, and other genes involved in reductive pathways such as *Gre2*, *Pkp1*, and *Ena1*, some of which are evolutionarily conserved in the yeast.

The expression of *Gst1*, *Gst2*, and *Gst3* encodes glutathione-S-transferase to protect the cells against reactive oxidants. Also, upregulation of *Cat3* (encoding catalase A) in the  $\Delta sre1$  mutant is highly significant in the hypoxic condition to degrade the  $H_2O_2$  to water, and the speculation for this is that  $\Delta sre1$  mutant may constantly be under more stress than the *wt* whenever the oxygen is limited [69]. In addition, *Hem13* expression appeared unchanged under hypoxic conditions either in the *wt* or  $\Delta sre1$  mutant; however, because of the strong connection between the Sre1p, ergosterol synthesis, and ionic homeostasis, the *Sit1*, *Ctr4*, *Fre7*, *Erg12*, and *Erg25* transcripts are usually drastically reduced in  $\Delta sre1$  mutants.

*C. neoformans* expresses the *Sre1* gene to sense oxygen and regulate sterol synthesis. This expression is a combination of endoplasmic reticulum membrane-bound Sre1p (homologue of mammalian sterol-response element-binding protein, SREBP) and its associated sterol- and oxygen-sensor activator (Scp1p orthologue of SCAP) with other possible accessory proteolytic enzymes such as a metalloprotein site-2-protease (Stp) and Kex2-like protease. Hypoxia (<3%  $O_2$  level) or

specifically low sterol level induces the proteolytic activation of Sre1p by Scp1p in the Golgi apparatus lumen and subsequently processed by Stp and Kex2 proteases to release a  $\approx 75$  kDa N-terminal transcription factor [69]. Any antifungal that blocks ergosterol biosynthesis will induce Sre1p activation; however, extremely low oxygen levels (0.2% O<sub>2</sub> level) will drastically slow down *C. neoformans* growth due to the slow activation of Sre1p [69]. The nuclear-translocation of this basic helix-loop-helix (bHLH) leucine zipper DNA-binding domain of N-terminal activated Sre1p induces the plethora of transcription factors that enable the fungus to cope with oxygen-limiting conditions alongside sterol, iron, and copper depletions [69-72].

The interdependence of oxygen, copper, and iron to facilitate the environmental survival of *C. neoformans* and cause infection in the human hypoxic tissues cannot be overemphasised. Heme is a cofactor for sterol production in fungi. The production of heme is iron-dependent, which is induced by hypoxia. Hence, it is unsurprising that  $\Delta sre1$  mutant, which failed to sense oxygen, is characterised by impaired ergosterol synthesis, iron, and copper uptake [69]. Under normal conditions,  $\Delta sre1$  mutants tend to accumulate more sterol intermediates while the ergosterol level is reduced to basal need. These sterol intermediates become higher than in the *wt* in hypoxic conditions as the ergosterol level reduces further because of the absence of *Sre1* expression. Thus, downstream accumulation of oxygen-dependent sterol intermediates under hypoxic conditions is necessitated in the  $\Delta sre1$  mutant except for some upstream oxygen-independent intermediates such as 24-methylene lanosterol, which seemed to be unperturbed in the mutant and *wt* [69].

Apart from the *Sre1* gene, Chun et al. established a hypoxia-sensitive trait in  $\Delta tco1$  mutant, a surprisingly hypermelanised avirulent strain with normal capsule production [73]. All mutants generated, such as  $\Delta sre1$ ,  $\Delta scp1$ ,  $\Delta stp1$ ,  $\Delta tco1$ , and  $\Delta sre1\Delta tco1$ , displayed normal growth in YPD at 37°C with no *in vitro* significant defect in the capsule formation but are sensitive to hypoxia condition, and the highest sensitivity is found in the double knockout mutant [68]. Furthermore, these mutants displayed a slight defect in melanin formation (except the  $\Delta tco1$  mutant with hyper-melanisation), defective to moderate tissue proliferation vis-à-vis dissemination, antifungal susceptibility (except  $\Delta tco1$  mutant), and impaired ergosterol biosynthesis (except  $\Delta tco1$  mutant) [68]. Enzymatically, the  $\Delta sre1$  mutant showed an insignificant defect in the activities of laccase, urease, and phospholipase, and the infection by this mutant failed to progress to terminal infection in an animal study due to limited brain lesions caused by poor growth in the brain though the mutant disseminated equally as the *wt* [69]. This *in vivo* weak infection may also be corroborated by the relatively smaller capsule size of this mutant *in vivo* [69].

Strictly, brain regions are endowed with self-secure barriers and nutrient stinginess to invading pathogens. Also, the outer regions of the brain, leptomeninges and cortex area are generally white with lower blood flow and iron content compared to the grey matter. Although Beard et al. observed a higher concentration of iron in the white matter, probably because of the preponderant transferrin circulation [74] yet the availability of this ferrous iron to the invading pathogen might not be corroborated. Furthermore, oxygen supply will be extremely low and stringently controlled in these brain regions because of the lower blood capillary network. Definitely, this self-care brain region might present a first-line defence against invading pathogens, including *C. neoformans*.

The phenotypic defects in the  $\Delta sre1$  mutants are similar to the observed defects in the  $\Delta cir1$ ,  $\Delta cuf1$  and  $\Delta cft1$  mutants, which further confirmed the interrelationship of the molecular oxygen, iron, and copper to facilitate the virulence traits of *C. neoformans* [43]. In fact, the *Sre1* transcript was downregulated in the  $\Delta cir1$  mutant, and the  $\Delta sre1$  mutant will not grow under iron-limiting conditions or in the presence of an iron chelator. However, adding ferritin to the iron-chelating media could restore the growth but not as much as the *wt* or reconstituted mutant [60,69].

Unsaturated acyl-CoA is a component of the membrane bilipid layer. The synthesis of this fatty acid around the endoplasmic reticulum is controlled by the availability of molecular oxygen. There is evidence of upregulation of the Ole1 transcription factor due to hypoxia in *C. neoformans*, but this regulation seemed to be Sre1-independent [68]. The *Ole1* encodes the endoplasmic reticulum membrane-bound  $\Delta^9$ -fatty acid desaturase in the yeast. This oxygen-dependent enzyme, in the presence of NADH-Cyt b<sub>5</sub> reductase, incorporates a double-bond into the saturated fatty acyl-CoA

through the diiron-oxo catalytic centre of the enzyme. Invariably, this enzymatic reaction is orchestrated by the ubiquitin/proteasome-mediated proteolytic activation of Spt23p and Mga2p and subsequent translocation of these dual-activators into the nucleus for the induction of *Ole1* transcript formation [75,76]. This activation is influenced by the low level of oxygen, low level of unsaturated fatty acids (or high level of saturated fatty acid), metal chelators, and carbon source. Explicitly, any event leading to iron depletion, such as transition metals like cobalt and nickel that compete with iron for heme, and metal chelators, which withhold iron from binding the heme, will reduce the oxygen-binding capacity of the heme. These events will induce the *Ole1* expression even under normoxic conditions.

*C. neoformans* can withstand excessive oxygen by expressing two loci, *Oxy1* and *Oxy2*, presumably linking hyperoxia-sensitivity to melanin production. The  $\Delta oxy1\Delta oxy2$  mutants are avirulent albino colonies with defective melanin production [77] and greater sensitivity to oxygen than  $\Delta oxy1$  mutant [78]. Emery et al. observed a lack of correlation between the *oxy* phenotype and melanin production, and it was concluded that the degree of melanin production is a defective predictor of hyperoxia resistance [78]. The reason is that *oxy2* defective loci in the *Oxy1* genetic background were characterised by defective melanin production and hyperoxia sensitivity, which means that the *Oxy2* locus is more important in conferring hyperoxia resistance. Though the antioxidant effect of melanin in *C. neoformans* might not be completely ruled out but Emery et al. predicted that the mechanism of defence against exogenous/endogenous neutral/reactive oxidant associated with hyperoxia stress in fungal aerobiosis solely relies on the interplay of *Oxy1* and *Oxy2* loci in the genetic make of *C. neoformans* [78].

Besides,  $\Delta oxy2$  mutants are defective in ferric/cupric reductase and high-affinity copper uptake. Even after reconstitution of the mutant to restore melanin production, most of the mutants retained hyperoxia sensitivity, interpreted as non-allelic, non-interfered, and unlinked loci between the *Mel* (for melanin locus) and *Oxy* loci (for hyperoxia locus). Nonetheless, the importance of melanin to withstand reactive oxidants cannot be overestimated. The interconnection between the *Oxy* and *Mel* loci seems elusive. Melanin and polymeric dopamine are effective cellular anionic antioxidants produced by *C. neoformans* against hypochlorite (produced by myeloperoxidase as found in inflammatory cells such as neutrophils) [79], superoxide ( $O_2^-$ ) and other reactive oxidants. However, activity against neutral oxidants such as  $H_2O_2$  is weak, especially in the presence of copper and oxygen [80], which by Fenton reaction generate reactive  $OH^-$  (hydroxyl ion),  $OH^\bullet$  (hydroxyl radical), and  $HOO^\bullet$  (hydroperoxyl radical) nucleophile that predisposes phagocytosed *C. neoformans* to oxidative arrest. Sometimes, perhaps, neutral oxidants can cross the cell wall and bypass the effect of melanin, which is why *C. neoformans* are highly susceptible to peroxides such as  $H_2O_2$ , *t*-BOOH, and COOH (for details on the phenotypic responses of different mutants of *Cryptococcus* against various quantitative external factors, check **Supplementary 3**). Whatever the case, *Oxy1* and *Oxy2* expression may be needed to resist hyperoxia-based melanin oxidation imposed by the endogenous oxidant from the leucocytes and macrophages.

#### 4.7. Carbon dioxide ( $CO_2$ )

Via a bicarbonate ( $HCO_3^-$ ) production, *C. neoformans* employs  $CO_2$  signalling sensors such as calmodulin (Cam1) and carbonic anhydrase (Can) to detect the  $CO_2$ -inducing cascade events leading to virulence factor formation, enhancing cell wall integrity, mating, resistance against excessive  $O_2$ ,  $CO_2$ , pH,  $Ca^{2+}$ , osmotic and oxidative stresses, toxigenic compounds, heavy metal toxicity, and antifungal drugs through direct activation of Cam1 or Can2-Cac1-cAMP/Pka cascade event. The *Can* gene encodes the families of four classes of carbonic anhydrase ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ), which are distributed differently in all living organisms [81], and the primary fungi transcript is *Can2* encoding a zinc metalloenzyme  $\beta$ -carbonic anhydrase that maintains equilibrium between  $CO_2/H_2O$  and  $HCO_3^-/H^+$ . Surprisingly, though *Can1* and *Can2* are found and expressed in *C. neoformans*, only *Can2* appeared to be the major transcript for  $\beta$ -carbonic anhydrase capable of expressing more of the enzyme at high  $CO_2$  (5%) than low  $CO_2$  (0.036%) [82].

The  $\Delta can2$  and  $\Delta can1\Delta can2$  mutants of serotypes A and D displayed growth defects in a CO<sub>2</sub>-dependent manner. At the same time, the overexpression of *Can2* impaired membrane integrity and made the cell susceptible to oxidative and genotoxic stress and antifungal drugs [82,83]. The mutant further displayed impaired decarboxylation reaction but exhibited an increased salvage pathway to produce pyrimidine via the upregulation of *Fur1* expression. The lack of several *Can2*-dependent genes, including *Fas1* (in lipogenesis), *Meu1* (in methionine salvage pathway), and *Gpb1* (in pheromone-response MAPK pathway for sexual differentiation) in this mutant warranted insufficient fatty acid production, impaired amino acid salvage pathway, and defective pheromone production for cell differentiation and filamentation, which are all responsible for defective growth of  $\Delta can2$  mutant incubated in ambient air with about 0.036% CO<sub>2</sub>. However, palmitate, among other fatty acids, appeared slightly effective at 2 mM to rescue some of these growth defects in the  $\Delta can2$  mutant [82]. This means that the ambient environment promotes the expression of the *Can2* gene for growth and survival, but this enzyme is dispensable in a CO<sub>2</sub>-rich environment such as human tissue and can still proliferate with adequate virulence production.

Apart from *Fas1*, *Meu1*, and *Gpb1*, other *Can2*-dependent genes include *Cfo2*, *Cas3*, and *Atf1*. Most of the *Can2*-dependent genes are majorly *ESR* genes that will not respond to the overexpressed *Can2* gene; this is very surprising. Kim et al. overexpressed the *Can2* gene in *C. neoformans* mutant, which eventually displayed poor cell wall/membrane integrity coupled with hypersensitivity to oxidative, genotoxic, and antifungal (azole and polyene), probably because of the preponderant accumulation of HCO<sub>3</sub><sup>-</sup>/H<sup>+</sup>, which may be perceived as stress index to the cell [83].

*Atf1p*, which is involved in diverse stress responses together with *Hog1p*, is slightly upregulated when *Can2* is repressed. Like *Hog1p*, *Atf1p* represses melanin and capsule formation to promote the expression of a group of genes involved in OSR, NSR, HTG, and OMSR (check **Supplementary 4** for as many details as possible for the transcription factor, kinase, protein, enzyme, and gene acronyms used in this review). Again, *Atf1p* induces the expression of genes involved in the thioredoxin system [84]; perhaps this is synergistically enhanced by the *Skn7* and *Yap4* expression against oxidative and nitrosative stress, respectively (for details on the phenotypic responses of different mutants of *Cryptococcus* against various quantitative external factors, check **Supplementary 3**). However, deletion of *Atf1* or *Yap4* in a mutant has no significant effect on the macrophage survival and virulence [84], just as *Can1* and *Can2*, with or without CO<sub>2</sub>, have no significant effect in modulating the *C. neoformans* virulence factors and response to osmotic shock, UV, temperature, oxidative and nitrosative stress [82]. Apparently, better survival was observed in the  $\Delta can2$  mutant than the wt in the CSF of the rabbit, and this mutant can only be recovered in the YPD supplied with a high level of CO<sub>2</sub>. Again, failure to observe any mating in the wt or  $\Delta can2$  mutant, *in vivo*, at a high CO<sub>2</sub> level showed that other factors contributed to *in vivo* mating of *C. neoformans* [82].

High CO<sub>2</sub> of about 5 – 6% in the human host induces capsule formation in cryptococcal cells, which is independent of *Cas3* or *Can2* expression but highly dependent on the *Cac1* expression for CO<sub>2</sub>-mediated capsule production [83,85]. Surprisingly, due to its negative effect on cellular activity, *C. neoformans* cannot keep up with the continuous production of bicarbonate because overexpression of the *Can2* gene under the *P<sub>ctr4</sub>* aggravated sensitivity to several oxidative- and osmotic-inducing agents such as H<sub>2</sub>O<sub>2</sub>, CdSO<sub>4</sub>, MMS, SDS, AmpB, FCZ, KCZ, and ICZ. However, with a genotoxic-inducing agent such as hydroxycarbamide/hydroxyurea, only a wt resistance was displayed [83].

#### 4.8. Sugar, Lipid, and Proteins (Amino Acids) Metabolisms

*C. neoformans* possess arrays of metabolic enzymes that help 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. *C. neoformans* secretes hydrolytic enzymes such as peptidases, lipases, and glycosidases to release amino acids, fatty acids, and sugars, respectively. The plethora of enzymes involved in these metabolic activities are 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 for growth, mating, cell differentiation, and virulence factors which are constantly under the regulation of cAMP-dependent signalling genes [86,87]. The  $\Delta gpr4$  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 [18]. However, their response to glucose remained unchanged, unlike the poor glucose response from  $\Delta hxt$  mutants. Despite a good response to glucose, the  $\Delta gpr4$  mutants still displayed impaired capsule formation and mating defect, both of which are recoverable in the presence of exogenous cAMP [86].

In a DNA microarray analysis, nearly 20% of total gene expression is dedicated to the transport and metabolism of carbohydrates and amino acids metabolism [88]. The expression of *Gpp2* encoding glycerol-3-phosphate phosphatase controls various metabolic systems as well as osmotic and cold shock responses. Deletion of this gene induced various permeases/transporters (*Aapp*, *Mup1p*, *Mup3p*, *Ena1p*, *Nha1p*, *Plb1p*, *Hxt1p*, *Stl1p*, *Uga4p*), significant facilitator transporter super-family genes, anti-oxidative enzymes (*Cat2p*, *Ccp1p*, and *Gst1p*), and membrane transporters (for sulphite, myo-inositol, and pantothenate). However, various genes involved in the redox process, the glycolytic pathway, and the TCA cycle are downregulated in this mutant [89]. More important is the induction of genes involved in sulphur-containing amino acid biosynthesis, such as *Cys3*, *Sul1*, *Soa1*, *Bds1*, *Met17*, and *Jlp1* in this mutant. The expression of *Gpp2* modulates the regulation of S-containing amino acids by facilitating the proteolytic degradation of Cys3p and subsequently reducing the sulphur assimilation [89,90].

Hitherto, the deletion of *Aap4* and *Aap5* allowed eugenol resistance in *C. neoformans*. However, deletion of *Gpp2* alters the biosynthetic regulation of Met and Cys due to the perpetual activation of amino acid permease protein (*Aapp*), which probably explains the increased sensitivity of the  $\Delta gpp2$  mutants to eugenol. This dire situation could not be rescued by exogenous proline; however, it is uncertain whether glycerol can suppress this defect or not [89]. Reasonably,  $\Delta hog1$  mutants might as well be sensitive to eugenol because *Aap* is also induced in *hog1* mutants [91]. Thus, osmotic stress response by glycerol in the yeast is essential, but Cys can feed the glutathione cycle to generate GSH for alternative osmotic stress response in a case where glycerol production is truncated in a mutant such as  $\Delta gpp2$ . This seemingly inversely proportional metabolic network was investigated further in the *C. neoformans wt* to be true because 1 M NaCl can downregulate *Cys3* and *Sul1* expression to some extent [89].

Furthermore, the activity of the Gpp2p is under the regulation of  $Ca^{2+}$  via the Cna1/Cnb1 cascade event. Just as in *A. nidulans* and *N. crassa*, deletion of *Cys3* in *C. neoformans* has been shown to reduce the expression of *Sul1* and both expressions are grossly repressed in the presence of S-containing amino acids (e.g., Met or Cys) in SD (synthetic dextrose) media more than the YPD [90]. This shows that media with low S-containing amino acids will likely induce *de novo* sulphur amino acid synthesis. Again, Cys seems better as a sulphur source than Met because methionine could be formed from cysteine in a transsulphurylation reaction [90]. Further investigation on  $\Delta cys3$  mutants showed that the mutants displayed a *wt* level of urease activity and capsule size yet attenuated for virulence in *Galleria mellonella*. Bad still, the experimental condition with Cys or Met in the culture media failed to support the melanin and phospholipase assay of the fungus [90].

The proteomic analysis that showed a concurrent induction of *Met3*, *Tef1*, *Gpd1*, *Ccp1*, *Rps0*, *Rps1*, *Tif1*, *Sub2*, *Tm8*, *Gpp2*, *Cna1*, and *Cnb1* has led to the proposal of a metabolic relationship between sulphur-containing amino acid biosynthesis, glycerol phosphatase (encoded by *Gpp2*), and calcineurin pathway (*Cna1/Cnb1*), which was further confirmed by protein-protein interactions [90]. Expression of *Cna1/Cnb1* motivates 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 while Gpp2p orchestrates inactivation of Cys3p by proteolytic degradation. A proposed mechanism is that inorganic sulphur induces *Cna1/Cnb1* activation to deactivate Gpp2p to promote Cys3p and activate downstream genes involved in S-containing amino acid biosynthesis. However, the presence of organic sulphur such as Met and Cys deactivates *Cna1/Cnb1*, and this activates Gpp2p to deactivate Cys3p and repress the downstream genes involved in S-containing amino acid biosynthesis [90].

Among the genes induced in  $\Delta cys3$  mutant are putative *Aro3* and *Aro4* involved in aromatic amino acids biosynthesis; *Jlp1* in sulfonate metabolism to provide  $SO_4^{2-}$  for Met5/10p; sulphiredoxin in oxidative stress response; and *Clr6* – encoding class I histone deacetylase (a transcriptional epigenetic repressor of *Mat2* – *Mat3* region and centromere for precise heterochromatin packaging during chromosomal segregation in fission yeast) [92], but the repression of *Clr6* in the *C. neoformans* *wt* promotes capsule formation and biosynthesis of various amino acids. With Maier et al.,

- ❖ *Clr6*, along with *Hog1*, *Mbs1*, *Ste12 $\alpha$* , and *Tup1*, is classified as a cluster of genes induced against reactive oxidants but repressor of respiratory 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 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 respiratory activities, ionic transporters, gluconeogenesis, and osmoregulatory process [93].

Transcriptome analysis  $\Delta pka1$  and  $\Delta pkr1$  mutants have been characterised with higher-level transcripts of genes involved in glycolysis/carbohydrate metabolism, TCA, amino acid metabolism, iron uptake, cell wall glycan formation, translational ribosomal proteins, translational initiation and proteins elongation, heat shock, protein and vesicular trafficking, UPR, cytoskeletal proteins, membrane transporters/permeases, cell surface and extracellular transmembrane proteins, oxidative and nitrosative stress response proteins, mitochondria membrane proteins, virulence factors, and phospholipid metabolism [94].

In addition, there is a characteristic increase in transporters/permeases such as sugar transporter, hexose transporter, glucose transporter, and maltose permease (all for sugar assimilation); ammonium permease and amino acid transporter (to assimilate nitrogen and amino acids, respectively); peroxisomal fatty acid transporter and ATP carrier (to assimilate phospholipase products), and many other residential permeases and ion-affinity coupled transporters, which are used for importing nutrients and micro-needed ions from the extracellular digestion. Therefore, through the interplay of *Pka1* and *Pkr1* expressions, cAMP plays one of the major central roles in coupling environmental sensing to metabolism, virulence, cell differentiation, mating, and growth.

*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, *C. neoformans* embarked on gluconeogenesis to supply sugars for metabolisms leading to titanisation and capsule formation. The interdependence of peroxisome, glyoxysome and cytoplasm to break down assimilated fatty acids ( $\beta$ -oxidation) to generate acetyl-CoA is essential to drive the glyoxylate cycle to generate oxaloacetate used in gluconeogenesis. Contrary to *C. albicans*, which showed up-regulated genes for key enzymes of gluconeogenesis [95], phagocytosed *C. neoformans* showed no upregulation of such enzymes [16]. However, transcriptional response and analysis of *C. neoformans* ingested by macrophage and amoeba showed that key enzymes like phosphoenolpyruvate carboxykinase and fructose-1,6-bisphosphatase were respectively upregulated [13]. The *Icl1* (encoding isocitrate lyase), *Mls1* (encoding malate synthase in glyoxylate cycle), and genes for  $\beta$ -oxidation enzymes are upregulated in *C. neoformans* recovered from murine macrophage [16]; however, *Mdh2* (encoding malate dehydrogenase) and genes for ergosterol biosynthesis were downregulated in the phagocytosed *C. neoformans* [13]. The findings of Rude et al. showed that though the *Icl1p* and the glyoxylate shunt are important for ATP production during fungal infection, still  $\Delta icl1$  mutants failed to display any apparent virulent defect in animal studies and produced a *wt* level of capsule and melanin without any defect in macrophage survival or phagocytic index [96].

In all these 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 [97]. Derengowski et al. have discovered a multiple-fold increase in the transcript level of *Hva1* in amoeba-phagocytosed cryptococcal cells [13] and the absence of this *Hva1p* unexpectedly increased the level of phosphoenolpyruvate kinase but definitely impaired the TCA due to reduced activity of 2-ketoglutarate dehydrogenase complex activity. This condition probably attenuates mitochondria oxidative reaction but favours sudden energy increased from preponderant floating of cytoplasmic NADPH via alternative pathways to produce ATP. This metabolic energy production shift favours cell proliferation and growth, hence the hypervirulence trait of  $\Delta hva1$  mutants in the murine model but not moths or worms [97]. This means the  $\Delta hva1$  virulence is temperature dependent. The exogenous supply of NADPH<sup>+</sup> further enhanced the higher tissue burden of this mutant in the spleen and brain than in the lungs [97]. Paradoxically, an extensive comparison of the virulence factor expression, 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, animal model, moths, and worms revealed no significant difference between  $\Delta hva1$  and  $\Delta hva1+Hva1$  mutants [97]. This displayed the importance of regulated metabolic systems in *C. neoformans* virulence.

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, impaired cell wall integrity, fragile cell membrane, osmotic stress hypersensitivity (due to impaired *Hog1* pathway), and failure to utilise mannose and fructose [98]. Insertional mutation T-DNA at the promoter site of *Pgi1* reduces the activity of *Pgip*, and in the presence of a 2% glucose supplement, this mutant tagged as LZM19 ( $\Delta pgi1$ ) is able to produce melanin due to the derepression of *Lac1*. Further work showed that an exogenous supply of cAMP could restore capsule deficiency in the LZM19 strain, but this repressed the *Lac1* expression [98].

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, *Snf1* is turned off by *Reg1-Glc7* protein phosphatase 1 (PP1) [99]. However, high glucose concentration activates *Cac1p* to produce cAMP, which interacts to activate *Pka* to repress the *H<sub>2</sub>C<sub>2</sub>* zinc-finger nuclear-translocated proteins *Msn2p* and *Msn4p* that are programmed to induce genes containing SRE-sequence promoters such as *Hxt1*, *Hog1*, *Ras1* and others, which are generally regarded as NSR, OSR, OMSR, ESR, GSR, HMSR, and CSR. In addition, the cAMP/*Pka* also inhibits the redundant upstream *Snf1p*-activation kinases, *Sak1*, *Tos3*, and *Elm1*, to put *Snf1* under repression in high glucose content.

High glucose deactivating *Snf1* leads to the activation of *Mig1p* by dephosphorylation to enhance the association of *Mig1p* with *Suc2p*. The *Mig1p* is a universal gene repressor in the yeasts [100], which binds the promoters of target genes, including the *Lac1* gene, to repress melanin biosynthesis as a response to glucose repression. In low glucose levels, however, the *Cac1* activity is reduced to lower the cAMP level, which invariably elevates the *Sak1* activity to activate *Snf1p* [101]. 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.

Canonically, despite glucose availability, mutation of *Pgi1* expression may have limited glucose catabolism and subsequently altered the ATP homeostasis. This condition favours *Snf1p* activation leading to the deactivation of *Mig1p* and subsequently derepresses *Lac1* for melanin production. Therefore, the involvement of the *Snf1-Mig1* regulatory pathway through nutrient homeostasis cannot be ruled out in the pathogenesis and virulence of *C. neoformans*. 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  $\Delta snf1$  mutants to AmpB relative to the *wt* response; however, the  $\Delta snf1$  mutants apparently showed no significant growth defect to AmpB and FCZ, but a very slight growth defect in the presence of RPM [102].

Generally, phenotypic defects of  $\Delta snf1$  mutants become conspicuous with increasing temperature. For example, melanin formation decreases above 30°C and ultimately becomes a non-melanised mutant at 37°C [102]. Surprisingly, though the transcript level of *Snf1* is practically increased in a glucose-limited or acetate medium, but there is no evidence of increased transcript in the cryptococcal cells recovered from the infected lungs. Besides,  $\Delta snf1$  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 [102]. This means the latter two transcription factors solely depend on the *Snf1* expression, especially in the glucose-limited medium. 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. The additional report showed that  $\Delta snf1$  mutants displayed a *wt Mls1* transcript irrespective of the media used [102]. The activation of *Hxt1p* in  $\Delta snf1$  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.

In addition to *Lac1* regulation, *Snf1* expression also affects the SOD activity. A drastic reduction of *Lac1* in  $\Delta snf1$  mutants cultivated in the glucose-limited medium indicates the *Lac1* expression being regulated by *Snf1p*. Similarly, the *Sod1* transcript is reduced in  $\Delta snf1$  mutants in a glucose-limited medium but not in the acetate medium. Irrespective of the medium, deletion of *Snf1* failed to significantly affect the expression of *Fhb1*, *Tps1*, *Glr1*, and *Skn7*, but *Lac2* and *Ssa1* transcripts are significantly increased in acetate media [102]. This means that in the glucose-limiting medium, *Snf1p* orchestrates the expression of *Lac1* to produce melanin and the expression of *Sod1* against oxidative stress. 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; after all,  $\Delta lac1$  mutants still cause infection in a murine model [102,103]. Unfortunately,  $\Delta lac1$  and  $\Delta lac1\Delta lac2$  mutants are totally *albino*. In contrast,  $\Delta lac2$  mutants are melanised in catecholamine-enriched media [104]. However, the elevated *Lac2* transcripts more than the *Lac1* in the presence of alternative carbon sources such as acetate [102] is reminiscent that *Lac2* may probably be involved in melanin production when unusual carbon sources weaken *Lac1* involvement. Therefore, by inference, catecholamine (such as *L-DOPA* and *norepinephrine*) metabolism into eumelanin may seem more complex than already revealed, especially in the presence of alternative carbon sources.

The role of *Acs1* expression encoding acetyl-CoA synthetase has also 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, coupled with concurrent elevated expression of different membrane transporters and stress response genes, which is indicative of a nutrient-limited environment and the route for alternative carbon sources as facilitated by the expression of *Snf1* [102]. Deleting *Acs1* yielded moderately attenuated mutants that cannot utilise alternative carbon sources such as acetate.

Sometimes *C. neoformans* can 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. In contrast, the  $\Delta hapX$  mutant and the *wt* showed similar growth in the glucose, sucrose, acetate, and ethanol, even in 0.01% SDS. Although *Hap* genes are iron-dependent regulatory genes, surprisingly, supplementing the media with hemin failed to improve the weak growth in *hap* mutants [40]. Because of this, iron-enrich media specifically improved 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 [46]. Apart from these elevated key enzymes, other accessory proteins such as ubiquinone, prohibitin (a membrane-bound chaperone stabilising the mitochondria proteins), and ATPase are equally pre-eminent [46]. 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. In fact, aconitase identified in *C. neoformans* seems to have paralogous encoding genes to probably enhance



its extracellular activities such as *mRNA* translation/stability – a function known for Irp1p. It could probably be that *C. neoformans* regulates excessive iron by converting this Irp1p to cytosolic aconitase, which fails to stabilise *mRNA* but promotes Irp2 ubiquitination and proteasomal degradation [105].

Further, into the regulatory roles of Hap3p and HapXp, these two transcription factors negatively modulate the expression of the orthologue *Lys4* in the LIM. This was confirmed by low detection of *Lys4* in  $\Delta hapX$  and  $\Delta hap3$  mutants cultured in the presence of 100  $\mu M$   $FeCl_3$ , but in the absence of  $FeCl_3$ , the expression of *Lys4* is well induced in either of these mutants [106]. Relative to the *wt*, the expression of *Lys4* remained unchanged in  $\Delta cir1$  mutants in either iron enrich media (IEM) or LIM. Like *Lys4*, *Leu1* expression was also similarly regulated by Hap3p and HapXp, either in the LIM or IEM. Low iron supports the expression of *Leu1* and *Lys4* in  $\Delta hap3$  and  $\Delta hapX$  mutants, but only a high level of iron favours the expression of *Leu1* in  $\Delta cir1$  mutant [107]. This means that Cir1p positively regulates this cytoplasmic located Leu1p in  $\Delta hap3$  and  $\Delta hapX$  mutants cultured in LIM but regulates negatively in IEM. There is speculation, however, that *Leu1* expression may be more under the regulation of Hap3p than HapXp expression because while  $\Delta hapX$  mutant produced a comparable *Leu1* transcript with the *wt*,  $\Delta hap3$  mutant produced a significantly more transcript of *Leu1* under the same IEM conditions [107]. 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 NCR.

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 availability of specific 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*). Analysis showed that *C. neoformans* possessed genes for lipase (encoded by *Tgl2* among at least three encoding genes) and enoyl-CoA hydratase [46,108]. 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* [108,109].

The lack of trypsin, chymotrypsin,  $\beta$ -galactosidase,  $\beta$ -glucuronidase, and  $\alpha$ -mannosidase by the species of this fungus showed that certain proteins and  $\beta$ -D-sugars might not be suitable in the culture media for *C. neoformans* [109]. However, many species of cryptococcal cells do possess esterase lipase (active against triacylglycerol with C4 – C8 acyl chain),  $\alpha$ -galactosidase,  $\alpha$ -glucosidase,  $\beta$ -glucosidase, N-acetyl- $\beta$ -glucosaminidase,  $\alpha$ -fucosidase, galactokinase, D-lactate dehydrogenase [46,109], which means that various  $\alpha$ -D-sugars, amino sugars, and disaccharides are suitable carbon sources for cryptococcal cells. Furthermore, since IEM elevates sterol biosynthesis, it can be speculated that fatty acid/sterol-formulated media could motivate sterol and phospholipid biosynthesis because of the possibility of expressing lipolytic and sterol biosynthetic enzymes under the iron influence.

In addition, lipid biosynthesis is an essential biochemical pathway leading to cell membrane integrity. The Mga2 is an orthologue transcription activating factor for the component of fatty acid synthesis and regulates *in vitro* normal growth at a range of temperatures. 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- $\beta$ -glucanase, and other relevant proteins are induced in this mutant [110]. 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  $\Delta mga2$  mutants are characterised with elevated expression of the homologues of small GTPase effectors, which control the polarisation growth, and the *Pak1*, which controls the filamentation growth. Therefore, it is possible that during this morphogenesis, Mga2 expression is repressed at  $\leq 30^\circ C$  for GTPases and *Pak1* expressions that will promote polarisation during growth, but this, however, will contradict the involvement of Mga2 in thermotolerance and morphogenesis as reported by Kraus et al. [110].

Besides, the dependency of *fas1* and *acc1* expressions on Mga1 for lipid and sterol biosynthesis will literally promote polarisation growth, mating, and haploid fruiting. Therefore, the background expression of Mga1 may have promoted polarisation and cell growth in  $\Delta mga2$  mutant.

Still, under the influence of iron, secretory vesicles (such as exocyst complex encoded by *Sec* gene), enzymes (such as glutamate dehydrogenase, *p*-aminobutyrate aminotransferase, proteasomes, ubiquitin ligase, RNA-processing enzymes, and mRNA splicing proteins), and transporters/permeases (for allantoin, urea, ammonia, and purine-cytosine) involving in nitrogen and amino acid metabolism including protein trafficking have been observed to be elevated under the IEM [46]. Not only these, iron influences several of the amino acid metabolism (anabolism and catabolism). In *S. cerevisiae*, iron probably orchestrates the binding of  $\alpha$ -isopropylmalate to homodimeric DNA-binding protein Leu3 (a repressor that, together with Gcn4, regulates amino acid biosynthesis) to regulate branched-chain amino acid biosynthesis, Aapp, nitrogen and carbohydrate metabolisms. The expression of Lys4p for Lys biosynthesis and Leu1p for Leu biosynthesis is iron-dependent because of the presence of conserved aconitase repeating motifs that bind Fe-S; and probably other amino acids biosynthesis proteins such as Met2p, Met3p, and Met6p for Met; Ilv2p for Ile and Val. Mutants generated from each of these transcription factors are avirulent in the MIMC.

Iron homeostasis in *C. neoformans* consists of arrays of gene clusters harmonising the acquisition and utilisation of iron, and this could be similar to the iron regulon of *S. cerevisiae* under the influence of Aft1 and Aft2 transcription factors. With NCR, certain nitrogen sources are not assimilated, perhaps for the reason of energy-costly 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 ( $\Delta gat201$ ,  $\Delta gat204$ ,  $\Delta bwc2$  or  $\Delta cir1$ ) failed to shut down totally the fungal growth in all the popular nitrogen sources [111]. This means that various amino acid permeases may still be activated to assimilate other nitrogen sources under later conditions, contrary to  $\Delta gat1$  mutants where the *Amt* and majority of the *Aap* gene expressions are impaired. However, Pro and Arg may probably 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  $\text{NH}_4^+$ . 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  $\Delta gat1$  mutants and *wt* [111]. 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  $\Delta gat1$  mutants in this medium. 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 the metabolism of His is also more complex, this may explain why  $\Delta gat1$  mutants had poor growth in a medium with each of these two amino acids as nitrogen sources.

The growth defect of  $\Delta gat1$  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*. Similar growth survival observed with the *wt* and  $\Delta gat1$  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 but at the same time, the survival of the  $\Delta gat1$  mutant in the same environment poses an investigation into the unique nitrogen sources suitable for such a mutant. Perhaps, such media may be rich in Pro or Arg as inferred from Lee et al. observation [111]. Notwithstanding, limited nitrogen sources in the environmental pigeon guano may have repressed the *Gat1* for the mating process that promotes propagule/basidiospore formation of the filamentous stage of this opportunistic human infectious fungus. Lee et al. had discovered that mating is greatly enhanced through the robust filament and basidia formation either in the unilateral or bilateral crossing of  $\Delta gat1$  mutants [111]. 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 moderately reduce the pathogenic efficiency

of *Δgat1* mutants compared to the *wt* [111]. This shows that the key virulence factor of *C. neoformans* is firmly under the influence of NCR.

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  $\text{NH}_4^+$  in each of the media [111]. This shows that  $\text{NH}_4^+$  represses capsule formation by activating NCR even in capsule-induced nitrogen sources, but, on the contrary, the presence of  $\text{NH}_4^+$  induces melanin formation. To buttress this, Lee et al. observed melanin formation in each of the aforementioned media when prepared along with  $\text{NH}_4^+$  except in the Pro medium, where a slight melanisation was observed even when  $\text{NH}_4^+$  is present [111]. Without  $\text{NH}_4^+$ , 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 [111]. 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  $\text{NH}_4^+$  represses capsule formation in the presence of different nitrogen sources, melanin is significantly induced.

It is shown that among the numerous amino acid permeases, *Aap2*, *Aap4*, *Aap5*, *Aap6*, and *Aap8* are more differentially expressed in the amino acid fortified SD media compared to the rich medium (YPD) [112,113], which means that NCR is highly induced in YPD compared to the SD. Without  $\text{NH}_4^+$  (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* [112]; also, under repressive NCR, *Aap2* and *Aap3* are induced in the presence of Lys-containing medium [106]. However, amino acid supplements in SD do repress the general amino acid control (GAAC) but promote the APC-superfamily membrane transporters/permeases (APC, AAP, AGCS, CCC, HAAAP, BCCT, SSS, NSS, NCS1, NCS2, and SulP families [114-116]).

Trp supplement alone in the SD could increase *Aap1* and *Aap2* transcripts by 3.5 and 3.8-fold; similarly, adding Trp+Met+His could improve *Aap4*, *Aap2*, *Aap8*, and *Aap5* transcripts by 3.3, 4.2, 7.5, and 21-fold respectively [112]. The transcription analysis further showed that NCR could repress *Aap2*, *Aap5*, and *Aap8* in the presence of  $\text{NH}_4^+$  but not *Aap4*, which means *Aap4* is sensitive to any nitrogen sources; however, *Aap1* and *Aap7* remained minimally expressed [112]. 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) [112].

The *Δaap1Δaap2* mutants are thermosensitive (at 37°C in synthetic media fortified with amino acids), hypocapsulated, and hypovirulent in the *G. mellonella* infection model [117]. 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/ $\text{NH}_4^+$  or in YPD; however, growth reduction was observed in liquid culture of *Δaap1* containing Met or Pro at 30 or 37°C [117]. Also, a liquid culture of *Δaap1Δaap2* mutant containing Gln or Arg displayed growth reduction at 30°C, and *Δaap8* mutant displayed growth reduction in media containing Met, Glu, and Trp.

Generally, *Δaap1Δaap2* mutants appeared to show defective growth in 60% of all culturable amino acid media (AAM), which means that these amino acid permeases are essential for growth at 37°C on amino acids media, just like *Aap4* and *Aap5*. However, *Aap1*, *Aap2*, *Aap6*, and *Aap8* appeared to be 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* [117]. Apart from being non-essential for virulence, none of the mutants seemed to be 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* [117].

From Martho et al.,  $\Delta aap2$ ,  $\Delta aap4$ ,  $\Delta aap5$ ,  $\Delta mup1$ ,  $\Delta mup3$ , and  $\Delta mup1\Delta mup3$  showed no sign of attenuated virulence in *G. mellonella*, but  $\Delta aap4\Delta aap5$  mutants are hypovirulent at 30 or 37°C [118]. Furthermore, virulence investigation in the murine model showed that mice inoculated with the *wt* and  $\Delta aap4$  and  $\Delta aap5$  died within a month, while the mice inoculated with  $\Delta aap4\Delta aap5$  mutants and PBS-treated strain survived with viable recovery colonies from their lungs and livers. In addition,  $\Delta aap2$ ,  $\Delta aap4$ ,  $\Delta aap5$ , and  $\Delta aap4\Delta aap5$  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 invariably shows that Aap4 and Aap5 are important permeases for thermotolerance, antifungal resistance, and oxidative stress response for tissue invasion, survival, and virulence in the animal model. By speculation, they are a potential target for therapeutic application. 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 at high temperatures except  $\Delta aap4\Delta aap5$ ,
- ❖ are not affected in AAM at 37°C and in capsule formation except  $\Delta aap1\Delta aap2$ ,  $\Delta aap4\Delta aap5$ , and  $\Delta mup1\Delta mup3$ ,
- ❖ are not sensitive to any stress agent except  $\Delta aap4\Delta aap5$ ,
- ❖ are virulent in *G. mellonella* except for  $\Delta aap1\Delta aap2$  and  $\Delta aap8$  (hypovirulent),  $\Delta aap4\Delta aap5$  (avirulent as well as in murine model),
- ❖ the *Aap1*, *Aap2*, *Aap3*, *Aap4*, and *Aap5* are very similar in the sequence homology, and each of the *Aaps* in the double mutants 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 repressed further from 30 to 37°C except for *Mup1*, which is induced further,
- ❖ irrespective of the growing media, there is no significant change in the growth of  $\Delta aap2$ ,  $\Delta aap4$ ,  $\Delta aap5$ ,  $\Delta mup1$ ,  $\Delta mup3$  and  $\Delta mup1\Delta mup3$  mutants at 30 or 37°C,
- ❖ the significant growth defect of  $\Delta aap4\Delta aap5$  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  $\Delta aap4\Delta aap5$  mutants,
- ❖ relative to  $\text{NH}_4^+$  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  $\text{NH}_4^+$  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* metabolises 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* gene,
- ❖ in all, growth is denser in L-amino acids containing media compared to corresponding D-amino acids media,



- ❖ pathologically,  $\Delta dao$  mutants of *C. neoformans* are virulent, but  $\Delta dao$  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,
- ❖ *L-Tyr* failed to dissolve at permissive pH for *C. neoformans* to grow,
- ❖ *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 in the presence of  $\geq 5$  mM  $H_2O_2$ ,
- ❖ unlike the  $\Delta aap4$  and  $\Delta aap5$  mutants, the growth defect of  $\Delta aap4\Delta aap5$  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*),
- ❖ by functions, *Aap2*, *Aap3*, *Mup1*, and *Mup5* are regarded as minor permeases while *Aap4* and *Aap5* are major permeases [117-119].

It is very surprising, therefore, to have observed the relevance of the Ras1 pathway in the control of amino acid permeases leading to thermotolerance. The  $\Delta ras1$  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  $\Delta ras1$  mutants [117]. The involvement of Ras1 in the assimilation of amino acids is a unique feature of this transcription factor apart from the popular thermotolerance that it is known for. This means that amino acid uptake may be part of the function of Ras1 to facilitate thermotolerance. Alternative carbon and nitrogen sources such as galactose and Pro may also induce *aap* expression. It is not surprising, thus, to observe growth reduction in  $\Delta ras1$  mutants cultured in either SD or synthetic galactose (SG) fortified with  $NH_4^+$  or Pro at either 30 or 37°C [117]. This shows the importance of Ras1p in the nutritional balance of *C. neoformans* against thermal stress. The controlling effect of Ras1p on the expression of *Aap* was further investigated by quantifying the expression level of each *Aap* transcript in  $\Delta ras1$  mutants. All *Aap* and *Mup* transcripts were repressed in  $\Delta ras1$  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 [117]. The exception of *Aap5* may suggest a different regulatory mechanism in addition to or different from Ras1 expression.

## 5. Secretory Hydrolytic Enzymes and Extracellular Vesicles in Cryptococcal Cells

Several extracellular hydrolytic enzymes from *C. neoformans* have been studied with diverse functions and are freely secreted under the regulatory N-terminal signal peptide or cleavage from the cell wall located GTI-anchored proteins or remained 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 GTI-anchor [120] yet the majority of the proteins involved in adhesion may require GTI-anchorage. For example, ectophosphatase is a membrane-surface hydrolytic enzyme that often releases phosphate from phosphothreonine substrate and is liable to be inhibited irreversibly by sodium orthovanadate. 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 [121].

Various hydrolytic enzymes, including endoproteinases, exoproteinases, and glycosidases, 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 fungus to penetrate the endothelial layer of BBB to establish infection [122]. 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* [123]. Likewise, Brueske discovered that media supplemented with  $\text{NH}_4^+$  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 [124].

The involvement of *C. neoformans* extracellular proteinases in the degradation of host immunological proteins has been demonstrated to be responsible for fungal invasion and dissemination. Convincingly, such proteinases could degrade  $\beta$ -casein, murine IgG<sub>1</sub>, bovine IgG, human C<sub>5</sub>, haemoglobin, and  $\gamma$ -globulin in media containing carbon and nitrogen sources [125,126]. 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* [127]. Proteases are released at permissive and non-permissive temperatures irrespective of their sources, but a positive correlation existed between the capsule size and the protease activity in the clinical isolates, which was not found in the environmental/bird-dropping isolates [128]. The majority of these proteinases are serine proteases having optimal activities between pH 7 – 8 at 37°C [125].

In another related study, aspartyl endopeptidase (encoded by putative *May1*) and carboxypeptidase D1 (encoded by putative *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 [129]. Specifically, the total proteolytic activity in the mutants lacking metalloendopeptidase/metalloproteinase (encoded by *Mpr1*), serine 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 unlike the  $\Delta$ *may1* and  $\Delta$ *cxd1* mutants, which showed significantly reduced activity of the extracellular proteases. Because it is a major secreted protease, it is 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 [129-131]. Mutation of these extracellular protease-encoding genes seemed not to affect melanin production, but with the fact that melanin synthesis is also a cell wall anchoring process enriched with mixed GTI-anchored proteins, chitin, chitosan, and glycan then, the deletion of *Prb1* had shown hypomelanisation in such a mutant [129].

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 production in pathogenic fungi such as *C. albicans* and *C. neoformans*, and this invariably inhibits fungal growth. Unlike Saquinavir, Nelfinavir, Lopinavir, Darunavir, Tipranavir, and Atazanavir, Amprenavir, Brexanavir, 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* [129-131].

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 to the killing by innate effector cells such as monocytes, neutrophils, and macrophages [130]. 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, unlike *C. albicans*, and again the transcriptional level of this enzyme is strategically independent of the media used [132].

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 this nickel-dependent functional protein complex [133]. Via the nickel/cobalt transporter (encoded by *Nic1*),  $\text{Ni}^{2+}$  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, *C. neoformans* deploys the pH-sensing signalling factors such as Rim21/Rim8/Rim20/Rim13 complex to counter the pH surge via the activation of Rim101 [18].

The  $\Delta ure1$  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  $\Delta ure1$  mutant, whether intranasally/intravenously, failed to show pulmonary distress, displayed delayed hydrocephalus and wasting, and significantly lived longer than the *wt*-infected mice [134]. Hydrocephalus and wasting are delayed manifestations of meningoencephalitis caused by  $\Delta ure1$  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 [134]. Notwithstanding, wholistic urease activation is essential for the microcapillary sequestration, dissemination, and brain invasion of *C. neoformans* because  $\Delta ure1$ ,  $\Delta ure7$ , and  $\Delta nic1$  mutants, which lacked urease activities, significantly displayed defective growth in the presence of urea with poor blood-to-brain invasion in an animal model [133,135]. Though urease seems to be essential to the dissemination, invasion, and survival of *C. neoformans in vivo* yet, clinically infectious urease-negative isolates capable of dissemination and invasion have been reported in AIDS patients [136,137], which means this enzyme may be playing second fiddle in the pathogenesis of *C. neoformans* [138].

Cryptococcal cells can assimilate nitrogenous bases and nucleosides to produce 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. Excessive nitrogenous compounds are degraded by ureases. The release of DNases tandemly correlated with the increased activity of ureases in most fungi except in a few isolates of *Endomycopsis fibuligera* and *Lipomyces starkeyi* [139].

Sod is another membrane residence anti-oxidative enzyme, which converts superoxide to  $\text{H}_2\text{O}_2$  and  $\text{O}_2$  and complements the effort of melanin against reactive oxidants. The expression of this enzyme increases with temperature to facilitate growth and thermotolerance, thereby contributing to the virulence of *C. neoformans* [140].

Phospholipases (PLs) are also secretory enzymes that 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 the integrity for survival against temperature, antifungals, oxidative stress, and cell wall destabilisers [22]. Moreover, the involvement of PLC as a component of PLs in the virulence expression, antifungal resistance, homeostasis and IP3 kinase activity, and cytokinesis displays the incredible functions of this membrane-associated enzyme [28,29].

Special attention is needed on some extracellular lipases capable of inducing antibodies in infected animal studies. Incredibly, Chen et al. discovered that 92% of the tested cryptococcal strains secreted butyrate and caprylate esterase lipase. At the same time, some also produced acid phosphatase, naphthol-AS-BI-phosphohydrolase, and  $\beta$ -glucosidase, and the sera of infected rodents were able to precipitate several of these secreted proteins [141].

Phosphatases are enzymes released to remove the phosphate group (dephosphorylation) from transcription factors that are usually activated/deactivated by phosphorylation through serine/threonine/tyrosine kinase. This controls the on/off regulations 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*, which facilitates fungal adhesion to monocytes, but deletion of this gene attenuates the fungus virulence [38,121].

Extracellular vesicles described as “virulence bags” are exosomal secretory vesicles containing microbodies and proteins, some of which are associated with virulence because of the presence of laccase, urease, and phosphatase in these “virulence bags” [12]. The proteomic analysis of the *C. neoformans* secretomes 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 [12]. 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 growth at a temperature above 30°C [142].

## 6. Conclusions and Perspectives

The significant involvement of different hydrolytic enzymes, transcription factors, and membrane proteins like pumps, permeases, and transporters was discussed in detail in this article. While each of these proteins plays an important role in the survival, adaptation, and pathogenesis of *C. neoformans* in animals and humans, some are redundant with barely known function. These attributes are also common in other invasive fungi responsible for candidiasis and aspergillosis. The multiplicity and redundancy of these proteins may be critical in catabolite repression to rescuing the fungi when in environment with alternative carbon sources, low oxygen, fluctuating pH, high salt contents, high level of oxidant, low phosphate content, low ammonium content, and low micronutrient elements. Ability to produce exocytic vesicles of microbodies, enzymes, and virulence proteins appears as alternative way of inducing cellular morphology, biofilm formation, cellular communication, filamentation, fruiting, and mating. We described these proteins as weapons that facilitate tissue invasion, persistence, and infection in animals and humans. With reference to the supplementary files, we further described the morphological and functional impairments associated with the deletion or alteration in the encoding genes for these proteins. Because of the functional similarities of these proteins with human cells, targeting any of them for antifungal drug discovery needs careful consideration that will preclude the consequence in the human cells.

**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.

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