Review

Transcription Factors and Intracellular Proteins Orchestrate Phenotypic Adaptation in *Cryptococcus Neoformans* for Survival and Human Infections

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

[1] Pathogenic Yeast Research Unit, Department of Biochemistry and Microbiology, University of the Free State, Bloemfontein, South Africa; 2014061341@ufs4life.ac.za
[1] Pathogenic Yeast Research Unit, Department of Biochemistry and Microbiology, University of the Free State, Bloemfontein, South Africa; SebolaiOM@ufs.ac.za
* Correspondence: SebolaiOM@ufs.ac.za; Tel.: +27-51-401-2004; foxxyphemmzy@gmail.com; Tel.: +1-781-957-7963

Abstract: Transcription factors are diverse intracellular proteins facilitating cellular responses to inducing factors via gene expression. Regulatory signalling cascades from the membrane proteins (sensors) to transcription factors (effectors) are paramount to accurate phenotypic display against external factors. This review examines several transcription factors germane to *Cryptococcus (C.) neoformans* adaptation and survival for human infection. These opportunistic pathogenic single-cell yeasts (fungi) possess several gene duplications and peculiar membrane proteins due to adaptive phenotypes and morphological plasticity. Consequently, hundreds of responsible pleiotropic genes have been studied to understand how these genes are induced, regulated, and coordinated. However, these findings are sparsely converged and interlinked, making it cumbersome to relate one gene to the other or group them by their functions. We reviewed several wide-ranging transcriptional analysis works associated with *C. neoformans* into comparable phenotypic traits that necessitate adaptation, survival, and human infection. We present a robust work that addresses several transcription factors and their inducing factors. Lastly, we converge, link, and group several of these factors according to their multifunctional expression pattern. We also provide adequate information on certain genes critical to this fungus, which could be explored pharmaceutically in drug targeting for more effective antifungal management.

Keywords: *Cryptococcus*; transcription factor; thermotolerance; virulence factors; antifungal; oxidative and osmotic stress; capsule; melanin; monokaryotic fruiting; mating and filamentation

1. Introduction

C. neoformans is an environmental opportunistic and obligate aerobe, capable of colonising various human systemic organs [1]. It is an encapsulated pathogenic fungus with a high degree of ecological adaptation. It is found in soil enriched with bird droppings and around trees. The probability of finding cryptococcal cells around woody and shrub plants such as Eucalyptus [2], Carob [3], Olive [4], Pine [3], *Platanus*, and *Prunus* [3] trees is evidenced. The soil environment is a complete and complex ecosystem that allows for the survival of all living organisms through competition and adaptation. As such, *C. neoformans* is among the saprophytes exposed to the good, bad, and ugly environmental conditions that invariably cumulate and translate into the phenotypic adaptation for human infection. Besides the harsh environmental conditions, *C. neoformans* are constantly in predatory association with the water soil amoeba, enabling the cryptococcal cells to develop peculiar features to survive these menace [5,6]. These persistence associations reasonably equipped cryptococcal cells to survive the predatory phagocytic amoeba and

 doi:10.20944/preprints202301.0461.v1

improve the cellular virulence factors and self-defence phenotypic traits [7-9]. In that case, surviving the mammalian residential monocytes, macrophages, and dendritic cells is a pathogenic feature of invading *C. neoformans*. To further bolster this possibility, *C. neoformans* pre-adapted in *Dictyostelium discoideum* became enlarged/titanised (giant cell) and melanised faster than the wild type (*wt*). In addition, acapsular/avirulent *C. neoformans* became virulent in the myosin-mutant soil amoeba [10]. This pattern of infection re-emphasises why the immunocompromised individual is predisposed to cryptococcosis. By spore-inhalation, the *C. neoformans* pathogen becomes invasive (cryptococcemia) from the lungs to other parts of the body tissues and organs, particularly the CNS, brain, bones, skin, prostate, and heart [11].

C. neoformans isolated from the soil contaminated with the pigeon dropping/plant niche are regarded as environmental isolates, while those from infected humans are known as clinical/blood isolates. However, among the serotypes A, D, and AD of *C. neoformans*, serotype A is the most common environmental and clinical/veterinary type with genotype similarity among the isolated strains such as VNI [12,13]. Early investigation showed that environmental isolates might be less virulent in the animal model compared to the clinical isolate [14]. However, a recent study has shown that the pathogenicity of the VNI strain, whether an environmental or a clinical isolate, was the same in the animal model [11].

Several virulence factors associated with the pathogenesis of *C. neoformans* have been studied and characterised. To enhance the strategical launching of each factor, the fungus compartmentalises the factors and releases the virulence factors according to the inducing cue. Cryptococcal cells produce capsules, which release the immunomodulatory capsular polysaccharides – glucuronoxylomannan (GXM) and galactoglucuronoxylomannan (GalGXM) that majorly alter the phagocytosis of this fungus by macrophages. In addition, the presence of a cell wall, which is made with chitin, can release melanin. The melanin may facilitate cell wall titanisation/thickening and the membrane clustering of other virulent factors and enzymes. The desire to produce melanin drives the neurotaxis of *C. neoformans* to the catecholamine-enriched tissue, such as the basal ganglia of the brain [15]. These functional virulence factors sequentially orchestrate the expression and secretion of extracellular hydrolytic enzymes, the sequestration of host metabolic pathways, the coordination of robust antioxidant mechanisms, the attenuation of host NO-induction and PAMPs-PRRs (pathogen-associated molecular patterns-pattern recognition receptors) killing system, the resistance of *C. neoformans* to Ca^{2+} and CO_2 influx, pH imbalance, nutrient, and oxygen deprivation. The constant modification of the dynamic components of the cryptococcal cell wall and membrane enables this pathogen to survive the environmental niche and host internal cellular homeostasis response [16].

Having highlighted some of the strategies launched by the cryptococcal cells for environmental adaptation, tissue invasion, and survival, it is paramount to harmonise how the transcription factors and intracellular proteins control each of these microbial phenotypic displays and, ultimately the release of various virulence factors. In this review, numerous works on the regulatory transcription factors of *C. neoformans* are critically examined and juxtaposed with fungal adaptation and survival during infection in the human host systems.

2. Regulatory Transcription Factors in Cryptococcal Cells

Various transcription factors are upregulated/downregulated when an amoeba or a macrophage engulfs cryptococcal cells. This happens because the infected cells launch a compulsory intracellular nutrient limitation and induce stress as a mode of pathogen attack. More than 525 inherent transcription factors are differentially upregulated in *C. neoformans* phagocytosed by macrophages [17,18], which is one of the reasons some basidiospores are constantly released non-lytically by vesicle secretion to promote cellular and tissue invasion, especially in the immunocompromised patients. These transcription

 doi:10.20944/preprints202301.0461.v1

factors cover different cellular systems, including translation, signal transduction, antioxidant induction, and carbon metabolism within the cryptococcal cells to facilitate cell dormancy, reproduction, infection, and escape the anti-cryptococcal-induced immune response [19-21]. In addition, the number of modulatory transcription factors expressed in *C. neoformans* when encountering stress from amoeba is usually more than that expressed in the macrophages purposely because of the concomitant effects of the environmental factors and predatory amoeba (with varying microbiota) as against the macrophages. Derengowski et al. reported more than double expressed genes in macrophages as expressed in Amoeba [8]. Several of these transcription factors are intrinsically linked to one another and the corresponding phenotypic expression [22-24]. Mutation (disruption/deletion) of such genes in cryptococcal cells produces defects that make them avirulent, easily phagocytosed, physiologically deformed, sterile, and easily killed. Paradoxically, mutation may sometimes enhance the survival, adaptation, and pathogenesis of cryptococcal infections (for details on the phenotypic display of different cryptococcal cell mutants, check **Supplementary 1,** and for details of cellular events that induced/repressed different membrane transporters and permeases in *C. neoformans*, check **Supplementary 2**).

Transcription factors are largely conserved and structurally similar in function among the cryptococcal species/strains; however, serotype-specific differences occur due to different environmental susceptibilities, evolution, and geolocation of each strain. This evolutionary divergence has brought about a discrepancy in the function of homologue transcription factors. Hicks et al. discovered that the Pka1 catalytic subunit of Pka is involved in the cAMP pathway major kinase function in serotype A and enhanced mating, haploid fruiting, capsule, and melanin formation, but Pka2 is engaged for the same purpose in serotype D [25]. The mating-type specific Pak (p21-activated protein kinase) homologues *Ste20α* in *MATα* and *Ste20**a*** in *MAT**a*** are allelic genes found within the congenic mating-type loci controlling the mating and pheromone formation in *C. neoformans*. While the *Ste* transcription factors contribute to the mating and filamentation of *MATα* in serotype A, the congenic *MAT**a*** is sterile with no pheromone and less virulence than *MATα* [26]. Further analysis showed that serotype A *MATa* mating-type locus lacked several mating-associated genes such as *Ste12α, Ste20α, Ste11α,* and *Mfα2*. This *MAT**a*** serotype A was later confirmed to contain the *Ste20**a*** gene, which is similar to serotype D (95% homology) but diverged from the *Ste20α* of serotypes A and D (67% homology) [26].

Significant virulence reduction was reported in *MATα* serotype D *Δste12α* mutant but not in serotype A mutant. Also, this *Ste12α* controlled other phenotypic expressions, such as capsule and melanin formation in serotype D, which further underpinned the molecular linkage between mating type and virulence in serotype D [27,28]. Furthermore, a relatively low mating frequency and hyphal formation in serotype D were observed when the *MATα Δste12α* mutant strain was convergently crossed with the *MAT**a*** strain [27]. This indicates the possibility of *Ste12* homologues in different mating-type strains. Furthermore, the strain with *Ste12α* reconstituted locus exhibited the *wt* phenotypic expressions such as capsular size, mating, phospholipase activity, and haploid fruiting in serotype D [27]. Though serotype A *Δste12α* showed a modest defect in mating but completely defective in haploid fruiting/sporulation like serotype D; however, unlike serotype D, this homologue gene seems to be redundant with no effect on the virulence of serotype A [29].

Naturally, serotype D (like the JEC21 strain) is resistant to fludioxonil (FDX) like *S. cerevisiae* because Hog1 phosphorylation that translocates this MAPK protein into the nucleus is not initiated in the presence of this fungicide; hence the Hog1 MAPK signalling remains inactive. However, serotype A (such as the H99 strain) is sensitive to FDX because the Hog1-specific nuclear phosphatases activate Hog1 via the Hog1 osmosensing pathway. The Hog1 protein, an apparent functional divergent mitogen-activated protein kinase encoded by the *Hog1* gene via the high osmolarity glycerol (HOG) pathway in response to high external osmotic stress, is a nuclear-localised dephosphorylation-activated protein regulated by the response regulator kinase Ssk1p. Optionally, it was proposed

 doi:10.20944/preprints202301.0461.v1

that Hog1-specific phosphatase could also be induced independent of Ssk1 but orchestrated by the response regulator phosphorelay histidine protein kinases, Tco1 and Tco2, in the yeast [30]. Generally, in its repressed state (phosphorylation), Hog1 prevents yeast virulence and mating by minimising the production of melanin, capsule, and mating pheromones. Contrarily, in its activated state (dephosphorylation), Hog1 mobilises the induction of stress response genes and the production of proteins against internally generated toxic molecules. Therefore, unless the *Hog1* gene is repressed or deleted in serotype A, it remains sensitive [31,32]. It was further concluded that FDX resistance is a dominant phenotype because serotype AD obtained by hybrid crossing of serotype A (FDX sensitive) and serotype D (FDX resistant) showed FDX resistance [31].

Similarly, deletion of the *Cna1* gene predisposes serotype B to a complete growth defect at 30°C. At 37°C, serotypes A and B, irrespective of mating strains, completely failed to grow and are rendered avirulent when *Cna1* is deleted. However, only *Δcna1* mutant of serotype A remained sensitive to osmotic stress induced by NaCl and LiCl [33] as well as FDX in a Hog1-independent manner [31]. In addition, there is a functional divergence of *Snf1* between serotype A strain H99 and serotype D strain JEC21. Generally, Snf1p facilitates virulence production, alternative carbon utilisation, stress tolerance, and thermotolerance. A high temperature is needed for H99 *Snf1* to function against stress response and the assimilation of alternative carbon sources, unlike strain JEC21 [34]. Likewise, disruption of *Snf1* in JEC21 predisposes the strain to amphotericin B (AmpB) but not in the H99 strain [20,34]. Check **Supplementary 4** for the meaning of the transcription factor, kinases, protein, and gene acronyms used in this review.

3. Regulatory Transcription Factors in Cryptococcal Cells Control Phenotypic Expression for Morphotypes, Adaptation, Survival, and Virulence

3.1. Cell Wall Chitin-Chitosan Components

C. neoformans overlays its cell wall matrix with chitin (a repeating unit of ***β***-1, 4 – *N*-acetyl-D-glucosamine). Chitin generally strengthens the cell walls and enables a rigid cellular shape [16,35]. With this, *C. neoformans* maintains a constantly balanced cellular turgor pressure against its cell wall and, together with capsules, helps to prevent desiccation injury when the soil is dry.

Though the presence of chitin and other unique cell wall components predispose the cryptococcal cells to the host PAMPs-PRRs (pathogen-associated molecular patterns-pattern recognition receptors) recognition to induce anti-cryptococcal defence strategies [36] but with the capsule formation and vesicular secretion of capsular components, *C. neoformans* can shield the chitin and circumvent the PAMPs effect [37,38]. Not only this, but *Cryptococcus* also has intrinsic chitin deacetylase encoded by seemingly redundant *Cda1*, *Cda2*, and *Cda3* functional genes (the fourth, *Fdp1*, remained undetermined), which can convert cell wall chitin produced by chitin synthase (encoded by *Chs3*), in the presence of its regulator (encoded by *Csr2*), to chitosan (a non-rigid soluble polymer) [39,40]. Interestingly, *C. neoformans* seems to prefer the *Cda1* gene, which is highly upregulated in mammalian lungs and primarily selected for fungal proliferation [16].

Eight genes encode chitin synthase, *viz Chs1* to *Chs8*, but only three chitin synthase regulators, *Csr1 – Csr3*, are functionally active. Unexplainably, as many as these encoding genes are in cryptococcal cells, a compensatory expression for each mutant rarely occurs except for *Δchs3*, where a slight increase in *Chs5* and *Chs7* was detected [39]. Furthermore, the Chs3-Csr2-Cda1 complex is particularly important for chitosan production for the cryptococcal cells to grow, disseminate, and invade the host at a physiological temperature [41,42]. Under vegetative growth, the expression of *Chs7* is entirely repressed, while *Chs6* is extremely and minimally detected in contrast to *Chs1* and *Chs4* expression. However, *Chs2*, *Chs3*, *Chs5*, and *Chs8* are highly induced during vegetative growth [39]. Surprisingly, none of these chitin-associated genes or regulators is essential for cryptococcal viability at 30°C. However, *Δchs3* and *Δcsr2* mutants are particularly hypersensitive to

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higher temperatures and cell wall stressors such as Calcofluor White (CFW), Congo red, SDS, and caffeine (for details on the phenotypic responses of different mutants of *Cryptococcus* against various quantitative external factors, check **Supplementary 3**).

Characteristically, all chitin mutants displayed similar phenotypic features as the *Δcsr1* and *Δcsr3*, except the *Δchs3* and *Δcsr2* mutants. These two mutants could produce melanin around their cell wall but failed to retain this pigmentation ("leaky mutants"), unlike other chitin mutants [39]. Furthermore, cytokinesis in these mutants is impaired and characterised by incomplete budding. The CFW staining revealed intense septal and uneven cell surface staining. The aggregated mutants are proportionally larger than the *wt*, and the *Δcsr2* mutant is even larger than the *Δchs2* mutant. The two mutants also failed to produce chitosan by 48 hours of incubation but instead accumulated a higher level of chitin than any other mutants and the *wt* [39]. This cryptococcal strategy of protecting its cell wall seems evolutionary to prevent predatory and host-released chitinase, maintain cell wall integrity and capsule width, ensure melanin production and attachment, and budding during vegetative growth [39,40,43].

3.2. Cell Morphotypes and Aneuploidy

Reports have shown that small-size (<2 μm) basidiospores produced during sexual reproduction in *Cryptococcus* are deeply embedded in the alveoli making it difficult to be dislodged from the mammalian lung through airway ciliary movement [44]. These spores are highly pathogenic in an immunocompromised murine model and resistant to high temperature, oxidative stress, sunlight, and desiccation [45-47]. Conversely, the filamentous (hyphae or pseudohyphae) morphotype of *Cryptococcus* is not as highly pathogenic as the spores but is literally adopted to survive environmental amoeba [48]. Conditionally, pseudohyphae growth is usually initiated under a nitrogen-limiting condition (that is seldomly observed in murine and human infection but regularly formed when co-culture with amoeba), and it is phenotypically longer in the diploid unisexual than haploid strain; however, this seems unconnected to cell ploidy. Lee et al. showed that Amt1p and Amt2p are required for pseudohyphal growth under nitrogen catabolite repressor (NCR), NH_4^+, but these hyphae lacked clamp connection that could have formed basidiospores (monokaryotic fruiting) [49]. Irrespective of the serotype, either *Δamt1* or *Δamt2* but not *Δamt1Δamt2* mutant can form pseudohyphae, which can fully develop back into the yeasts in yeast extract peptone dextrose (YPD) media (yeast ↔ pseudohyphae). Still, the colonies are wrinkled at the edges when cultured in invasive agar growth [49]. Formation of filaments in *Cryptococcus* seems to be microevolution depending on the host interaction but is unlikely to be significant in virulence [50-52]; however, this has been shown to improve agar adherence and invasion [49]. Thus, the reversible yeast-pseudohyphal switch may probably be responsible for the infection latency observed in humans.

Titanisation (cell giant) is another yeast-like morphotype, which has been linked to the accumulation of chitin within the cell wall to withstand several host-derived stresses [53-55]. Giant cells are conspicuously found in alveoli of the mammalian pulmonary system during infection, growing from 15 – 100 μm in size with polyploid genomes that can degenerate into haploid and aneuploid progenies; the bases of genetic variability, pathogen dormancy, and reactivation [56-58]. The formation of aneuploid spores during the sexual or non-sexual reproduction of *MAT**a*** and *MATα* is the foundation of the infectious propagule of *Cryptococcus* [59]. Aneuploidy is a common phenomenon in the environmental strains to withstand external stress as well as the clinical isolates to initiate adaptation, survival, and infection [60,61].

However, aneuploidy is a rare event in *C. neoformans*. Out of the 75 basidiospores examined after crossing *Δcbk1:Nat* mutant with *Δcna1:Neo* mutant with both genes being far from each other on the serotype A genomic contigs; the *MAT**a*** and MAT*α* alleles segregated independently with 37.3% *wt* (Nat^S::Neo^S), 30.7% *cbk1* (Nat^R::Neo^S), 30.7% *cna1* (Nat^S::Neo^R), and 1.35% *cbk1cna1* double mutant (Nat^R::*cna1*:Neo^R) *(Nat - nourseothricin acetyltransferase; Neo – neomycin; S – sensitive; R – resistant)* [62]. Further analysis showed

 doi:10.20944/preprints202301.0461.v1

that this aneuploidy progeny with *cbk1:Nat*^{R}*::cna1:Neo*^{R} double mutant resistant strain displayed *Δcbk1* phenotypic morphotype as a dominant allele because of the occurrence of heterozygous *CNA1* locus *(Cna1/Δcna1:Neo)*. This indicates a parallel function of Ram and Cna1 pathways to regulate cellular morphology at physiological temperature.

The catalytic subunit of Cna1p has been shown to relocate to ER-associated puncta and budding neck at 37^{o}C. This thermal stress temperature induces a preponderant *m*RNA accumulation in the ER that attracts processing bodies (PBs) and stress granules (SGs). At the ER-associated puncta, a greater proportion of Cna1p localisation has been found with PBs decapping enzyme encoded by *Dcp1* and SGs poly(A)-binding protein constituent encoded by *Pub1* [63]. This co-localisation is to ease the thermal stress, which causes *m*RNA accumulation. The *Ram*-associated genes such as *Cbk1, Kic1, Mob2, Sog2,* and *Tao3* are also crucial for normal cell polarisation, karyokinesis, and cytokinesis. The *ram* mutants displayed hyperpolarisation and incomplete cytokinesis leading to cell aggregation that forms hyper-elongated pseudohyphal, though karyokinesis leading to septate between the dividing cells appeared normal in these mutants [62]. This phenomenon is well displayed by each *ram* gene mutant (*Δram)*, with actin being localised at the bud tips, unlike the *wt* where actins are well distributed throughout the cell for proper cell segregation. The mislocalisation of actin in the *ram* mutants has been traced to the absence of *Cbk1* and *Kic1* gene products at the punctate and septa structures of actively dividing cells (cytokinesis) [62,64]. The septal localisation of the Kic1 has been speculated to be controlled by other *Ram* genes, such as *Tao3* [62]. The point mutation in the *Mob2* gene usually found in *C. gattii* may be responsible for the pseudohyphal strain with incomplete mating when crossed with the *wt,* and this displayed normal mating hyphae and basidia formation but no sporulation [49]. Natural single nucleotide difference in *Mob2* of *C. gattii* genes may have produced differentiated 37^{o}C thermosensitive pseudohyphal strains and progenies, which failed to form yeast in YPD, and are more sensitive to Cna1 pathway inhibitor (FK-506) than the *C. neoformans* pseudohyphae [49].

Further investigation revealed that Ram protein kinases could interact to facilitate MAPK activity leading to complete and successful cytokinesis. By expressing *C. neoformans Cbk1, Kic1,* and *Mob2* genes in *S. cerevisiae*, the yeast two-hybrid growth assays revealed a strong protein-protein interaction between Cbk1::Mob2, Cbk1::Kic1, and Kic1::Kic1 but a weak interaction between Mob2::Kic1 [62]. Despite this hybrid expression of the Ram kinases, none of the cryptococcal *Cbk1, Kic1,* and *Mob2* genes could complement the corresponding cytokinesis defect in *S. cerevisiae ram* mutants; neither was the homologues of *S. pombe* complemented *ram* defective *S. cerevisiae* mutants as well [62,65]. Therefore, proteins from generally related organisms may structurally resemble but are functionally divergent sometimes, regulating different components of the pathways.

3.3. Capsule: Capsular Polysaccharides and Glycoproteins

The polysaccharide capsules afford *Cryptococcus* species to avoid and survive the attack of phagocytic cells like soil amoeba and mammalian macrophages, dendritic cells, and neutrophils [66]. The phenotypic switch in *C. neoformans* induced by immediate environmental stresses such as a change in pH, high CO_2 level, iron and nutrient limitation, and antifungal has been shown to affect the resultant biophysical and biochemical natures of capsular GXM. This eventually allows the cryptococcal cells to survive different environmental conditions and brings about structural heterogeneity and antigenic variation [67-69]. Subtly, this invariably enables the cryptococcal cells to evade the physiological immune attack launched against this pathogen. This could be very deleterious and become aggravated, especially when the cryptococcal infection is being treated in immunocompromised patients. Coupled with the antifungal effect, there may be selective pressure and indirectly cause a phenotypic switch within the infected strain that can cross the blood-brain barrier (BBB), resulting in intracranial pressure. This is the cause of high morbidity and mortality in patients with meningoencephalitis [37,70].

 doi:10.20944/preprints202301.0461.v1

Capsular components are immunomodulatory extracellular complex polysaccharides produced as a radial whitish cloud around the cryptococcal cells **(Figure 1)** and constantly shed as systemic antigens in the infected patients, which can be assessed from urine, serum, and cerebrospinal fluid (CSF). This enables the cells to suppress the host immune response, withstand the phagocytosis in the systemic milieus, and promote intracellular survival in the macrophages [1,71,72]. *In vivo* capsule production is usually induced by low iron content, physiological temperature, and CO_2 [67,73]. The presence of capsular components such as glucuronoxylomannans (GXM), galactoxylomannans (GalXM), glucuronoxylomanogalactan (GXMGal), and a minor glycoprotein component known as mannoproteins (Mp) have been found in the systemic circulation, causing the shedding of L-selectin adhesion and integrin proteins from the surface of leukocytes such as neutrophils [74,75]. This shedding prevents polymorphonuclear leukocyte (PMN)-endothelial interaction and extravasation of the granulocytes into the inflammatory tissue.

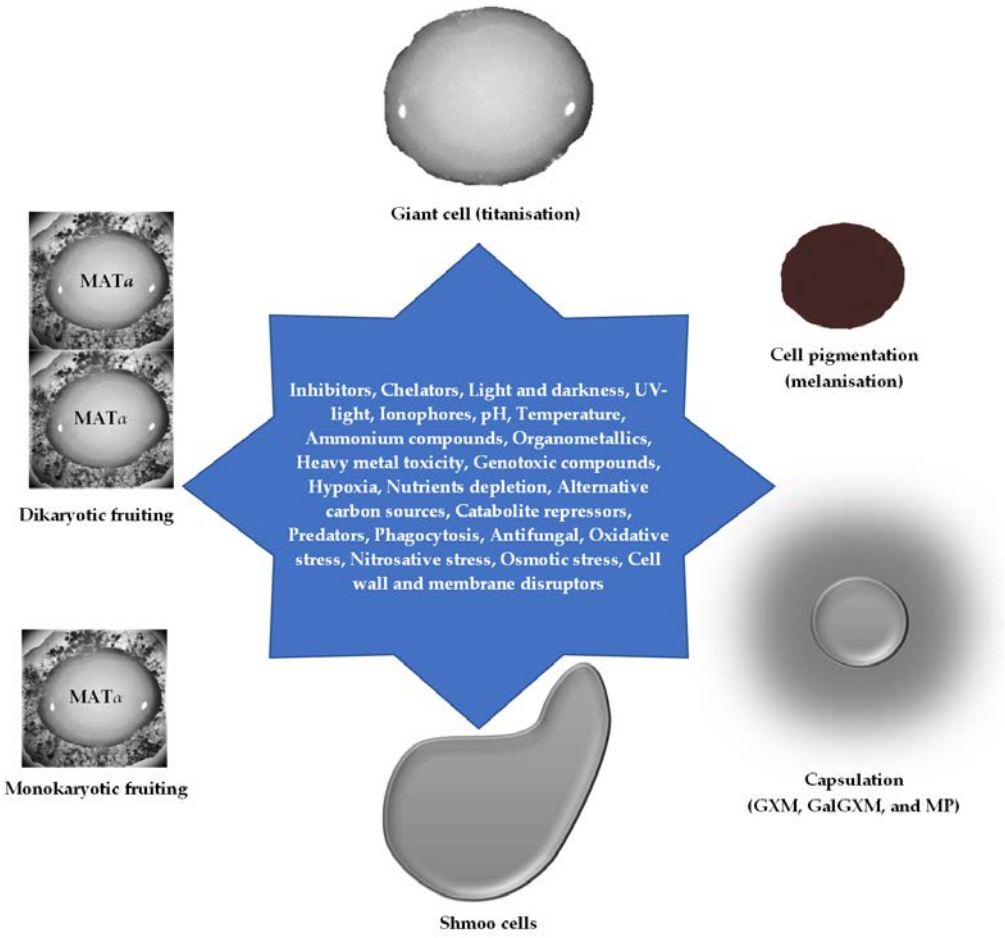

Figure 1. Exogenous activities that promote the expression and regulation of transcription factors in *C. neoformans* for adaptation, survival, mating, and pathogenesis. Many of these external factors include common environmental factors and interactions, as listed at the center of the figure above. These factors interact with the fungi cell wall, interpret by the specialised cell membrane proteins, and relay the message as signals via transcription factors to induce specific and non-specific genes in an attempt to subvert and resist invading factors. Overwhelming situations could force the fungi into some phenotypic cell shapes, including titanisation (relatively bigger cell size compared to the wild type), pigmentation (melanisation), capsulation (source of antigenic factors), and shmoo-like cell formation (unusual cell morphotype).

Furthermore, these capsular components are chemotactic, downregulating the surface expression of the TNFα receptor from the PMN. Though the expression of cell-adhe-

 doi:10.20944/preprints202301.0461.v1

sion molecules such as CD11b and CD15 integrins are stimulated in the presence of capsular components yet, tissue inflammation and granulocyte infiltration are inhibited [75]. In addition to reducing pro-inflammatory cytokines, GXM also promotes the uncoupling activity of NF-κB to regulate growth, apoptosis, and inflammation in macrophages [76]. More dangerously, macrophage proliferation can be altered in a capsule-independent manner to promote cell cycle disruption and chromosomal aberration, known as aneuploidy in the macrophage. This type of systemic alteration, which is hitherto commonly found in bacterial and viral infections, has been suggested as a way of promoting tumorigenesis in fungi infection [76].

This effect is very similar to chemoattractants, such as formylmethionylleucylphenylalanine (FMLP) and IL-8, which promote pro-inflammation when located in the extravascular space or inhibit inflammation when situated within the intravascular space. Together with IL-8, GXM stimulates the endogenous production of IL-10 and IL-1β from the monocytes, which down-regulates pro-inflammatory cytokines to block PMN infiltration into the site of tissue inflammation caused by *C. neoformans* infection [72,77]. Substantially, the systemic concentration of cryptococcal GXM in untreated infected patients ranges from 250 – 500 μg/mL [72]. This is high enough to downplay the critical roles of pro-inflammatory cytokines from the peripheral blood monocytes.

Furthermore, the immunocontrol ability of cryptococcal cells, which resides in the expression of capsular polysaccharides during infection, differs by serotypes. For example, the dynamic structural complexity of the cryptococcal GXM reduces from serotypes C, B, A, to D [78]; however, the capsular diameter reduces from serotypes D, A, C, to B. This feature conferred a higher immunomodulatory effect on serotype B GXM than other serotype capsular components. To support this, Fonseca et al. reported that serotype B GXM, which has the least molecular mass, was the most potent activator of immunocellular responses such as NO production from macrophages and expression of toll-like receptors following the activation and nucleus translocation of NF-κB. On the contrary, this induction was independent of the resultant polysaccharide negative charges of the GXM [79].

It has been shown that while *C. neoformans* var. *neoformans* (serotypes A and D) culture filtrate antigen (CneF), which is majorly capsular GXM, induced chemotaxis and chemokinesis responses of hPMN (human polymorphonuclear leukocyte), the var. *gattii* (serotypes B and C) failed to stimulate but inhibit the hPMN responses to the chemoattractants [80]. This, therefore, means that PMN infiltration may be higher in the var. *neoformans* (serotypes A and D) immunocompromised infected patients than the var. *gattii* (serotypes B and C) immunocompetent infected patients. This implies that while the cryptococcal antigenemia may be almost completely cleared from the immunocompromised infected patients due to higher leukocyte mobilisation bolstered with antifungals, the clearance level in the immunocompetent may be inefficient due to lower leukocyte infiltration, despite the body resistance. This may also explain, in part, why var. *neoformans* serotypes A and D prefer immunocompromised patients to immunocompetent individuals. It is reasonable to think that the more the cryptococcal secretomes stay in the body, the higher the tendency of antibody identification and attack, which may happen in immunocompetent patients, unlike immunocompromised patients where the fungi secretomes are quickly cleared due to higher leukocyte mobilisation.

Hog1 plays an insignificant role in capsule formation in serotype D, unlike serotype A, where a mutation in the *Hog1* gene enhances capsule formation [32]. Multiple mutated genes (*Δgpa1, Δcac1,* and *Δpka1)* in the *Δhog1* background mutant completely hindered capsule production, just like when each gene was mutated alone or doubly mutated with *Hog1* [32]. This shows that *Hog1* expression must have prevented certain cAMP/Pka upstream cascade events that facilitated capsule production. To this effect, *Δssk1, Δpbs2,* and *Δhog1* mutants have been characterised by hypercapsulation; however, *Δtco* mutant only showed *wt* capsule production [30]. In fact, the four major capsule-associated genes

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(*Cap10*, *Cap59*, *Cap60*, and *Cap64*) are significantly upregulated in *Δhog1* and *Δssk1* mutants more than the *wt* but not in the *Δskn7* mutant [81]. Because of the difficulty in deleting *Ypd1*, it is uncertain whether the transcription factor from this gene plays a role in capsule formation. Notwithstanding, Lee et al. observed an increased capsule volume of *Δhog1Δypd1* mutant similar to *Δhog1*, which is more significant than the *wt* [82].

D'Souza et al. discovered that while disruption of the *Pka1* gene led to sterility and failure to produce capsule and melanin in serotype A, mutation of *Pkr1* failed to overshadow the hypercapsulation and hypervirulent of the *wt* strain in an animal model [83]. This means that if cAMP is a potent inhibitor of Pkr1, then repressing/inducing the *Pkr1* gene, even supplying cAMP exogenously, will not correct the *Δpka1* defects. However, *Pkr1* exhibited an epistatic effect on the *Pka1* with a similar phenotypic appearance as the *Δpka1* mutant. Furthermore, this sterile and avirulent double mutated *Δpkr1Δpka1* strain showed that the catalytic subunit of Pka encoded by the *Pka1* gene is a downstream functional/catalytic protein of the regulatory subunit of Pka encoded by the *Pkr1* gene [83].

Mutation of *Gpa1* is characterised by a phenotypic defect in mating and reduced production of melanin and capsule due to impaired function of the G*α*-protein. However, the phenotypic features of attenuated G*α*-protein-deficient avirulent sterile strain were restored by exogenous cAMP supply [84]. This means that cAMP is a direct secondary messenger signalling factor to the Gpa1. Unlike acapsular and avirulent *Δgpa1* mutants, *Δpkr1Δgpa1* double mutant and *Δpkr1* mutant significantly produced larger capsule sizes with higher cell volume than the *wt* in the iron-limiting capsule-inducing media. This is due to the elevated and constitutive activation of Pka, with capsule size being larger in the doubly mutated strain, *Δpkr1Δgpa1*, than *Δpkr1* mutant [83,84]. Such *pkr1* mutation may be one of the unusual normal and natural transcriptional regulations in response to temperature to activate other more relevant factors. Indeed, some clinical isolates, characterised by enlarged cell volume, hypercapsulation, and melanisation, have been reported to infect immunocompetent individuals. These isolates were inexplicably larger in the infected tissues than in the *in vitro* culture [85,86]. Furthermore, mutation of the *pkr1* gene showed increased virulence, lethality, tissue burden, and survival of this mutant in animal studies compared to the isogenic/*wt* strains [83]. Analysis from other G-protein encoding genes showed that *Δgpb1*, *Δgpa2*, *Δgpa3*, *Δgpa2Δgpa3*, *Δgpg1*, *Δgpg2*, and *Δgpg1 Δgpg2* mutants displayed normal melanin and capsule formation and that all displayed normal/*wt* virulence but *Δgpa2Δgpa3* mutant exhibited attenuated virulence due to longer surviving period of the inoculated mice [87].

Serotype A *MATα Δste12α* mutant consistently produced less capsule when compared to the *wt* or reconstituted strain [29] because Ste12*α* protein is a Hog1 repressing factor that activates cAMP/Pka pathways for capsule and melanin production [32]. Ordinarily, the expression rate of virulence-associated genes such as *Cap59*, *Cap60*, *Cap64*, and *Lac1* – associated with melanin production are time-dependent, and their expression becomes conspicuous as glucose depletes. *Cap60* gene, for example, increased the capsule size by 2-fold compared to the *wt* at 6-hour incubation in the low-iron media (LIM) [88]. However, with serial analysis of gene expression (SAGE), the tagging of other capsule genes, *Cap10*, *Cap59*, and *Cap64*, was not as significant as *Cap60* irrespective of the iron level [88].

The stationary phase of *C. neoformans* will usually have a larger capsule [68] and higher melanin production [89] compared to the exponential phase when the glucose level is very high. In serotype D, *Δste12α* mutants are characterised with a low level of *Cap* and *Lac1* gene expression compared to the *wt* over the course of growth using a promoter-coupling reporter gene [27]. With galactose as an expression inducer, overexpression of Ste12*α* promotes *Cap* and *Lac1* gene expression in the *Δste12α* mutant better than the glucose. Unbelievably, by juxtaposing the relative effect of *Ste12α* when deleted or overexpressed, Chang et al. could not observe any significant difference in the capsule and melanin production when assessed on the agar culture [27]. This means that serotype D

 doi:10.20944/preprints202301.0461.v1

*MATα*Δ*ste12α* mutant may have encountered other environmental cues that facilitate alternative subways to subtly overshadow the phenotypic defects of deleting *Ste12α* when the mutant is cultured in agar media. In another way, deleting *Ste12α* may not be substantial enough to shut down the significant number of cooperative genes that facilitate virulence in *C. neoformans*, considering different environmental factors.

The *Cas* transcripts are homologs of *Cap64* confirmed to be involved in the positional linkages of xylose and *O*-acetylation in the mannan backbone. Among the putative homologues identified, *Cas3*, *Cas31*, *Cas32*, *Cas33*, *Cas34*, and *Cas35*, none was shown to be actively involved in the CO_2-mediated capsule formation like *Cap64*, but by speculation, they may be involved in capsule assembly and configuration, [90]. Though high CO_2 promotes capsule formation, no capsule was formed in the *Δcac1* mutant despite active *Cas* and *Can2* gene expression/overexpression because capsule production is CO_2-activated Cac1-dependent [91].

3.4. Melanin

The production of melanin is one of the critical environmental adaptive features of *C. neoformans*, and this is usually regulated by glucose and nitrogen catabolite repressions. Generally, low glucose induces melanin production in all cryptococcal strains, but nitrogen repression is strain-specific [89,92]. Through environmental sensing, *C. neoformans* can synthesise this heterologous and hydrophobic chromogenic polyphenol molecule within 4 – 48 hours under the influence of complexly regulated transcription factors using different exogenous molecules such as diphenolic/indole compounds [93], pigeon excreta [94], bacterial-presenting homogentisate [95], bacterial-presenting dopamine [96], catecholamine precursors (*L*-DOPA, dopamine, methyl-DOPA, epinephrine, norepinephrine, homovanillate, 5-hydroxylindolacetate, serotonin, catechol) [97,98], 1,8-dihydroxynaphthalene (DHN) [99], aminophenol and diaminobenzene compounds [92], and γ-glutaminyl-3,4-dihydroxybenzene (GDHB) [100]. In addition, other exogenous substrates have been well-characterised in different types of fungi [101]. These sources determined the eventual colour, chemical, and physical characteristics of melanin [102,103]; however, among the various reported colours – red, yellow, green, and purple; brown and black (eumelanin) are the major pigmentations [104].

This cell wall-associated biosynthetic process mostly involves the enzymatic conversion of exogenous catecholamine-intermediates into eumelanin [102]. Though melanin and capsular components are closely associated at the cell surface, evidence shows that the two components are independently regulated and differently shuttled to the cell wall. While melanin production is a cell surface synthesis, the components of the capsules are produced in the cytoplasm and transported in the vesicles for cell wall fusion; nevertheless, the heterologous nature of melanin particles can impose physical changes on the capsular size [102]. Notwithstanding, *C. neoformans* use molecular sorting mechanisms such as the endosomal sorting complex required for transport (ESCRT) to transport polysaccharides and conjugated molecules.

The Snf7p has been identified as a critical operator of ESCRT. In conjunction with other tagging chaperones and vacuolar proteins such as Vps20/25, Snf7p forms multivesicular bodies transported to the specific organelle, such as the plasma membrane, where the vesicular components can be directly fused or released extracellularly (exosome). Furthermore, ESCRTs have been implicated in other signalling events, such as the Rim101 pathway during the pH-sensing by the fungi [105]. The Vps20/25-Snf7 complex forms a bridge connecting the endosomal ESCRTs I, II, and III to the Rim20/13/101.

As a sequel to this, Godinho et al. mutated *Snf7* and discovered impaired molecular trafficking of capsular and melanin components, and as such, the mutant displayed impaired polysaccharide secretion, capsule, and melanin formation, especially at physiological temperature with a complete loss of virulence in an intranasal model of murine cryptococcosis [106]. In addition, the depigmentation of the var. *gattii* appeared more intense in this mutant than the var. *neoformans* as temperature progressed from 30 to 37°C in either

 doi:10.20944/preprints202301.0461.v1

Niger or *L*-DOPA agar [106]. Furthermore, this mutant showed defective growth at pH > 7.5 and in the presence of $LiCl_2$ solution with concomitant reduction of *Rim101* expression; however, there is no significant effect on the phospholipase B (PLB) and urease activities [106].

C. neoformans produce melanin to protect and elongate propagules life span [107] and to survive antifungal/antimicrobial effects [108,109], oxidants [110], UV-light, engulfment/phagocytosis (camouflage) [98,111], heat (42 – 47°C)/cold (-20°C) [112], chelators/heavy metals [113,114], acid hydrolysis [102,115], and fungal cell wall denaturants [116]; however, melanised *C. neoformans* are susceptible to melanin-binding fungicidal like trifluoperazine [117]. Very importantly, melanin can sequestrate microbicidal peptides and reactive oxygen species generated by the macrophages against the internalised/phagocytosed *C. neoformans*, thereby promoting survival and pathogenesis [118].

Interestingly, *C. neoformans* strategically refused to cluster the Cu/Zn-Sod (encoded by the *Sod1* gene) together with melanin produced from laccase activity (encoded by *Lac1* and *Lac2* genes). The two components are actively involved in oxidative stress response and are induced by exogenous copper, iron, and calcium but are repressed by glucose, nitrogen, and high temperature [73,119]. While Sod1p is restricted to the membrane lipid raft (enriched with sphingolipid and ergosterol), laccase is shuttled to the cell wall, where melanin is produced [120]. Therefore, melanin performs the first-line defence against the cell wall oxidants, while Sod1p is positioned as a second-line defence against the cell membrane oxidants.

Under the glucose-repressed *Lac1*/*Lac2* transcription regulatory factors, *C. neoformans* expresses an iron-containing laccase enzyme similar to the copper-containing phenol/diphenol oxidase [121,122]. This enzyme is an analogue of tyrosinase in higher animals, which converts various exogenous phenolic substrates to pigmented compound, melanin, via auto-polymerization of quinine-like molecules [89,121]. The *Lac1* gene seems more vital than the *Lac2* transcript in laccase production [123]. The *Lac1* gene is expressed as laccase, while the second laccase (75% homology) is produced from the adjacent *Lac2* gene [123]. Four major transcription factors, Bzp4, Usv101, Mbs1, and Hob1, have been identified to be involved in the induction and regulation of the *Lac1* gene [124]. Low glucose level facilitates cytoplasm-nucleus trans-localisation of Bzp4 and Usv101; and, together with the residential nuclear transcription factor, Mbs1, initiate the transcription of the *Lac1* gene [124]. Conversely, Hog1 provides a repressive regulation of *Bzp4* and *Lac1* to coordinate melanin production. The *Lac1* expression was particularly shown to be upregulated in *Δssk1*, *Δhog1*, and *Δskn7* mutants [81]. In the early investigation, *Snf5* and *Mbf1* have been implicated in regulating laccase transcription, poor growth in non-glucose media, bilateral sterility, and mating defect [125] (for details on the phenotypic display of different cryptococcal cell mutants, check **Supplementary 1**). Also, because melanin production is anchored by the cell wall chitin and governed by the cytoplasmic ionic homeostasis, *Chs3*, *Ccc2*, and *Atx1* have been further identified as important transcripts for melanisation [125].

Two oppositely regulated signalling pathways govern melanin production – cAMP/Pka and Hog1 [32,84]. Low glucose concentration activates cAMP/Pka-dependent pathways while repressing the Hog1 signalling pathways. This crosstalk significantly induced the *Lac1* gene for melanin production. In serotype A, deletion of the *Hog1* gene effectively restored melanin production in hitherto non-melanised *Δgpa1*, *Δcac1*, or *Δpka1* mutant within 2 days in 0.1% glucose media. However, *Hog1* seemed not to have a significant effect on the melanin production in serotype D because mutation of the *Hog1* gene failed to restore melanin production in *Δpka2* mutant [32]. Because of the close association of Hog1 with the cAMP/Pka signalling cascade, it can be deduced that *Hog1* gene expression may have negatively hindered Pka downstream activity in melanin production. From the two-component system of the Hog1 pathway, deletion of *Tco* genes did not contribute significantly to melanisation; however, *Δtco1* or *Δtco1Δtoc2* mutant showed enhanced

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melanisation at 37°C even in up to 2% glucose media, and this melanin content was considerably higher than the *Δhog1* mutant [30]. This shows that Tco1 kinase is a key repressor of *Lac1* expression, just like Ssk1, Pbs2, Hog1, and Skn7 proteins but complementing the *Δtco1* mutant (*Δtco1::Tco1*) reduced the melanin to the *wt* level.

The Ypd1 is also a component of the Hog1 pathway, which regulates melanin and capsule formation. At 0.1% glucose, *Δypd1Δhog1* mutant produced a highly significant level of melanin just like *Δhog1*, *Δssk1* and *Δskn7* mutants at 30°C, and at 37°C, only the *Δypd1Δhog1* and *Δskn7* retained the melanin [82]. Similarly, Bahn et al. reported comparable melanin formation in the *wt*, *Δhog1*, *Δpbs2*, *Δssk1*, and *Δskn7* mutants at 37°C [30]. In 1.0% glucose medium, *Δssk1*, *Δhog1*, and *Δskn7* mutants melanised as *wt*, but all mutants, including the *wt*, failed to retain this melanin at 37°C. Surprisingly, the deletion of *Ypd1* represses melanin formation in the *Δhog1* background mutant irrespective of the temperature [82]. Contrary to this, *Δhog1*, *Δpbs2*, *Δssk1*, and *Δskn7* mutants still retained pigmentation in 1.0% glucose media at 37°C while melanin formation had already been lost in the *wt* and reconstituted *Δssk1*. Yet, in 2% glucose media, pigmentations are still found in these same set of mutants with significant melanisation in the *Δskn7* than the rest of the mutants [30]. Because of the regulatory effect of Hog1p on the expression of *Mbs1*, then Song et al. showed significant melanin and capsule defect in *Δcac1* compared to *Δmbs1* mutants at 37°C, notwithstanding *Δmbs1* mutant still showed a reduced virulence with low degree tissue fungal burden and titanisation compared to the *wt* or complemented *Δmbs1* mutant (*Δmbs1::Mbs1*) [126].

While it is reasonable to summate that increasing cAMP production would elevate melanin synthesis, D'Souza et al. showed that high levels of cAMP led to overexpression of Pka1 and repression of melanin production as compared to the *wt* [83]. However, capsule production appeared highly induced [83]. This means that capsule production may be more critical to virulence than melanin. Though the same upstream regulatory factors may control melanin and capsule productions, different transcription factors are involved in the terminal responses. Evidence exists that melanin is produced in the infected tissues but not as much as produced *in vitro;* nonetheless, the fact that larger capsules have been produced in the infected tissues than culture media means that capsules are more involved in the *C. neoformans in vivo* virulence and pathogenesis than melanin. Notwithstanding, an appreciable level of melanin is necessary for effective virulence, tissue invasion, antifungal resistance, and macrophage survival within the infected tissues [127,128].

Ironically, the presence of melanin in the infected tissues has been proposed with uncertainty. Liu et al. discovered that laccase-dependent catecholamine oxidative products such as pyrrole-2,3,5-tricarboxylic and pyrrole-2,3-dicarboxylic acids rather than melanin might be produced in the mouse brain during infection to induce oxidative cytotoxic effects within the infected tissue [129]. The accrued evidence came from the work of Ito et al. that dopamine-*o*-quinone is one of the reactive catecholamine oxidative intermediates, which are formed during the synthesis of melanin, and this intermediate strongly attacks the sulfhydryl groups of protein cysteine [130] and can be further oxidised to pyrrole acids in the presence of alkaline peroxide [131,132]. Therefore, this contrary discovery means that if melanin is actually produced during pathogenesis, then it may be stringently controlled to induce moderate pigmentation against oxidative damage. At the same time, most laccase products are diverted into making heterologous quinone-like derivatives, which are cytotoxic catecholamine oxidative intermediate products.

Mutation of the *Pka1* gene in *C. neoformans* usually leads to sterility and avirulent because the strain failed to produce melanin and capsules; however, overexpression of Ste12α in the *Δpka1* mutant restored the mating but was unable to restore the virulence of *Δpka1* mutant [83]. Contrarily, the mutation of the *Pkr1* gene, encoding the regulatory Pka subunit protein, failed to significantly affect the melanin production in serotype A *MATα*, unlike the mutation of *Gpa1*. Interestingly, it was shown that the *Δpkr1Δgpa1* double mutant produced a comparable level of melanin to the *wt* better than the *Δgpa1* mutant [83].

 doi:10.20944/preprints202301.0461.v1

This indicates that melanin and capsule production is under the multifactorial transcription factors downstream of the Pka protein. Furthermore, mutation of the *Crg1* gene has been shown to enhance melanin production in the *MATα* strain, which depends on the *Ste12α* expression. This mutation directly increased the virulence in the animal study in a Cpk1-independent manner [133].

Luberto et al. exposed the vital role of the *Ipc1*/*Aur1* gene encoding inositol-phosphorylceramide synthase 1, which catalyses the formation of membrane-associated complex sphingolipids called inositol-phosphorylceramide (IPC) and diacylglycerol (DAG) from phytoceramides and phosphatidylinositol (PI) in fungi. The DAG is an essential second messenger of mitogen and protein kinase *c* (Pkc) activation. Under P_{gal7}-regulated inducible expression, it was shown that while the glucose-repressing condition reduced melanin production by 60%, the galactose-inducing condition increased melanin production by 80% [134]. Furthermore, this repressive condition impaired the virulence, survival rate, *in vivo* growth, replication, and cellular diffusion of infectious strain H99 in immunocompromised animal and macrophage-like cell line models [134]. Again, because of the increased melanin production in the *hog1* deleted background mutant, Ko et al. discovered that upregulation of *Ipc1* in the *Δssk1* and *Δhog1* mutants might synergistically enhance melanin production in *C. neoformans* [81].

3.5. Heterokaryotic Mating/Conjugation, Filamentation, and Sporulation/Haploid Fruiting

Like melanin and capsule formation in serotype D, Hog1 is less involved in mating in serotype D. Mutation in the *Hog1* gene enhances mating filamentation and cell fusion in serotype A. Mating formations are strongly induced because mutation of the *Hog1* gene re-activates yeast Ste4 analogue Gpb1 MAPK pathway to produce more pheromones from the induction of *Mfα1* gene hence mutation of *Hog1* gene will not improve mating defect in *Δgpb1* mutant unlike *Δras1*, *Δgpa1*, *Δcac1*, and *Δpka1* mutants [32]. Notwithstanding, the *Δras1* mutants displayed defective mating, reduced *in vivo* viability and virulence with growth defect at 37°C [135]. The appearance of prototrophic progeny from unilateral mating of the two complementing auxotrophic mutants showed that Ras1 is required for cell fusion and maintenance of heterokaryotic/diploid cell formation [135]. The replacement of the H99 *wt Ras1* gene background with a site-specific mutated ***ras1***^{Q67L} tagged as the dominant gene evidently induced excessive haploid filamentation after 4 weeks of incubation at 25°C [135]. So, besides controlling cell fusion and dikaryotic cell formation, Ras1 also promotes haploid fruiting filamentation under nutrient starvation.

The second Ras protein, Ras2p, is expressed at a low level, and its absence caused no defect in filamentation, cell differentiation, and virulence factor production [136]. Furthermore, the double mutant *Δras1Δras2* strain exhibited poorer temperature-dependent growth than either of the single mutant, and the overexpression of Ras2 entirely suppressed the mating defect but partially suppressed the actin polarisation defect in the budding of *Δras1* mutant at higher temperature [136]. The observation that defects in mating, filamentation, basidia, and basidiospores formation in *Δras1* mutant can be partially suppressed by exogenous cAMP or fully suppressed by the overexpression of Gpb1 (a pheromone-sensing MAPK signalling element) under the control of serotype D P_{gal7} in glucose/galactose enriched media showed that Ras protein functions upstream of pheromone-response MAPK signalling pathway and this is the reason Ras1 activation will not suppress the mating defects in *Δgpa1* and *Δgpb1* mutants. Ras1 coordinates the downstream activation of Gpb1 and Ste12*α* to regulate haploid fruiting, but then, constitutive activation of Ras1p will not induce filamentation/haploid fruiting during starvation in *Δgpb1* and *Δste12α* mutants except if each of these genes is re-introduced [135]. The fact that cAMP and Gpb1 failed to suppress the growth defect at 37°C in *Δras1* mutant showed that Ras1 regulates the vegetative growth of *C. neoformans* at 37°C in a separate pathway independent of cAMP, osmotic-rescue sorbitol, and MAPK pheromone sensor [135].

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Reconstituting *Δras1* mutant (*Δras1::Ras1*) restored the virulence and induced the haploid fruiting (cell differentiation), filamentation, agar invasion, and sporulation in nitrogen-limited media. Selectively, exogenous cAMP that restored mating in the *Δgpa1* mutant only partially suppressed mating in the *Δras1* mutant and restored agar adherence as well in this mutant (agar adherence by cAMP-dependent pathway). However, because the dominant ***ras1***^{Q67L} allele induced a mixture of yeast and hyphae filament at 25°C, only a mutant of this dominant allele could adhere, invade, and retain the invasion in the presence/absence of cAMP (cAMP-independent pathway) [135]. This shows that cAMP, the primary target of Gpa1, is an auxiliary downstream effector regulator of Ras1 function because *Δras1* mutant apparently showed no significant defect in melanin and capsule production at 30°C in an iron-limiting media when compared to the *wt*. After all, evidence shows a parallel expression pattern of *ras*-dependent and cAMP-dependent genes in a DNA microarray analysis [135,137].

C. neoformans produced G proteins in the form of 3G*α* (Gpa), 1G*β* (Gpb), and 2G*γ* (Gpg), which are needed for pheromone production, mating, and virulence. There are two putative mating-specific-pheromone-receptor encoding genes in *C. neoformans* – *Cpr1* and *Cpr2*, which are homologues of yeast *Ste3α*/***a***. During the pheromone-sensing event, pheromone-activated receptor Cpr1*α* interacts with Gpa2 or Gpa3, and the Gpa2 interacts with the heterotrimer G protein (Gpb1-Gpg1/Gpg2) and Rgs1 domain of Crg1. However, coupling Gpa3 to Crg1, which does occur during pheromone induction, may not be via Gpb1 [87]. Though all the G proteins interact with Ste3, Gpb1, and Crg1 *in vitro*, only Gpa2 has a strong *in vivo* interaction. Crg1, on the other hand, can act as a pheromone desensitiser via Rgs protein to disengage Gpa2 or Gpa3, thereby inhibiting mating/cell fusion. The Gpa2 GAP activity is greatly enhanced by Crg1 for conserved active involvement in pheromone response and mating. No observable pheromone response when the *MATα wt*, *Δgpa2*, *Δgpa3* or *Δgpa2Δgpa3* mutant was crossed with *MAT****a*** *Δcrg1* mutant; however, shmoos cells with conjugation tubes were observed in the *MAT****a*** *Δcrg1* mutant crossed with *MATα Δgpa3* or *Δgpa2Δgpa3* mutant. This shows that the latter mutants produced pheromones, and the significantly high pheromone production by the *MATα Δgpa2Δgpa3* is similar to *MATα Δcrg1* mutant that stimulated a large number of shmoos cells [87]. Further analysis showed the importance of Gpa3 in pheromone response (conjugation tube formation), while Gpa2 is important in pheromone production, reaffirming the coupling of Gpa2 to Gpb1. Unlike the *MATα* strain, the phenotypic changes accompanying the deletion of *Gpa* in the *MAT****a*** strain are less conspicuous; however, this may affect the conjugation tube stimulated from the *MATα Δcrg1* when crossed with *MAT****a*** *Δgpa2*, *Δgpa3*, *Δgpa2Δgpa3*, and the *wt*. Nevertheless, in general, the conjugation tube induced in the *Δcrg1* mutant, whether *MATα* or *MAT****a***, is rationally higher when crossed with either *MATα Δgpa2Δgpa3* or *MAT****a*** *Δgpa2Δgpa3* mutant.

The significant regulatory contribution of Gpa2 and Gpa3 to mating, dikaryotic filamentation, basidia, and basidiospores increased considerably compared to the Gpa1. Therefore, mating mutant, either *MATα* or *MAT****a***, with the deletion of *Gpa1*, significantly increased mating with the *wt MAT****a*** or *MATα*, respectively. However, unilateral mating seemed to be attenuated with MATα/***a*** of *Δgpa2Δgpa3* x *MAT****a***/*α* of the *wt* respectively but completely abrogated in bilateral mating *MATα Δgpa2Δgpa3* x *MAT****a*** *Δgpa2Δgpa3* [87]. On the other hand, the unilateral mating of *MATα Δcrg1Δgpa2* or *Δcrg1Δgpa3* with the *wt MAT****a*** is also defective, unlike the unilateral mating of *MATα Δcrg1* with the *wt*. There is an *in vitro* interaction of Gpg1 and Gpg2 with Gpb1 to exercise pheromone response. No pheromone response was detected in the *MATα Δgpg1*, *Δgpg2*, and *Δgpg1Δgpg2* mutants when crossed with *MAT****a*** *Δcrg1* mutant; however, the formation of conjugation tubes appeared more promising on the *MATα Δcrg1* mutant when crossed with *MAT****a*** *Δgpg1* and *Δgpg2* [87]. In addition, unilateral mating of *MATα*/***a*** of *Δgpg1* was attenuated while *MATα*/***a*** of *Δgpg2* and *MATα Δgpg1Δgpg2* were utterly sterile, just like *Δgpb1*.

In addition, the pheromone-responsive Cpk1 MAPK inherently enhances mating in the *MATα Δcrg1* mutant [133] because Crg1 is a negative regulator of mating and when

 doi:10.20944/preprints202301.0461.v1

studied in a confrontational assay with *MAT**a*** *Δssk1*, *Δpbs1*, and *Δhog1* mutants produced more pheromones than the *wt*, but with *Δskn7* mutant, no filament was induced [30]. Similarly, bilateral mating between the *MATα* and *MAT**a*** mutants of *Δssk1*, *Δpbs2*, *Δhog1*, and *Δskn7* was significantly enhanced in V8 media at pH 5.0 incubated at 25°C in the dark and even when co-cultured with *Δcac1* mutant [30]. This shows that transcription factors of the Hog1 pathway are potent repressors of mating pheromones.

Confrontational assays are best done in the dark because light induces some mating repressing genes such as *Crk1*. *C. neoformans* senses light via the putative membrane photoreceptor, Cwc1p (a blue light chromophore-binding protein) that conjugates with another transcription factor, Cwc2p, to form an oligomeric protein that migrates to the nucleus to induce various light-dependent transcription factors that majorly suppressed pheromone production and filamentation. The Crk1, for example, is a protein kinase that is induced in the light to suppress Mat2, Znf2, and Sxi1*α* that are involved in bisexual mating; however, its effect in monokaryotic fruiting is ambiguous because unisexual mating remained attenuated in the *Δcrk1* mutant and even when overexpressed [138].

Though the G-protein *β*-subunit (Gpb1) and MAP kinase (Cpk1) are not mating-strain specific factors [139,140], unlike Sxi1*α*, Ste11*α*/***a***, Ste12*α*/***a***, Ste20*α*/***a***, and Mf*α*/***a***, which are mating-type specific [26,141] (check **Supplementary 1** for mating-type specific factors) but mutation of *Gpb1* completely abrogated mating while its overexpression induced conjugation tubes and cell fusion through the regulation of pheromones secretion via the upstream regulation of Cpk1 MAPK cascade event irrespective of the mating-type. This regulation is, however, independent of the Ste12*α* factor [140] but highly dependent on Ste20*α* expression [142]. In fact, overexpression of the G protein *β*-subunit Gpb1 could not rescue the mating defect of *Δste20α* [142-144].

Furthermore, Ste50p is a vital adaptor protein for Ste11 autophosphorylation to regulate pheromone-dependent Cpk1 MAPK sexual reproduction in any serotype of *Cryptococcus* and other yeasts. However, unlike other yeasts, Ste50 expression is not involved in any stress response, titan cell formation, and virulence factors as controlled by Hog, Ras, and cAMP/Pka cascade pathways in *C. neoformans* [145]. Therefore, it is not surprising to observe cell fusion and pheromone-inducing mating defects in *Δste50* mutant due to the repression of the *Mfα1* pheromone gene. Not only this but even the mating enhancing *Δcrg1* mutant also showed severe impairment in pheromone production and filamentation when *Ste50* (the same for *Aca1*) is deleted in this background mutant [145]. This means that neither *MATα Δste50Δcrg1* nor MAT***a*** *Δcrg1* mutant will form any conjugation tube when confronted.

In the same way, none of *MAT**a*** *Δste50Δcrg1* and *MATα Δcrg1* mutants will form any conjugation tube. Therefore, in the pheromone-response MAPK pathway, Ste50 appears to be a downstream functional protein to Crg1, while Aca1 acts as an upstream regulatory protein (check mitogen activation pathways in **Supplementary 2**). In addition, the deletion of *Ste50* fails to affect pheromone-independent enhanced melanin production from *Δcrg1* mutants.

The Gpb1 is another pheromone-sensing transcription factor regulating mating and haploid/monokaryotic fruiting via the Cpk1 MAPK signalling cascade. The Gpa1 senses the nutrients to regulate mating, filamentation, and virulence via the cAMP signalling cascade. No wonder the pre-suppose phenotypic defects of *Δgpa1* mutant are suppressed by cAMP cascade events [84]; however, virulence expression (melanin and capsule production) is independent of the Gpb1 factor [140]. Contrarily, the G*α*-Gpa1-cAMP regulatory cascade cannot suppress the mating defect incurred from *gpb1* mutation. This indicates the separate role of Gpa1 and Gpb1 in *C. neoformans.* Furthermore, overexpression of the G*β*-subunit, which associates with the *γ*-subunit of Gpb1, enhances conjugation tube formation in *MATα* and *MAT**a*** mating strains [139]. This confirmed that the G*βγ*-complex subunit of the Gpb1 protein constitutes an important activation factor in mating, but this may probably be antagonised by the G*α*-subunit of the Gpb1.

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Lengeler et al. overexpressed *Gpb1* and *Ste12α* in a serotype A *MAT**a*** and discovered that Ste12α induced filament formation while Gpb1 failed to induce filamentation [26]. This shows that serotype A *MAT**a*** might probably be a sterile mating strain. Nevertheless, the existence of diploid/aneuploid hybrid serotype AD might have demonstrated that fertile serotype A *MATα* with *MAT**a*** mating type may naturally exist, albeit with a very low mating efficiency. This is more evidenced because analysis showed that the hybrid AD is heterologous with *MATα* and *MAT**a*** loci derived from parent serotypes D and A, respectively [146]. Naturally, mating still occurs in serotype D *MATα* and *MAT**a*** mating types; however, most isolated serotype A, whether clinical/environmental, are *MATα*. Being the most prevalent aetiology of cryptococcosis, virulent serotype A has been proposed as an asexual propagule because the serotype A *MATα* can mate with serotype D *MAT**a*** to produce serotype AD; however, the rare serotype A *MAT**a*** cannot cross-fertile with serotypes A and D *MATα* mating-strain. In fact, Lengeler et al. could not recover any fertile serotype A *MAT**a*** mating strain even from the self-fertilised serotype AD basidiospores [146].

In another scenario, overexpression of Ste12α could not rescue the mating defect in *Δgpb1* mutant [29] in the same way overexpression of Gpb1 could not rescue the mating defect in *Δste20α* [142]. However, overexpression of MAP kinase Cpk1 or Ste11α restored the mating defect not only in the *Δste20α* but also in the *Δpak1* mutants [142]. Hierarchically, the flow of MAP kinase activation proceeds from the inducing activity of Gpb1 and the terminal activity of Ste12, Gpb1→Ste20α→Ste11α→Cpk1→Mat2→Ste12 (check mitogen activation pathways in **Supplementary 2**). This explains why overexpression of Cpk1 or Ste11α can rescue the mating defect in *Δste20α* mutant because they are functional downstream PAK kinases to Ste20α; however, monokaryotic fruiting defect in *MATα Δste20* and *Δpak1* mutants cannot be rescued by the overexpression of either Cpk1 or Ste11α MAP kinase but Ste12α [142]. This shows that Ste12 is a multifunctional transcription factor robust enough to circumvent the mating roles of Ste20 and Pak1 and relay the information from the pheromone-sensing factor Gpb1 to the MAP kinase Cpk1 to promote mating and monokaryotic fruiting.

Further works showed that "Ste" transcription factors were majorly involved in MAP kinase signalling events to regulate haploid fruiting and filamentation on the filament agar but not primarily engaged in mating and virulence [28,29]. Contrarily, Chang et al. showed that serotype D *Δste12α* mutant was marked with a lower virulence as compared to the *wt;* however, overexpression of this gene enhances hyphal projection with fertile basidiospores in serotype D *MATα Δste12α* when co-cultured with *MAT**a*** on a V-8 juice agar [27]. This mating expression displayed by the *Δste12α* progeny is similar to the *wt* serotype D *MATα* X *MAT**a*** mating progeny but with significantly reduced viable basidia [27]. Nevertheless, the deletion of *Ste12α* seemed not to impede hyphal formation and mating in the serotype D strain, but haploid fruiting is usually impaired, just as found in serotype A.

cAMP orchestrates the activation of Pka1 to regulate mating and filamentation. Generally, *Δgpa1* and *Δpka1* mutants are sterile and failed to produce basidia/basidiospores when co-cultured with serotype D *MAT**a*** strain; however, exogenous cAMP restored the mating defect in the *Δgpa1* just like how the virulence would have been restored. Although the Pka is a downstream functional protein to cAMP, exogenous cAMP could not restore mating defect in *Δpka1* [83]. Notwithstanding, because Pka targets *Ste12α* expression, among others, to induce filamentation, then overexpression of *Ste12α* was shown to restore mating and dikaryotic/haploid filamentation with preponderant basidiospores in serotype A *MATα Δpka1* mutant co-cultured with serotype D *MAT**a***. Emphatically, the interplay of Pka1 and Ste12α to initiate filament differentiation is well characterised in the nitrogen-limiting filamentation media. Compared to the wt, the overexpression of Ste12α in *Δpka1* mutant produced elongated filaments in the nitrogen-limiting filamentation liquid culture that failed to form basidia/basidiospores; however, overexpression of Ste12α in the *wt* had less elongation but formed basidia [83]. Thus, this filament differentiation may, in addition to the Pka1, require further environmental cues to produce basidia. On

 doi:10.20944/preprints202301.0461.v1

this note, the acquisition of iron from the iron-rich medium may pose ionic stress in *C. neoformans*, which induces several genes encoding calcium-calmodulin-calcineurin signalling components to facilitate cell wall integrity, growth at 37°C, mating, haploid fruiting, and virulence [88,147].

The regulatory (R) and catalytic (C) subunits of Pka are sequential and functional proteins downstream of G*α*-Gpa1-cAMP-Pka(R)-Pka(C) cascade events to promote differentiation and virulence in *C. neoformans*. Mutation of *Pkr1* showed no effect on the mating in the *wt* strain but restored mating in otherwise non-mating serotype A *MATα Δgpa1* mutant when co-culture with the serotype D *MAT**a*** mating strain; however, *Δpkr1Δpka1* double mutant failed to restore mating [83]. This means that Pka(C) is the last functional protein in this cascade event through which other regulatory factors will be activated for mating and virulence production, including the regulatory feedback inhibition of cAMP production. Literarily, exogenous glucose increases cAMP production, which inhibits *Pkr1* expression to activate Pka. However, at the threshold level, Pka inhibits further production of cAMP to keep the intracellular level of this second messenger. Thus, mutated *pka1* strains accumulated cAMP production in the glucose-rich media, but the *Δpkr1* mutant negated the overproduction of intracellular cAMP to prove that the Pka autoregulates cAMP production, possibly by activating the phosphodiesterase, Pde1 [83], to hydrolyse cAMP to AMP [148].

The Tco transcription factors are expressed as multiple regulatory sensor kinases such as Tco1, Tco2, Tco3, Tco4, Tco5, and Tco7. These sensor kinases control the response regulators such as Ssk1 (an upstream signalling component modulating the Pbs2-Hog1 MAPK-dependent phenotypes) and Skn7 (a Pbs2-Hog1 MAPK-independent regulator) [149]. Among the Tco kinases, Tco1 and Tco2 are the most significant kinases. Surprisingly, there is increasing evidence of the regulatory effect of Tco kinases in mating. The majority of the *Δtco* mutants displayed normal mating like the *wt*. However, mating was found completely defective in the bilateral mutant crossing of *Δtco1 (Δtco1 MATα* x *Δtco1 MAT**a**)*, and a reduced mating was also observed with a unilateral mutant crossing of *Δtco1 (Δtco1 MATα* x *MAT**a**)* [30]. Further investigation showed a relatively efficient dikaryotic cell fusion in bilateral mutant crossing, though with heterologous sizes and uneven filamentation compared to the *wt* or unilateral mutant crossing [30]. This means that Tco1 is highly essential for cell viability and dikaryotic stability but not necessarily for cell fusion. Mating is also enhanced in the *Δhog1*, *Δpbs2*, and *Δssk1* mutants, making them negative modulators of mating.

C. neoformans prefer mating in an ambient environment with about 0.036% CO_2 because higher CO_2 (4 – 10%) naturally inhibits mating in cryptococcal cells by preventing the expression of *Mfα1*; however, deletion of the *Can2* gene rescues this mating inhibition [150]. Similar to *Can2* gene deletion, mating is highly promoted in *Δhog1* and *Δcrg1* mutants in the ambient environment but less efficiently, and deletion of the *Hog1* gene partially restores mating in the presence of high CO_2 while *Δcrg1* mutant failed to engage in bilateral mating in the presence of high CO_2 [32,150].

C. neoformans deploy carbonic anhydrase (encoded by *Can* genes) to harness the CO_2 as HCO_3^- and H^+ in the presence of water molecule, and the HCO_3^- are used in regulating other transcription factors such as Cac1 or metabolic reaction involving carboxylation such as lipogenesis [150]. Similar to serotype A *MATα* x *MAT**a***, high CO_2 disrupted the bilateral mating of *MATα Δcan1* x *MAT**a** Δcan1* and unilateral mating of *MATα Δcan2* x *MAT**a*** but not in the bilateral mating of *MATα Δcan2* x *MAT**a** Δcan2* [150]. Despite the good state of the dikaryotic cells in the *MATα Δcan2* x *MAT**a** Δcan2* crossing filaments, the basidia and basidiospore formations are highly impaired, which means that Can2 is highly important for sporulation. Being the primary encoding transcript, accumulation of HCO_3^- by *Can2* expression caused by the high level of CO_2 was proposed as the basis of mating inhibition but not the H^+. So, the absence of Can2 drastically reduces the cytoplasmic HCO_3^- to the level only generated nonenzymatically, probably via aquaporin/water channel (encoded by *Aqp1*/*Aqy* gene) hydration of the diffused CO_2, and this can support

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vegetative growth and promote mating in the bilateral crossing. Further analysis showed that high CO_2 arrests the cell fusion in the *wt* while a normal cell fusion was displayed in the *Δcan2* but with impaired sporulation [150]. Just as high CO_2 rescues growth and mating defects in *Δcan2* and *Δcan1Δcan2* mutants, the same way exogenous cAMP (≈1.0 mM) rescued the mating defects in *Δgpa1*, *Δaca1*, and *Δcac1* mutants; and again, as accumulated HCO_3^- disrupted cell fusion in the *wt* or basidia formation in the *Δcan2* mutant, the same way the excessive supply of exogenous cAMP (10 mM) will arrest the cell fusion [150]. So, it is not unusual to predict the inevitable involvement of a Can2-mediated HCO_3^- dependent Cac1p activation for basidia, basidiospore, and mature sporulation in *C. neoformans* in the same way it was discovered in *C. albicans* [151].

On the contrary, the effect of CO_2-sensing linking to pheromone production is less pronounced in serotype D mating compared to serotype A. Pheromone and mating defective serotype D mutants such as *Δste11*, *Δste7*, and *Δcpk1* highly exhibited defective unilateral mating and pheromone production in low and high CO_2 levels [150]. Thus, mating could be pheromone-dependent like *MATα-MAT**a*** bisexual development and pheromone-independent like *MATα* unisexual development. Mat2 is a high-mobility group (HMG)-box homologue of Prf1, recognising various PREs to promote bisexual reproduction of the fungi. It is a negative regulator of unisexual development, and its deletion favours hyphal formation and monokaryotic filamentation under the regulation of Znf2 and Cna1/Cnb1 [152]. This condition is favoured by high copper, calcium, and temperature at 37°C but not magnesium, zinc, and iron. The presence of FK506 (Cna1/Cnb1 inhibitor) and bathocuproine disulfonate (BCS) (copper chelator) will prevent hyphal formation.

Transposable genomes and transgene repeats do cause genomic instability and mutation during meiosis; hence *C. neoformans* employs RNA*i* silencing pathways using a quelling model to defend the genome against these transposons. Among the conserved regulatory genes for this purpose are *Rdp1*, *Ago1*, *Dcr1*, *Znf3*, *Qip1*, *Cpr2*, and *Fzc28*. All these transcription factors favour sex-induced pheromone-dependent and mitosis-induced silencing except Cpr2 and Fzc28, which are not involved in yeast-hyphae formation during mitosis-induced silencing [153]. Transposon interacts with the DNA template during replication as a tandem to induce RNA*i*, which begins with the deployment of Qde3 to resolve the secondary structure of the tandem, promote the transcription, and enable the formation of *ds*-genome by the Qde1. The duplex genome, made up of *passenger* and *guide* strands, is shortened to 21 – 25 nucleotides *si*RNA by the redundant dicer activity of RNase III encoded by the *Dcl1* and *Dcl2* genes [154]. As a result, the *passenger* strand is nicked and degraded by the *slicer* and Qip activities of Qde2.

In contrast, the guide strand, by complementary pairing, identifies other homologous *m*RNA and targets them for Qde2-dependent sequence-specific degradation [155]. This degradation is orchestrated by the binding of Ago1p to the *si*RNA duplex to activate the RNA-induced silencing complex (RISC) [156]. By so doing, *C. neoformans* maintain genomic integrity during sexual development and yeast-hyphae formation (vegetative growth) at the post-transcriptional level and exclude the interfering short-sequence RNAs or other interfering transposons.

3.6. Cell Wall and Membrane Integrity: Sensitivity to Temperature, Radiation/Light, Salinity, pH, Antifungal, Genotoxicants, Reactive Radicals (Oxidative and Nitrosative Stress), and Quorum-Sensing Molecules

The cell wall components are majorly glucan fibrils, chitin, and melanin. The unique biochemical process and synthesis at the cell wall have made it a potential site for drug targets against cryptococcal cells. Cell wall integrity is highly important to environmental and common stress survival, adaptation, and development. It has been earlier confirmed that 1 M of NaCl was strong enough to disrupt cell wall integrity and impose stress on the cell membrane, thereby reducing the capsule size [157] (for details on the phenotypic responses of different mutants of *Cryptococcus* against various quantitative osmolytes and oxidants, check **Supplementary 3**). The cell wall is the first point of contact to various

 doi:10.20944/preprints202301.0461.v1

environmental effects (**Figure 1**), and it is enriched with various sensory proteins to relay the message for the recruitment of transcription factors. These factors, with other messengers, induce gene expression for nutrient assimilation, environmental adaptation, and survival. The *Hog1* gene is crucial to cellular response against various environmental cues such as temperature, radiation, oxidative stress, and salinity. This gene island encodes p38-like MAP kinases known as Hog1 MAP kinases in the yeast [158].

Different serotypes shared the same conserved structural functions of the *Hog1* gene yet displayed distinctive roles in response to temperature. Serotype A *Δhog1* mutant was sensitive to peroxide and temperature at 40°C but not at 37°C or 30°C; however, no peroxide and temperature sensitivity growth defect in serotype D *Δhog1* mutant [32]. The *Δhog1* mutants of the two serotypes were sensitive to UV radiation between 480 – 720 J/m^2, and more than 1.0 M chloride salts are needed to significantly alter their growth [32] (for details on the phenotypic responses of different mutants of *Cryptococcus* against various quantitative external factors, check **Supplementary 3**).

In addition to the Hog1 pathway, *C. neoformans* synergistically harnesses arrays of other signalling pathways such as Ras/Cdc, cAMP/Pka, Cam/Cna, Pkc/Mpk, Rho/Mpk, and Rim against various environmental and common stress agents (reviewed in [149]). There is evidence of interconnections among these signalling pathways. For example, methylglyoxal (MG) is a ubiquitous reduced form of pyruvate in fast metabolic cells, imposing stress against *C. neoformans*. Mutants lacking Hog1 or any cAMP/Pka pathway components are variously sensitive to MG, but *Δras1* mutants showed no observable changes against this stress-inducing intermediate metabolite. Further analysis showed that *tco2* was downregulated in mutants lacking Hog1 and cAMP/Pka pathways; however, this double-hybrid histidine kinase transcript was slightly upregulated in *Δras1* mutants – a condition that conferred a *wt* resistance to *Δras1* mutants against MG effect [137]. In another scenario, *C. neoformans* may deploy one or two strategic pathways for a particular resistance while other pathways are routed against other stress to avoid futile cycles, metabolic redundancy, and excessive energy dispensary. For example, genotoxic and oxidative resistance is jointly controlled by the Ras and Hog pathways, thermotolerance is controlled by the Ras and Can pathways, osmotic resistance is jointly controlled by the cAMP/Pka and Hog pathways, common and heavy metal stress resistance is controlled by Ras pathway, cell wall stress resistance and integrity are jointly controlled by the Pkc and Ras pathways, and antifungal resistance is jointly controlled by cAMP/Pka, Hog, and Ras pathways in a differentially repressed/induced mode to elevate/depress ergosterol biosynthesis – a condition that respectively facilitates resistance to azole/polyene antifungal agents [137]. Special attention has been drawn to the independent regulation of cell wall integrity by the Aca1 and Ras1 pathways and how the double deletion *Δaca1Δras1* mutants usually displayed higher sensitivity to cell wall disruptors compared to *Δaca1* or *Δras1* [137]. The *Δaca1* mutants usually display phenotypic appearances similar to *Δras1* mutants but distant from other cAMP family components, *Δgpa1*, *Δcac1*, *Δpka1*, and *Δpka2*.

Surprisingly, this fungus and other related human pathogenic fungi have developed a plethora of transcription factors associated with each pathway to ensure independent or complementary control to resist the imposing cytotoxic agents. Notwithstanding, mutants of different transcription factors deficient may respond similarly to the same stress agents. This means the involved signalling pathways must have shared some common routes at the upstream/downstream regulatory points. On the other hand, the lack of one transcription factor in a mutant can be complemented by other factors. For example, MG had no effect on the *Δras1* mutant because the presence of the cAMP/Pka pathway stimulates the expression of *tco2* for Hog1 pathway induction; however, *Δaca1*, *Δgpa1*, *Δcac1*, and *Δhog1* mutants are hypersensitive to MG while *Δpka1* and *Δpka1Δpka2* are slightly sensitive. This is further corroborated by the susceptibility of *Δaca1Δras1* and *Δcac1Δras1* mutants to MG [137] (check **Supplementary 3** for phenotypic responses of different mutants).

Among the well-studied transcription factors that regulate temperature are Ras proteins (Ras1p and Ras2p). In every stressful condition, especially under nitrogen starvation,

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C. neoformans prefers to express *Ras1* while silencing or minimising the *Ras2* expression. Most of the Ras-dependent genes of *C. neoformans* are unique and bear no orthologues with other yeast, just like the cAMP-dependent genes, but among the evolutionarily conserved few genes that are involved in the regulation of Rho-GTPase and activation of Cdc42 via Cdc24 are *Pxl1, Rdi1,* and *Bem3* [137]. Some of these genes have been implicated in the full virulence and gene repair in *C. neoformans* (for details of cellular events that induced/repressed different transcription factors for full virulence and gene repair in *C. neoformans,* check **Supplementary 2**). Through the guanine nucleotide exchange effector Cdc24, Ras1 is activated to mediate thermotolerance and infection in the infected hosts. In terms of the oxidative and genotoxic stress responses, *Δcdc24* and *Δras1* mutants responded similarly, which supported the downstream roles of Cdc24p to Ras1p [137,159]. The *Δras2* mutants, on the other hand, displayed the *wt* features against most of the stresses, which shows a minor role against stress; however, careful observation showed a slight sensitivity to amphotericin B (AmpB), fludioxonil (FDX), and *tert*-butyl hydroperoxide (*t*-BOOH) agents [137] (check **Supplementary 3** for phenotypic responses of different mutants to antifungals).

The avirulent nature of *Δras1* mutants is basically due to defective growth at 37°C because, together with *Δras2* mutant, there are no observable differences in virulence expression compared to the *wt* [135]. The growth of *Δras1* mutants decreased sharply at a temperature higher than 30°C. Still, the mutants were not quickly killed even at 39°C for 24 hours and transferring this mutant into YPD culture at non-permissive temperature survived with efficient growth [135]. Unfortunately, the temperature-dependent growth defect imposed by the deletion of *Ras1* (*Δras1*) appeared irreversible even in the presence of 1 M sorbitol or 1 – 50 mM cAMP unless the mutant is complemented with the *wt Ras1* gene (*Δras1::Ras1*) [135]. Like *Δcan1* mutants, *Δras1* mutants are also sensitive to immunosuppressive agents FK-506 and CsA at temperatures >30°C because both transcription factors modulate thermotolerance. The *Δcna1* mutants are sensitive to NaCl and LiCl, but *Δras1* mutants showed a *wt* phenotype with NaCl and LiCl [135] (check **Supplementary 3** for phenotypic responses of different mutants to different salt concentrations). Again, this shows osmotolerance control of Cna1 and Ras1 in different and distinctive pathways. Waugh et al. overexpressed the *Ras2* in the *ras1* background mutant under the *Gdp1* promoter and *Ura5* selectable marker and discovered that both transcription factors overlapped in their functions [136]. Furthermore, overexpressed *Ras2* can partially restore some of the phenotypic defects associated with *Δras1* mutants; hence both may be redundantly functional.

Contrarily, *Rho1 wt* expression under *Gal1* promoter in the *C. neoformans Δade2Δura5* auxotrophic mutant showed that the overexpression of this Ras-related superfamily small G proteins had no significant impact on the growth of this mutant at 30 and 37°C in glucose- or galactose-enriched media. Again, this overexpression showed no effect on pneumocandin B_0 inhibition and the activity level of cell wall glucan synthase [160]. Attempt to create *Δrho1* mutants failed because Rho1 is highly required for growth; therefore, the *rho1* gene replacement mutant was created with deregulated ***rho1***^{E41I} allele and discovered that the glucan synthase activity remained unaltered while the mutant failed to grow at 37°C, but this condition could be rescued with 1 M sorbitol at 25°C, and such mutant could survive 37°C thereafter [160]. Furthermore, because Pkc1 is a downstream functional protein of Rho1, any alteration with this transcription factor may nullify the thermosensitivity of the ***rho1***^{E41I} auxotrophic mutant [161].

Similarly, *rho1* mutants like ***rho1***^{G15V} survived quite well at 25 and 30°C, even at 37°C, but were hypersensitive to 39°C, which could not be rescued with sorbitol except by complementing the mutated allele. The ***rho1***^{Q64L}, however, showed no significant growth defect at all tested temperatures [162]. These two mutants, including *Δrho10*, which is nevertheless hypersensitive to 37°C, constitutively activate Mpk1 independent of the heat shock/stress at 24 or 39°C contrary to 39°C-induced Mpk1 activation by Rho1p in the *wt*,

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Δrho11, *Δrho10Δrho11*, and *Δrho10::Rho10* [162]. This shows that uncoordinated constitutive hyperphosphorylation of Mpk1 in *Δrho10* and point mutated mutants (***rho1***^{G15V} and ***rho1***^{Q64L}) at 24 and 39°C predisposed these mutants to growth defects observed at temperature >37°C, which means that the Rho transcriptional factors play an essential role in the Pkc1 pathway for cell wall integrity against stress. Moreover, Rho10 and Rho11 may have opposite roles regarding Mpk1 phosphorylation in the Pkc1 signalling pathway. In terms of virulence, all the mutants produced a *wt* melanin level, but the cell body size and the capsule diameter of the point mutated *rho1* mutants are significantly lower than the *wt* [162].

The Pkc1 encodes protein kinase *c* with pleiotropic effects on other transcription factors to regulate defence mechanisms against oxidative and nitrosative stress, osmotic imbalance, high temperature, cell wall disruptors, and anti-virulence agents. In addition, it maintains chitin and chitosan localisation within the cell wall and melanin production and attachment. Among the popular putative and probably membrane glycoprotein sensors are Slg1p, Wsc2p, and Mid2p, which initiate the activation of Pkc1 via Ipc1p, which eventually phosphorylates Bck1, Mkk2, and Mpk1 for subsequent activation of different Pkc1-dependent pathways (reviewed in [149]). This activation has been strongly correlated with the presence of oxidative and nitrosative stress agents, but the downstream Bck1, Mkk2, and Mpk1 appeared dispensable because their mutants displayed *wt* resistance against oxidative and nitrosative agents [163] except against SDS (a membrane destabiliser) and Congo red (cell wall/membrane stressor) [164]. The growth of *Δbck1* and *Δmkk2* mutants at 30 and 37°C was significantly considerable compared to the *wt* except at 39°C, where a significant defect was observed. This defect could be restored with 1 M sorbitol [164]. Being a protein kinase, Pkc1 phosphorylates Bck1p (MAPKKK) that activates Mkk2p (MAPKK) in a cascade event, Pkc1→Bck1p→Mkk1p/Mkk2p→Mpk1/Slt2p; however, the activation of Bck1p could also be from Rho1 or Rho11 [149]. This phosphorylation has been observed to be thwarted in *Δmkk2* and *Δbck1* mutants at a permissive and non-permissive temperature but not in the *Δpkc1* mutants [162], which means that activation of Bck1p and Mkk2p is highly important in the Mpk1p phosphorylation against thermal stress; however, Pkc1 seems dispensable [162].

By interpretation, other transcription factors, such as activated Rho1p and Rho11p, are promoted by guanine nucleotide exchange factors (*GDP↔GTP*) and GEFs (such as Rom1p and Rom2p/Rom20p/Rom21p) may be activating the Bck1p in the *Δpck1* mutants in thermal stress response. Surprisingly, repression of Pkc1 by dephosphorylation appeared to favour capsule formation, but capsules are not adequately attached to the cell wall because of the impaired cell wall integrity caused by the Pkc1 dephosphorylation [163]. Therefore, apart from the integral function of the Rho1-Pkc1 cascade pathway to regulate cell wall biogenesis, Mpt5p and Ssd1p are potential alternative transcriptional factors for initiating cell wall biogenesis [164]. Further investigation showed the possible involvement of three GEFs homologues (Rom2, Rom20, and Rom21) and four GTPase-activating proteins, GAPs (Lrg1, Bag7, Bem3, and Rga1), in the regulation of *C. neoformans* Rho1, Rho10, and Rho11 regulatory pathway for the Pkc1-mediated cell wall biogenesis [164].

In addition, small heat shock proteins (Hsp) are induced due to temperature changes to facilitate thermotolerance (check **Supplementary 2** for detailed transcription factors induced/repressed by temperature). Reports showed that *Δpka1* mutants are more heat-tolerant than the *wt* and *Δpkr1* mutants. On this point, it was demonstrated that the *Δpka1*, *Δpka2*, and *Δova1* mutants subjected to heat shock at 50°C for as high as 30 min still survived and remained viable better than the *Δpkr1* and the *wt* when cultured at 30°C in YPD [165]. The Hsp10, Hsp12, Hsp60, Hsp70, Hsp90, Hsp122, Sks2, and Gre2, are particularly under the influence of cAMP, and their expression seems induced in *Δpka1* mutants irrespective of the stress or nutrient availability; however, the *Pkp1* gene encoding dehydrogenase kinase is negatively controlled by Pka1 expression (as induced by Gpa1 or Aca1 in the cAMP pathway). This scenario was well examined, and discovered that Cac1 is not

 doi:10.20944/preprints202301.0461.v1

involved in the repression of *Pkp1* [137]. Therefore, an unaltered expression of Pkp1 in the *Δcac1* mutants may be the reason *Δpkp1* mutants displayed hypocapsulation (less capsulation) similar to *Δcac1* mutants while the *Δgre2*, *Δhsp12*, *Δhsp122*, and *Δhsp12Δhsp122* are characterised with a *wt* capsule production. Nevertheless, *Δpkp1* mutants deviated from *Δcac1* in terms of melanin production. All the mutants of these cAMP-dependent genes displayed *wt* melanin production in the *L*-DOPA (3,4-dihydroxyphenylalanine) media fortified with 0.1 – 0.3% glucose irrespective of the temperature, but *Δcac1* mutants are hypomelanised [137]. Regarding osmotic stress, cell wall/membrane stress, oxidative stress, genotoxic stress, MG, azoles, and FDX, each of the mutants displayed similar phenotypes as mutants from the cAMP pathway except *Δaca1* [137]. This means that though oxidative and thermotolerance genes seem induced in *Δpka1* mutants, this condition could not contribute to or improve the attenuated virulence in this mutant.

Together, the basal production of these hypoxic-responsive genes prepares the fungus against temperature, antifungals, and hypoxia. Therefore, the repression of Pkp1 may probably increase the availability of reducing equivalent needed for reductive pathways such as ergosterol biosynthesis, usually induced by the hypoxic condition. However, despite the involvement of cAMP in capsule and melanin production, none of these genes participates in the virulence of *C. neoformans* [137]. Notwithstanding, each of the mutants (*Δgre2*, *Δhsp12*, *Δhsp122*, *Δhsp12Δhsp122*, and *Δpkp1*) are slight to moderately sensitive to heavy-metal toxicity and that *Δhsp12*, *Δhsp122*, and *Δhsp12Δhsp122* are more sensitivity to AmpB than their corresponding *wts*, unlike the *Δgre2* and *Δpkp1* that showed a *wt* sensitivity level to AmpB under normoxic or osmotic condition. This observation indicated that *Hsp12* and *Hsp122* expression might be redundant against AmpB resistant [137]. Besides the cAMP influence on these transcription factors, the expression of Hsp12 and Hsp122 seemed to be Hog1-dependent. Complete repression of these transcripts was observed in *Δhog1* and *Δssk1* mutants, unlike the *Δskn7* mutant that showed comparable expression to *wt*. On the other hand, the basal expression of Gre2 is slightly repressed in the *Δhog1* and *Δssk1* mutants, but the *Δskn7* mutant displayed a *wt* expression of Gre2 [137].

The *Cnn1* gene encodes 34-amino acid helix-turn loosely conserved protein containing 16 tandem copies of tetratricopeptide-repeat (TPR). These polypeptides associate together to form complex regulatory proteins by protein-protein interaction to coordinate different cellular processes. These processes include cell cycle/division, DNA replication, RNA transcriptional repression and splicing, protein trafficking and kinesin-mediated intracellular cargo, signal transduction, stress response, peroxisome and mitochondrion biogenesis, protein kinase R (PKR, a *ds*RNA-activated kinase) inhibition, and neurogenesis [166-170]. The *Cnn1* is a homologue of the *S. cerevisiae Clf1* gene [171] and an orthologue of the *Crn* gene that controls *Drosophila melanogaster* neurogenesis, normal cell proliferation, and embryo development [172].

In yeast, this protein engages in 5′-pre-*m*RNA splicing, cell cycle control and progression, and DNA replication (by modulating the activity of Orc2p while initiating DNA replication). Furthermore, as a U2-snRNP component, it associates with Syf1, Syf2, Mod2, and U1-Prp40 to displace the branchpoint binding protein (BBP) known as splicing factor 1 (SF1) and forms a pre-spliceosome complex, which attracts the U4/U6.5 tri-snRNP. An active spliceosome is formed when the incoming U4/U6.5 ribonucleosome displaces the U1-Prp40 to orchestrate the *m*RNA splicing and cell cycle progression [173]. The absence of Clf1p together with any other TPR-containing proteins such as Prp1p/Zer1p, Prp4p, Prp6p, Prp9p, Prp11p, Prp13p, Cdc16p, Cdc23, Cdc28p, and Nuc2+p in the yeast usually promotes *ts* mutants, defective poly(A)-RNA nuclear export, G2 cell cycle arrest and S phase delay transition, and G2→M growth transition arrest [171,174].

Intriguingly, a genetic lesion resulting in the deletion of Lys^{217} residue from Cnn1p of B-4551 serotype A *MATα* strain of *C. neoformans* isolated from the chronic granulomatous lesion in the nasal cavity of a cat (feline) is the basis for the temperature sensitivity of this natural strain [175,176]. This strain can only grow to the optimum temperature of 35°C *in vitro*, characterised by short hyphae but normal melanisation and capsulation; however,

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the strain failed to cause systemic infection [176]. Notwithstanding, reconstitution of this strain with the *wt Cnn1* gene restored the growth at 37°C with systemic infection [175]. The eukaryotic expression of Cnn1 or its homologues is iron-dependent [88], performing different structural and functional cellular activities but related to achieving a common goal in the cell. The *Cnn1* gene seems to be specific to *C. neoformans* because complementing the *ΔCnn1* mutant with *S. cerevisiae Clf1* restored the thermosensitivity; however, *C. neoformans Cnn1* transcript could not rescue the *Δclf1 S. cerevisiae* mutant [175].

Ras-signalling cascade plays a significant role in cryptococcal thermotolerance. This GTP-activated protein (Ras1 and Ras2) effectively interacts with other effector proteins, such as Aca1, to synthesise cAMP and MAPK signalling cascade components. The *Δras1* mutants are viable at 25°C with normal melanin and capsule productions even at 30°C; however, this mutant failed to grow at 37°C, and the *in vivo* virulence is drastically reduced [135]. Moreover, though Ras1 signal is a Gpa1cAMP-dependent (nutrient-regulated sensor) and Gpb1-MAPK (pheromone-responsive) signalling event yet, exogenous cAMP and expression of MAPK could not restore the thermotolerance defect in *Δras1* mutant [135] but overexpression of Ras2 partially suppressed the high-temperature growth defect in *Δras1* mutant [136]. Therefore, together with the Cna1 signalling cascade, the thermotolerance ability of *C. neoformans* is controlled by the Ras1 cascade event but with distinct regulatory pathways. No wonder the Cna1 inhibitors, cyclosporine A (CsA) and tacrolimus (FK-506/Fujimycin/Prograf/FR900506), which are popularly used as an immunosuppressant in organ transplant patients, inhibited the growth of *Δras1* mutant even at 30°C [135]. The poor physiological thermotolerance of this mutant and the blocking of the calcineurin pathway complement the lethality of this drug. This calcineurin is a Ca^{2+}-calmodulin-dependent (Cam1) serine/threonine protein phosphatase that activates the nuclear translocation of Crz1p via dephosphorylation to activate other transcription factors against environmental stress and induces virulence expression. Besides, *Cna1* expression is induced in high temperature and other stress indicators to activate P-bodies and stress granules RNP transcription factors such as Puf4p, Lhp1p, Pbp1p, Pab1p, and Gwo1p (check **Supplementary 2** for events that induce P-bodies and stress granules RNP transcription factor).

Cam1-Cnb1 complex activates the Cna1p to cause the activation of Crz1p by dephosphorylation for nuclear translocation to coordinate cell wall integrity via the expression of *Chs5* – 7. Park et al. discovered that deletion of *Cna1* or *Crz1* repressed *Chs5* and *Chs6* at 37°C [177]. The *Chs7* expression remains unchanged in *Δcna1* compared to the *wt* but significantly reduced in *Δcrz1* mutant [177]. Similarly, transcription factors like Puf4p, Pbp1p, Tif3p, Vts1p, and Gwo1p in the P-bodies and probably Lhp1p, Gcd2p, and Anb1p in the stress granules are also activated by dephosphorylation via active Cna1p and all act within the cytoplasm to orchestrate virulence, thermotolerance, and sexual reproduction [177]. Thus, evidence exists that Lhp1p, and yet unknown transcription factors may be an indirect target of Cna1p or are probably activated by PB/GS-unrecruited Cna1p; conversely, Puf4p and Pbp1p are directly activated by the PB/GS-translocated Cna1p [177]. It is important to note that Lhp1p and Puf4p are located differently but are activated in parallel to Crz1p by Cna1 for additive function in thermotolerance and virulence expression. Strategically, Pbp1p with Crz1p activation seems to perform opposite roles against heat stress because the thermosensitivity of *Δcrz1Δpuf4* or *Δcrz1Δlhp1* mutants is usually higher than the individual mutants while *Δcrz1Δpbp1* mutants lie generally in between the *Δcrz1* and *Δpbp1* mutants [177]. To bolster the parallel regulation and additive phenotypic effects of Crz1p and these *m*RNA-binding proteins, every double mutant of *Δcrz1Δpuf4*, *Δcrz1Δlhp1*, and *Δcrz1Δpbp1* consistently exhibits higher attenuated virulence than their corresponding individual mutants (check **Supplementary 1** for detail). Regarding mating and hyphal formation, sexual reproduction is more severely defective in *Δcna1* than the *Δpbp1* mutants but with marked reduced *Mfα1* expression in the *Δpbp1* than the

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Δcna1 mutant during bilateral mating assay [177]. This shows that the phosphatase activity of Cna1p also controls the phosphate level of Pbp1p to regulate pheromone production, sexual reproduction, hyphal formation, and elongation.

The Mga2p is another temperature-regulated transcription activator protein and an orthologue of the components of fatty acid biosynthesis. With WSC domain orthologue Slg1, Mga2p constitutes the cell surface proteins essential for heat-stress perturbation signal control via the Pkc-Mpk MAPK pathway. Apart from these two transcription factors, Pps1, Thr4, Glt1, Dur3, Lys2, Rim15, Pma1, Chs6, Chl1, Clc1, Mdr1, Rds1, and Smg1 are highly involved in the thermotolerance of *C. neoformans* [178] (check **Supplementary 2**). These putative proteins are clustered as WSC domain proteins, chitin synthase, trehalose-associated enzymes, glycan-forming enzymes, proteases, amino acid permease and oxidase, multidrug-resistant proteins, pentose phosphate pathway enzymes, DNA helicase, oxidoreductases, peroxidase, and catalase. Specifically, genes involved in ribosomal, amino acids (such as isoleucine or valine) and pyrimidine biosynthesis are repressed at 37°C than 25°C [178], which re-iterates that cell development is not favoured at higher temperatures but rather *C. neoformans* divert resources to adaptation and survival. Surprisingly, deletion of *Ilv2* and *Ura5*, especially in serotype A, still produced high-temperature growth defects [179], which means stress-accumulated intermediates must have developed at higher temperature in these mutants leading to non-viable cell growth.

Drugs such as CsA, FK-506, and Rapamycin (RPM) are potential antifungals; however, due to their immunosuppressive effect on the calcineurin signal transduction and T-cell activation, non-immunosuppressant analogues are recommended. These drugs have an affinity for Frr1p (a homologue of Fkbp12 prolyl isomerase encoded by the *Frr1* gene in *C. neoformans*), and the drug-Frr1p complex targets Tor1-like kinase to interrupt necessary MAPK needed for cell cycle ($G_1 \rightarrow S$ phase) and signal transductions (such as calcineurin signalling event). Therefore, any mutation in either *Frr1* and *Tor1* will confer resistance against immunosuppressants without affecting the fungi growth, prototrophy, mating, sporulation, cell differentiation, and virulence [180]. Reports have shown that *C. neoformans* is resistant to FK-506 and its non-immunosuppressive analogue (*L*-685,818) at 24°C but sensitive at 37°C; even the FCZ-resistant strains are also susceptible at this temperature [181]. The CsA, FK-506, and *L*-685,818 are active against pulmonary cryptococcosis but not against cryptococcal meningitis. These drugs are relatively bigger to cross the BBB, and their immunosuppression outweighs the *in vivo* antifungal action at 37°C. This, therefore, calls for further investigation into the use of non-immunosuppressive agents as a potential antifungal.

C. neoformans displays multiple copies of gene-encoding peptidyl-prolyl *cis-trans* isomerases (PPIases), also known as rotamases/foldases, including cyclophilins A (encoded by *Cpa1* and *Cpa2*), parvulin (encoded by *Ess1*), and FK-506 binding proteins (Fkbps). These enzymes catalyse the isomerisation of *cis-trans* peptide bonds preceding prolyl residue and bring about protein folding and conformational change [182,183]. Deletion of *Cpa1* alone or with *Cpa2* produced *ts* mutants with attenuated virulence, unlike the *Δcpa2* mutants [184]. The double mutant, *Δcpa1Δcpa2,* is resistant to the CsA effect just like a site-specific mutation on the Cna1p conferred resistance against CsA [33]. This shows a link between the Cna1p and Cpa1p-Cpa2p in the same way Cna1p is connected to Tor1p. Neither *Δcpa1* nor *Δcpa2* mutant displayed any defect in unilateral mating and formation of filament, basidia, and basidiospores; however, these phenotypic defects are manifested in *Δcpa1Δcpa2* in a similar way to *Δcna1* mutant but with a bilateral mating defect. Though Cpa1p and Cpa2p may differ in their structure and function, evidence shows synergistic functions may exist between them. From Wang et al., only the *Δcpa1Δcpa2* mutant displayed defective capsule and melanin formation in a glucose-limiting medium, and only the *Δcap1* and *Δcpa2* mutants displayed hypersensitivity to CsA stress with mating defect but not *Δcpa1Δcpa2* mutant [184].

 doi:10.20944/preprints202301.0461.v1

The *Ess1* gene, on the other hand, was found to be dispensable for cell growth, mating pheromone response, haploid fruiting, and capsule formation; however, *Ess1* expression is highly important for melanin and urease activity and for this reason, *Δess1* mutants displayed impaired virulence in murine model cryptococcosis [185]. It is important to note further that *Δess1* mutants appeared to have delayed growth in standard medium without any PPIase inhibitor. It is very likely that each component of the PPIase compensates for one another in an overlapping function though there may not be a full replacement. Since the viability of these mutants above 30°C is Cna1-dependent, *in vitro* assays must be maintained below 30°C in these mutants due to ineffective Cna1p. Both *Δcpa1* and *Δcpa2* mutants are found with Fkbp12 expression, which is insufficient to confer resistance against CsA, and again, *Δess1* mutant may have produced Cpa1 and Cpa2 but also insufficient against CsA at 25 – 28°C [184,185]. The *Δess1* mutant showed no response to the FK-520 analogue at 25°C (check **Supplementary 3** for mutant responses to FK-520). Surprisingly, both works concluded that the reconstitution of the mutants, *Δcpa1::Cpa1//Δcpa2::Cpa2* and *Δess1::Ess1,* appeared incomplete as the phenotypic traits were still less than the corresponding *wts*.

DNA topoisomerase I, encoded by the *Top1* gene in fungi, is an enzyme in genome replication and transcription. This enzyme features a unique fungal pocket sequence region, which is not found in mammalian topoisomerase I. Under the influence of inducible promoter, Del Poeta et al. demonstrated that moderate overexpression of *Top1* gene predisposed serotype A strain H99 to heat shock, γ-ray, and camptothecin (a topoisomerase inhibitor) as compared to the isogenic strain [186]; though capsule and melanin production remained unchanged. Previous work to generate *S. cerevisiae Δtop1* mutant promoted the resistance of this strain against camptothecin; however, the *wt* strains were sensitive to this antifungal, possibly by accumulating preponderant levels of cleaved DNA duplexes, which topoisomerase failed to ligate because of camptothecin. This invariably leads to a cytotoxic effect [187,188]. Contrarily, Jiang et al. had shown the essentiality of the *Top1* gene for the survival and full virulence of *C. albicans wt* compared to the *Δtop1/Top1* heterozygote mutant [189]. Meanwhile, the impaired expression of this nucleus-located repairing enzyme in *C. neoformans* serotype A showed no effect on the toxicity of dicationic aromatic compounds (DACs) such as *bis*-benzimidazoles, carbazoles, furans, and pentamidine analogues, which are novel antifungal agents and that such strain was fully pathogenic. However, the reported initial stress response experienced by *C. neoformans* H99 strain in the presence of these antifungals may probably depend on the initial low expression level of the *Top1* gene, which gradually increased over a period [186].

DNA topoisomerase has been proposed as a target enzyme for drug inhibition against various infectious agents, including cryptococcal cells [190]. *C. neoformans* possesses the second *Top2* gene for DNA topoisomerase 2, but then there is no evidence of functional replacement/complementarity between the *Top1* and *Top2* genes [186]. Strategically, *C. neoformans* serotype A may likely engage in a housekeeping regulation of the production of topoisomerase, even under the induce-promoter system, to avoid initial stress from antifungals, radiation effect, and heat shock. This may also be tantamount to the expression of genes involved in producing heat-shock protein (*Hsp70*) and melanin (*Lac1*) to cope with heat shock and radiation, respectively.

The UV-light incidence on *C. neoformans* can inhibit mating or haploid fruiting; in the same way, it will induce DNA damage in the yeast via the production of cyclobutane pyrimidine dimers (CPDs) or photoconjugate products. Such photoconjugates include the formation of dipyrimidine from adjacent thymine or cytosine bases, 6 – 4 pyrimidine–pyrimidone or 6 – 4 pyrimidine–pyrimidinone, and Dewar pyrimidinone isomers of 6 – 4 photoproducts. In each case, failure of the DNA repair enzymes (repairsome) and photolyase to repair these damages can lead to a double-strand breakage due to base mismatch, thereby impairing the mating and haploid fruiting. This has been shown by Idnurm et al. that dark incubation favoured mating and hyphal formation more than light/illuminated

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incubation irrespective of the mating type, with evidence of hyper-hyphal formation in either bilateral or unilateral crossing involving *Δbwc1* mutant in the V8 media at pH 7.0 [191].

Furthermore, light signalling/transducer homologues, including Ops1, Phy1, and Bwc1, were identified in *C. neoformans*. The *Δops1*, *Δphy1*, and *Δops1Δphy1* mutants showed no effect on the mating and haploid fruiting in the presence of light, unlike the *Δbwc1* mutant, which is sensitive to light [191]. However, mutation of these genes (single, double, or triple) has no significant effect on the virulence factors of *C. neoformans*. *Bwc1* and *Bwc2* are the two major photo-inducing genes in *C. neoformans*. The Bwc1p significantly controls the cell fusion and filament development when the *Δbwc1* mutant is crossed with either *ade2* or *lys1* auxotrophic *Bwc1 wt* [191]. Similar to *Neurospora crassa* Wc-1 protein, blue light induces the non-zinc finger DNA binding protein Bwc1p to regulate cell fusion and repress hyphal development in *C. neoformans* serotype A or D [191]. This step inhibits further growth pending favourable conditions to preserve the integrity of the genome. Unlike Bwc1p, Bwc2p appeared to have the PAS and zinc-finger DNA binding domain, like *N. crassa* Wc-2. The Bwc1p perceives the photon as a light sensor and induces a conformational change, which the transcription factor Bwc2p can recognise to favour protein-protein interaction.

The ability of *C. neoformans* to replicate and proliferate within the micro-acidic phagolysosome of macrophages is partly controlled by the *Ipc1* expression. The outcome from Luberto et al. showed that downregulation of the *Ipc1* gene under the influence of glucose-repressing P_{gal7}*::Ipc1* induction expression reduced the exponential proliferation of strain H99 at pH 4.0 in a macrophage-like cell line, but galactose-inducing condition stimulated the rate of replication even than the *wt* strain [134]. The implication is that glucose-enriched tissues like CNS, which have 50 – 60% of systemic glucose (800 μg/mL), may impact early stress on *C. neoformans* to prevent proliferation. Nevertheless, the effect of *Top1* and many other transcription factors may come to play to oppose this stress and promote cell replication.

Strategically, *C. neoformans* demobilises the activation of the cAMP/Pka-Rim101 signalling pathway in the acidic medium (such as phagosomes) because this pathway is generally activated in the neutral-to-alkaline media. The acidic nature of the phagosomes usually excludes glucose to maintain low osmolarity. This condition favours the activation of cryptococcal cAMP/Pka sensors, which will not initiate the Rim101 cascade event but Gpa1. The induction of the *Gpa1* gene and the concurrent release of DAG to activate Pkc favour the *Lac1* and *Cap* genes for the formation of melanin and capsule, respectively. Together with chitin expression, this virulence enables the cryptococcal cells to withstand the acidic phagosomes and promote endosomal and lysosomal survival of *C. neoformans* within the phagolysosome. Consequentially, titanisation of the phagocytosed cryptococcal cells is initiated – because of the chitin formation, leading to phagosome rupturing. Here, the cell can then utilise the cytoplasmic glucose of the macrophage for metabolism and growth while initiating the time-dependent macrophage apoptosis. Indeed, *C. neoformans* is a battle-ready pathogen with numerous counter-attacking accessory genes.

C. neoformans prefers an acidic environment to an alkali medium. Gradual reduction of H^+ in a culture medium induces *Ena1* expression. From **Supplementary 3,** *Δena1* and *Δcna1* mutants are hypersensitive to alkali pH [192]. This shows that the two separate pathways regulated by ATP and Ca^{2+}, respectively, are involved in the survival of *C. neoformans* under elevated pH. Though *Ena1* expression has been speculated to be induced for long-term hyperosmotic adaptation under the influence of *Hog1* expression; however, the results from Idnurm et al. showed that there is no evidence of increasing *Ena1* expression under the influence of elevated salinity of monovalent salts of Li^+, Na^+, and K^+ or divalent salts of Ca^{2+} or even 1 M sorbitol but somewhat under the alkali medium when Na^+ or K^+ is low [192]. Besides, the expression of *Ena1* could be tightly regulated by Can1, Hog1, and Rim101 [192]. The *Nha1* expression, on the other hand, was induced by osmotic

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stress in probably a Hog1-dependent pathway and plays a role in survival under high K^+ osmotic stress and acidic conditions [193].

Plc1p is another important membrane-localised protein that releases phospholipase B from the glycosylphosphatidylinositol (GTI)-anchor. A *Δplc1* mutant is excessively susceptible to azoles, 5-flucytosine (5-FC), and AmpB due to defective cell wall integrity in this mutant [194]. Similarly, U73122 ((1-[6-[((17*β*)-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione)) is a PLC and PLA2 synthetic inhibitor that prevents the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) to two intracellular second messengers, 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG activates Ca^{2+}-dependent Pkc that modulates other MAPKs to restore cell wall integrity, which is usually perturbed by high temperature and cell wall disruptors. In contrast, IP3 binds the ER to release storage Ca^{2+} for Ca^{2+}- and calmodulin-regulated signalling pathways, which means that Plc1 activity contributes significantly to calmodulin-activation but to calcineurin-activation, its contribution is insignificant [195]. Back to the synthetic phospholipase inhibitor, incubating *C. neoformans* in the presence of U73122 at 2.5 μM prevented the growth for over 12 hours, and the effect was more devastating as the temperature increased; however, the analogue of this inhibitor (U73343) displayed no antifungal effect [194].

Further studies have shown again that *Ipc1* expression may encourage sphingolipid interaction with the membrane acyl chains and ergosterol to promote a more compact, resilient, and less permeable bilipid layer surrounding the cryptococcal cells [134]. This excludes extracellular solutes and prevents ionic perturbation while maintaining intracellular homeostasis. In addition, repression of the *Ipc1* gene delayed macrophage toxicity and disruption, extracellular predominance, and tissue invasion [1,134]. Similarly, iron absorption encouraged ergosterol biosynthesis to maintain membrane integrity. Deficiency in iron acquisition has been shown to influence fungi susceptibility to antifungal drugs [196]. Though *Δcft1* and *Δcft2* mutants displayed normal growth on YPD, but these mutants have been characterised with impaired ergosterol biosynthesis, and that *Δcft1* is more sensitive to miconazole (MCZ) and AmpB than the *Δcft2* (check **Supplementary 1** for detail). Therefore, drug targeting against the functional iron permease, especially the Cft1p, may be an additional benefit to the current antifungal drugs.

Till now, the most widely used azole antifungal, such as fluconazole, itraconazole (ICZ), and voriconazole, target one or two enzymes in the ergosterol biosynthesis. These enzymes may be oxygen-dependent/independent. The motif behind this is that in all circumstances, cryptococcal cells modify the membrane components to survive every environmental impediment. In doing so, azole drugs can easily block the deployment of such key enzymes needed for membrane sterol production. Erg11 encodes oxygen-dependent lanosterol-14*α*-demethylase in the production of ergosterol. This enzyme is the target of azole drugs. The Mbs1 negatively regulates basal expression of *Erg11* and, when deleted, increases the azole resistance; however, the resistance against polyene is drastically reduced as par *Δert1, Δjjj1, Δhcm1* and *Δecm22* [126,197] (mutant responses to polyene drugs are highlighted in **Supplementary 3**). Also, Hob1 is another negative regulator of *Erg11,* and its deletion remarkably increased the basal expression of *Erg2* and *Erg11*. However, under sterol depletion, *Δhob1* mutants are characterised by a remarkable reduction in the induction of *Erg2, Erg3, Erg5, Erg11,* and *Erg25* involved in ergosterol biosynthesis [197]. It is, therefore, possible that *C. neoformans* partially represses *Hob1* expression to elevate ergosterol accessory enzymes in the *wts*.

Unlike Hob1, Sre1 is a positive key regulator of ergosterol synthesis. It is probably possible that the FCZ resistance observed in *Δhob1* mutant results from the background expression of *Sre1* to rescue impaired ergosterol synthesis in *Δhob1* mutants. Surprisingly, while *Δhob1* mutants are hyper-resistant to FCZ but hypersensitive to AmpB, the *Δsre1* mutants are hyper-resistant to AmpB but hypersensitive to FCZ (mutant responses to FCZ are highlighted in **Supplementary 3**). Realistically, the expression of *Sre1* in *Δhob1* mutant induces most of the oxygen-dependent enzymes for sterol biosynthesis, including *Erg1,*

Erg2, Erg3, Erg5, Erg7, Erg11, and *Erg25,* in the presence of FCZ [197]. Notwithstanding, *Sre1* expression can also regulate and control the expression of other oxygen-independent enzymes involved in the upstream catalysis of ergosterol intermediate formation, and this includes the expression of *Erg6, Erg10,* and *Erg13* [198]. To this extent, Sre1 and Hob1 expressions may be playing complementary *tête-à-tête* roles against the oxidative, osmotic, genotoxic, ER, cell wall, and membrane stress by jointly regulating the ergosterol biosynthesis [197]. Consequentially, *Δstp1* and *Δscp1* mutants are unequivocally susceptible to FCZ even in the normoxic conditions (normal atmospheric oxygen concentration, 20 – 21% or physiological oxygen concentration, 2 – 3%) at 30°C; not only FCZ but also ICZ and voriconazole [198]. Convincingly, reconstituting this mutant with the *wt Sre1* gene restored normal growth even under the inhibitory concentration of the antifungals. Paradoxically, oxygen availability is insufficient to prevent the growth defect displayed by the *Δsre1* mutant in the azole-containing media. With this sterol biosynthesis and proper incorporation of melanin and chitin, cell wall integrity is confirmed to withstand antifungal and increasing temperatures.

Also, the cell wall integrity and antifungal are jointly controlled by the *Mpk1* and *Can* expression. The *Δmpk1* mutants are attenuated for virulence with poor cell wall integrity and are thermosensitive and susceptible to antifungals such as nikkomycin Z (which inhibits chitin synthase) and caspofungin (CpF, which inhibits *β*-1,3-glucan synthase) (other mutants are discussed in **Supplementary 1**) [199]. Surprisingly, disruption of calcineurin function activates the expression of a single-copy *Fks1* homologue to produce *β*-1,3-glucan synthase, which is essential for cell viability in a low-iron medium [200]. Based on this, capsule induction is expected to increase; however, *ΔcanA* and *ΔcanB* mutants showed no significant difference in their capsule sizes whether cultured in a low or enriched iron media [88]. Thus, by inference, calcineurin function may not be essential for capsule formation.

Apart from *Sre1* expression controlling ergosterol biosynthesis, disruption of the *Hog1* components and *Skn7* independently controls ergosterol synthesis to promote antifungal resistance. The *Δhog1* and *Δssk1* mutants showed upregulation of major *Erg* genes such as *Erg4, Erg5, Erg6, Erg11, Erg20, Erg25,* and *Erg28* with a concomitant preponderance of cellular ergosterol content, while *Erg8, Erg10, Erg13, Egr26, Erg27, Hmg2,* and *Idi1* are more upregulated in *Δssk1* mutant; however, *Δskn7* showed no upregulation of these genes [81]. Because Ko et al. discovered a significantly higher level of cellular sterol in *Δssk2* (MAPKKK) and *Δpbs2* (MAPKK) than in the *wt* and *Δskn7* mutant, it is therefore concluded that constitutive phosphorylation of Hog1p represses ergosterol biosynthesis under normal conditions [81]. To this effect, all four mutants are hypersensitive to AmpB, but *Δskn7* showed a *wt* resistance level. Contrarily, all four mutants, together with *Δskn7*, are KCZ- and FCZ-resistant, but to imidazole (ICZ) at low concentration, a *wt*-resistance was displayed (mutant responses to antifungals are highlighted in **Supplementary 2**). This outcome has presented a differential response of the Hog1 pathway to different antifungals. The resistance observed with the *Δskn7* mutant was purely aberration and certainly not *Erg*-dependent resistance because there was no significant difference in the expression of *Erg* genes in this mutant compared to the *wt.* However, an observation from Wormley et al. showed that *Skn7* might be responsible for the expression of thioredoxin reductase [201] – an enzyme released against oxidative stress.

To mollify this observation, Bahn et al. discovered that mutation of *Skn7* showed no significant effect on the sensitivity to H_2O_2, UV-irradiation at 720 J/m^2, high temperature, methylglyoxal (MG), and high salt solution (especially KCl); however, *Δssk1, Δpbs2,* and *Δhog1* mutants are sensitive to all these factors except FDX [30]. Precisely, the *in vitro* flocculating *Δskn7* mutants of *C. neoformans* var. *grubii* (serotype A) are highly susceptible to 0.025 mM *t*-BOOH, 1 M NaCl, and significantly less virulent due to oxidative arrest; nevertheless, the non-flocculating *Δskn7* serotype D mutants displayed a *wt* similar stress, antifungal, and adaptations to *t*-BOOH, 1.5 M NaCl, AmpB, and 38°C [202]. The Skn7 function against oxidative stress is independent of Trx2 and Glr1 but induces Trr1 and

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Sod1 to maintain intracellular redox balance [201]. Because *Δskn7* mutant can still deploy other oxidative and nitrosative stress regulatory/response groups of genes (*OSR* and *NSR* genes), its pathogenesis and survival in the macrophage-killing assay appeared unaltered [201]. Intraendothelial survival, virulence, and brain and lung colony recovery of *Δsnk7* serotype D mutants appeared significantly reduced compared to the isogenic control, but the tissue adherence and fungaemia are similar to the *wt* [202].

The dependence of Hog1 activation on Ssk1p is very paramount in *C. neoformans.* Most times, *Δssk1* mutants display similar phenotypic defects as *Δhog1* and *Δpbs2* mutants; however, Ssk1 plays a less significant role in osmosensing pathways because, in the *Δssk1* mutant, Hog1p can still be phosphorylated to a lesser extent in the presence of 1 M NaCl but not in the presence of FDX or MG [30]; no wonder *Δssk1* mutant displayed a *wt* phenotype against 1 M NaCl and 1.5 M KCl in YPD [82]. Paradoxically, Ssk1p is needed to keep Hog1p constitutively phosphorylated under normal conditions. However, in the absence of the second two-component system response regulator, Skn7, Hog1 exhibited a more progressive time-dependent dephosphorylation in 20 μg/mL FDX than the *wt*. This Hog1 dephosphorylation and activation in *Δskn7* are, however, similar to the *wt* in the presence of 1 M NaCl and 20 mM MG [30]. The observation that *Δhog1, Δssk1, and Δpbs2* mutants are hypersensitive to MG but hyper-resistant to FDX (while *Δskn7* mutant showed opposite phenotypic features) showed that dephosphorylation of the Hog1p is differentially regulated towards different osmotic, oxidative, and antifungal stress. In addition, *Δssk1* and *Δpbs2* shared similar phenotypic defects with respect to H_2O_2, UV-radiation, and FDX (**Supplementary 3** highlights responses of several mutants to various quantified factors).

The roles of the thioredoxin system (Trx1 and Trx2) in maintaining the cytosolic redox-equilibrium glutathione reductase activity in *C. neoformans* deserve high commendation. Functionally, the two proteins appear redundant and can replace each other to enhance growth, promote membrane integrity, antioxidative, antinitrosative, and influence the virulence of this pathogen. The induction of Trx1 in the presence of H_2O_2, Trx1 and Trx2 in the presence of *t*-BOOH, and Trx1 and Trx2 in the presence of $NaNO_2$ confirmed the antioxidative and antinitrosative roles of thioredoxin proteins. The *Δtrx1* and *Δtrx1Δtrx2* mutants exhibited severe growth defects due to increased levels of oxidised glutathione compared to the total glutathione level, but the *Δtrx2* mutant only showed comparable *wt* growth phenotypes [203]. Therefore, *trx1* expression is highly important in macrophage survival and virulence. Likewise, the expression of thiol peroxidase Tsa1, Tsa3, and Tsa4 in the presence of H_2O_2, *t*-BOOH, and NO at 37°C also complements the roles of the thioredoxin system. Among these, Tsa1 appeared to play the most significant role against oxidative and nitrosative stress to bring about thermotolerance and virulence in the mouse model [204]. Unfortunately, while an exogenous ascorbate supply restored all the growth defects in *Δtsa* mutants, neither ascorbate nor dithiothreitol could easily restore the growth defects in the *Δtrx* mutants (check mutants in **Supplementary 1** and **3** for other responses to different oxidants).

Glutathione peroxidase is another plausible cytoplasmic localised antioxidative enzyme encoded by *Gpx1* and *Gpx2*. These redundant transcripts are differentially induced in response to stress agents but have no impact on virulence expression. Missall et al. discovered that only the *Gpx2* is induced in response to H_2O_2 while the two transcription factors are induced in the presence of *t*-BOOH and cumene hydroperoxide (COOH) but are repressed with NO stress in the *wt* [205]. Interestingly, H_2O_2 can induce *Gpx1* in the *Δgpx2* mutant as potential transcriptional compensation, but *t*-BOOH failed to induce *Gpx2* in the *Δgpx1* mutant showing that the *Δgpx1* mutant is particularly sensitive to *t*-BOOH without any compensation from the *Gpx2* [205].

Another well-studied *C. neoformans* antioxidative enzyme is Mn-SOD, an important component of the mitochondrial antioxidant defence system encoded by the *Sod2* gene. From Giles et al. analysis, *Δsod2* mutants showed increased sensitivity to superoxide rad-

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icals generated by Antimycin A or Paraquat™ (N,N′-dimethyl-4,4′-bipyridinium dichloride or Methyl Viologen) with O_2-dependent temperature-sensitive (*ts*) growth defect. However, these phenotypic defects were observed to be restored with increased exogenous supplementation of 20 mM ascorbate or 200 μM Mn-salen-type ligand (a Jacobsen's catalyst known as N,N'-bis(3,5-di-*tert*-butylsalicylidene)-1,2-cyclohexanediaminomanganese(III) chloride) at 30°C but not 37°C [206]. Surprisingly, an equal concentration of inorganic $MnCl_2$ failed to restore this *ts* phenotypic defect, and these restoring concentrations appeared to inhibit the *wt* growth at 37°C [206]. Contrarily, Narasipura et al. achieved progressive growth of the *Δsod2* mutant in the presence of 5 mM and 10 mM of ascorbate at 37°C, and this growth was comparable to the *wt* at 37°C [207].

Regarding the electron transport chain in mitochondria, the two antioxidative enzymes, Ccp1p and Aox1p, are very important. For example, *C. neoformans* cultured in the yeast nitrogen base (YNB) at pH 4.0 and 30°C are moderately inhibited by 1 mM H_2O_2 but highly exacerbated in the presence of 0.5 μg/mL of Antimycin A (an inhibitor of Ccp1p-mediated electron transport chain) or slightly inhibited in the presence of 2 mM of SHAM (an inhibitor of Aox1p-mediated electron transport chain). Additively, the combination of the two inhibitors prevents the growth of *C. neoformans* either in the presence or absence of H_2O_2 [208]. This shows the significant role of Ccp1p in the antioxidative effect against H_2O_2; nevertheless, the supportive role of the Aox1p-mediated pathway is highly commendable, especially in reducing the preponderant formation of ROS during redox reactions. Furthermore, the *wt* strain had shown early downregulation of *Aox1* transcript, which remained unperturbed when exposed to exogenous H_2O_2 effect, unlike its functionally redundant *Ccp1* transcript that was later upregulated [208]. On the other hand, the presence of 0.25 μg/mL of FCCP (4-trifluoromethoxycarbonylcyanide phenylhydrazone), a protonophore (proton translocator/ionophore) that dissipates the H^+ gradient across the inner mitochondrial membrane, has no effect on *C. neoformans* but a 50% growth reduction when added with 1 mM H_2O_2. Contrarily, 0.5 μg/mL of FCCP is sufficient to moderately reduce the growth of *C. neoformans,* unlike *S. cerevisiae* [209] and *C. albicans* [210]; and when combined with 1 mM H_2O_2 drastically inhibited the growth [208]. This demonstrated mitochondria oxidative phosphorylation pathways against oxidative stress imposed by exogenous peroxide, perhaps by providing ATP for reparative and homeostasis cellular processes after a series of oxidative damages. One such is proteasomal ubiquitin-dependent protein catabolism, which actively involves Ubc8p against oxidative stress imposed by H_2O_2 [81].

Structurally, Mn-salen is a less complex structure than Mn-porphyrin and possesses a high-valent central Mn in a nitrogen and oxygen donor environment [211]. This condition may have improved cellular entry and redox participation of the Mn better than the inorganic salt or a more complex Mn-porphyrin. Due to its function in maintaining the steady-state cellular endogenous reactive oxidants, *Δsod2* mutants in an Mn-salen medium are non-viable in aerobic conditions at 37°C for 24 hours because of the accumulated reactive oxidants [206]. This is not surprising because *C. neoformans* is a facultative aerobe; however, the *Δsod2* mutants are viable under the anaerobic condition at 37°C and can as well survive ambient conditions when transferred to 25°C [206]. Deletion of the *Sod2* gene has no significant effect on capsule production, melanin formation, and urease activity; however, surviving the oxidative attack by the macrophage is impossible in the absence of *Sod2*, and this is the reason Giles et al. could not recover the *Δsod2* mutant from either the lung or brain in murine inhalation model of cryptococcosis (MIMC) [206]. Thus, this is one of the antioxidative enzymes not linked to virulence *per se* but to survival, adaptation, and infection in the host.

Contrarily, Narasipura et al. discovered that the killing of the *Δsod2* mutants is necessarily not from the oxidative attack by the phagocytes but from the high oxygen environment [207]. This observation is true for the *in vitro* system but achieving such a high level of O_2 *in vivo* may be difficult. Therefore, the major killing of the *Δsod2* mutants may still be from the oxidative attack by the macrophage. There was a slight difference in the

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growth of *Δsod2* mutants at 30°C in the Narasipura (at 95% air) and Giles (no percentage stated) works. Convergently, when Giles et al. incubated the mutant at 37°C under anaerobic conditions and transferred the mutant to a condition as low as 25°C, viable growth was observed, which is as good as the growth Narasipura et al. observed at 30°C. However, *Δsod2* practically remained unviable at 30°C, making the mutant isolation difficult, unlike 25°C [206]. In the presence or absence of O_2, *Δsod2* and *Δsod1Δsod2* mutants displayed similar *ts* growth defect at 37°C contrary to *Δsod1* [207]. In addition to the oxidative attack by oxygen, *Δsod2* and *Δsod1Δsod2* mutants are equally hypersensitive to all common superoxide- and osmotic-inducing agents (check **Supplementary 3** for other responses); however, the cytosolic *sod1* mutants behaved alike except against Paraquat™ where a slight sensitivity was observed [207]. It was further discovered that *Δsod2* and *Δsod1Δsod2* viability reduced drastically in the stationary phase nutrient limitation study. The *Δsod2* mutants are completely avirulent in the murine inhalation study, but the virulence is attenuated in the murine intravenous study. The *Δsod1Δsod2* mutants, on the other hand, lost their virulence completely with murine inhalation/intravenous model of cryptococcosis with a complete fungi clearance in the lungs; however, traces of this mutant were recovered from the brain after 120 days post-infection. The analysis further showed that these mutants were the same as the inocula mutants lacking Sod1p and Sod2p [207].

Unlike thioredoxin, peroxidase, and dismutase systems, the catalase system seems to play a less significant role against oxidative stress and virulence factors. Among the four *Cat* genes identified in *C. neoformans, Cat1* and *Cat3* (encoding putative spore formation-specific catalase), *Cat2* (encoding putative peroxisomal catalase), and *Cat4* (encoding putative cytosolic catalase), none of the mutants (*Δcat1, Δcat2, Δcat3, Δcat4, and Δcat1Δcat2Δcat3Δcat4*) showed any phenotypical defect related to oxidative stress irrespective of the temperature, just as observed in *S. cerevisiae* [212]. Furthermore, it was shown that none of the *Cat* genes is required for survival, infection, and virulence expression; however, *Cat4* seemed to be required for mating and spore viability.

Out of the seven different hybrids of sensor histidine kinases (Tco1, Tco2, Tco3, Tco4, Tco5, Tco6, and Tco7), Tco1 and Tco2 are the most studied two-component phosphorelay system expressing sensor kinases. In tandem with Ypd1p, it activates response regulator kinase Skn7p in the nucleus or Ssk1p in the cytoplasm [213]. The *C. neoformans* Ypd1 is an intermediate and dynamic phosphorelay histidine-containing phosphotransfer protein (HPt) and a structural homolog of yeast HPt protein. The Ssk1 kinase activates the Ssk2-Pbs2-Hog1 MAPK cascade event, and this subsequently induces a plethora of downstream target genes for various cellular activities, including cell wall/membrane stress response, sterol formation, virulence factors, and cell differentiation and filamentation [32,81]. Furthermore, the disruption of *Tco* and *Hog1* genes differentially predisposes *C. neoformans* to antifungal, osmotic shock, and membrane disruptors.

5-FC induces the regulation of several genes under the control of Tco kinases and the Hog1 pathway. These genes are majorly involved in signal transduction, cell cycle, replication, translation, ribosomal maturation, and post-translational processing. Since 5-FC is a pyrimidine analogue, there is a possibility that when incorporated during replication/transcription, it will accumulate in either truncated or non-functional transcripts, which may invariably produce biologically toxic proteins and compromise the induction of genes majorly involved in the quality control of DNA, RNA, and protein production. The *Δtco1* mutant is sensitive to 5-FC, but *Δtco2* is more resistant to 5-FC even more than the *Δssk1, Δssk2, Δpbs2, Δhog1,* and *Δskn7* mutants under the same conditions. Unequivocally, the *Δtco2* resistance against 5-FC was not attributed to a natural alteration in the putative genes encoding cytosine deaminase (encoded by *Fcy1*), cytosine permease (encoded by *Fcy2*, *Fcy3*, and *Fcy4*), or UPRT (encoded by *Fur1*) but a metabolic alteration was suspected rather than the transcription factors [126].

The unique *wt* upregulation of *Ste14* transcript encoding isoprenylcysteine carboxylmethyltransferase (ICMT) [214], which in addition to palmitoylation, prenylates the CAAX motif of Ras1p for proper membrane localisation to promote functional GTPase

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and thermotolerance trait deserves closer attention to unravel the 5-FC-resistance. So, for this connection, it is possible that active and membrane-located Ras1p promotes resistance to 5-FC in a similar way that Atf1p promotes 5-FC- resistance [91]. Previously, none of the hybrid sensor kinases was differentially involved in the resistance/sensitivity to AmpB, FCZ, ketoconazole, itraconazole, osmotic shock, UV irradiation, and high temperature (as high as 40°C) except the *Δtco2* mutant that showed hypersensitivity to AmpB and 1.5 M KCl [30,81].

Though Ypd1p is an intermediate HPt, its roles in the viability of *C. neoformans* are inevitable. The initial attempt to delete this gene in a *wt* was unsuccessful but from the *Δhog1* background mutant [82]. Changing its promoter to a copper regulatory promoter (P_{ctr4}) showed that it regulates the growth in a Hog1-dependent manner. The P_{ctr4}*::Ypd1* strain grew under the influence of BCS but not $CuSO_4$, meaning that $CuSO_4$ is a repressor; however, the P_{ctr4}*::Ypd1* strain in *Δhog1* background grew in both BCS- and $CuSO_4$-containing media [82]. Characteristically, *Δhog1Δypd1* and *Δhog1* mutants displayed osmosensitivity to 1.5 M KCl but not 1.0 M NaCl solution, unlike *Δskn7*, which is hypersensitive to 1.0 M NaCl. Basically, Ypd1p controls the Hog1-dependent pathway to maintain membrane integrity against perturbation caused by osmolytes. This HPt protein can as well function independently of the Hog1 pathway to confer resistance to oxidative stress caused by diamide (thiol-specific oxidant) and H_2O_2. The *Δhog1Δypd1* mutant conspicuously showed hyper-resistance against azole better than *Δssk1*, *Δhog1*, and *Δskn7* mutants (check **Supplementary 3** for other mutant responses). This may reflect the additive repression of *Ssk1* and *Skn7* expression in the *Δhog1Δypd1* double mutant to promote the expression of the *Erg11* gene.

Oxidative stress imposed by H_2O_2 induces specific stress regulatory genes, which outnumber the specific genes induced by osmotic and antifungal stress, probably because H_2O_2 produces reactive radicals (oxidants). This means that *C. neoformans* possesses a unique genome-wide regulatory expression profile induced by oxidative stress. This dynamic expression profile has been streamlined into the array of genes involved in signal transduction, membrane transport (ionic, solutes, and secondary metabolites), metabolism, transcription, post-translational modification, and ubiquitin-regulatory proteins with full dependence on the *Hog1*, *Ssk1*, and *Skn7* expressions [81] (check **Supplementary 2** additional information). Just as *Hog1* and *Ssk1* expressions control ergosterol biosynthesis, evidence showed that Ubc6-2 might also be involved in ergosterol synthesis because *Δubc6-2* mutants are resistant to FCZ but sensitive to AmpB. However, these mutants failed to show any increased susceptibility to osmotic and oxidative stress except for a slight sensitivity to $CdSO_4$ and FDX [81] (check **Supplementary 3** for further information).

This seems contrary to the ubiquitin-proteasome system in *S. cerevisiae*, where Ubc7p/Qri8p selectively degraded unphosphorylated Ssk1p two-component system that activated Ssk2p MAPKKK during hyperosmotic stress [215]. Though the *Δubi4* mutant of *S. cerevisiae* was hypersensitive to H_2O_2 with evidence of respiratory-induced basal production of Ubi4 in the *wt* [216], it was the *Δubc8* mutant of *C. neoformans* that showed hypersensitive to H_2O_2 [81]. The production of Ubi4p has been further shown to be induced by starvation, $CdSO_4$, genotoxic compounds, and heat shock [216]. This indicates that the roles of the ubiquitin-conjugating system are highly laudable in the antioxidative and antiosmotic stress strategies of *C. neoformans* as well. Among the downregulating genes in the *wt* exposed to oxidative- and osmotic-stress are translational and ribosomal structure-expressing genes, but these arrays of genes are unaffected in *Δhog1* mutant [81].

Further analysis showed the implication of *mbs1* expression in the resistance against 5-FC and potentially involved in thermotolerance. 5-FC induces *mbs1* transcripts in the *Δtco1* mutant, unlike *Δtco2* and *Δhog1* mutants, where the basal transcript was slightly higher than the 5-FC-induced transcript [126]. By interpretation, the interplay between Tco2p and Hog1p to regulate *Mbs1* basal transcription promotes 5-FC resistance. Nevertheless, Ko et al. had projected redundant and distinct roles of Tco1 and Tco2 kinases in modulating the Hog1-dependent phenotypic expression [81]. There is a suspicion that

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mbs1 expression, proposed to be controlled by Tco2p and Hog1p, could be modulating ergosterol synthesis in *C. neoformans* because the *Δmbs1* mutant was AmpB-sensitive but resistant to ketoconazole and FCZ through its controlling expression of *Erg11* [81]. The basal transcriptional level of *Erg11* and the cellular ergosterol level are quite higher in the *Δmbs1* mutant, just as in the *Δhog1* mutant, than in the *wt* [81]. Therefore, the low level of the membrane sterol may be the reason for polyene susceptibility, while the high activity level of lanosterol 14*α*-demethylase (encoded by *Erg11*) promotes azole resistance. The same reason predisposes *Δmbs1* and *Δhog1* mutants to membrane perturbation by SDS and osmotic stress caused by NaCl and KCl in the absence of glucose [126]. Therefore, in addition to antifungal resistance and potential thermotolerance, Mbs1p also ensures membrane stability against osmotic stress.

Because of the close association of Mbs1p with the nucleic acid to maintain DNA integrity and repair during cell growth, the *Δmbs1* mutant displayed high susceptibility to genotoxic agents such as hydroxyurea (HU) just like *Δhog1* mutant, unlike *Δtco1* and *Δtco2* mutants. However, the *Δmbs1* mutant showed resistance against methylmethanesulfonate (MMS) and thiabendazole (TBZ) but a slight resistance against TBZ in the *Δtco1* and *Δtco2* mutants [126]. Since Tco1 and Tco2 are upstream of Hog1, their presence may slightly negate TBZ-resistance, so if Hog1 and Tco2 modulatory effect on the Mbs1 is repressed, then this condition will favour TBZ-resistance. However, house-keeping expression of *Mbs1* (together with *Rad9* and *Ddc1* expression as discovered in the yeast) may still be needed to initiate a complete transcriptional response of *Rnr2* (encoding ribonucleotide reductase) during DNA damage checkpoint control initiated by genotoxic agents such as MMS [217]. The connection among these three transcription factors is evidenced in the similar susceptibility of the *Δtco2*, *Δtco1Δtco2*, *Δssk1*, *Δhog1*, and *Δtbs1* mutants to H_2O_2 and similar resistance to diamide; however, *Δtco1*, *Δtco3*, *Δtco4*, *Δtco5*, and *Δtco7* mutants showed a *wt*-resistance to H_2O_2 but *Δtco1* only slightly resistant to diamide [30,126]. Thus, the resistance to diamide might be because it is an exogenous oxidant, which failed to produce reactive radicals during the redox process, unlike the H_2O_2 that is endogenously produced with concomitant reactive radicals.

Remarkably, resistance against FDX and MG appeared to be controlled by the two-component system. Mutation of *Tco1* improves MG resistance while *Δtco2* and *Δtco1Δtco2* mutants are highly sensitive, however, reconstituted *Δtco2* mutants showed a high MG tolerance as high as *Δtco1*, *Δtco3*, *Δtco4*, and *Δtco5* but *Δtco7* and reconstituted *ΔTco1* mutants showed a *wt* MG-tolerance [30]. MG, therefore, represses *Tco* genes, but the *Tco2* gene is induced for concerted resistance effect. Just as found in the mutation of the *Hog1* pathway, FDX-tolerance is highly improved in *Δtco1*, *Δtco1Δtco2*, and moderately improved in *Δtco2* mutants but restoring the corresponding *Tco* gene into the *Δtco1* and *Δtco2* mutants drastically reduced the FDX-tolerance to the *wt* susceptibility level, which *Δtco3*, *Δtco4*, *Δtco5*, and *Δtco7* mutants naturally displayed [30]. This observation further confirmed the biological importance of Tco1 and Tco2 sensor kinases on the Hog1 regulation compared to other hybrids of sensor histidine kinase. By interpretation, the Hog1 pathway must naturally be repressed perpetually for the yeast to withstand FDX. This means that though the *Tco* genes may be less involved against the oxidative and osmotic stresses, but their roles in FDX are inevitable as they are the upstream regulatory sensors that can modulate Hog1 MAPK activity and, in so doing, negate FDX resistance.

The evidence of consistent early or late dephosphorylation of Hog1p when exposed to NaCl, H_2O_2, MG, and FDX in *Δtco1*, *Δtco2*, and *Δtco1Δtco2* mutants showed that Hog1 phosphorylation may still be initiated by some other subcellular activation systems sensitive to the same environmental cue as Tco sensor kinases or, yet some undiscovered MAPKKKK that may agitate Ssk2 kinase. Specifically, Hog1p is dephosphorylated early when *Δtco1* mutants are exposed to 1 M NaCl, MG, and FDX and when *Δtco2* mutants are exposed to MG and FDX. However, late dephosphorylation of the Hog1p is observed in *Δtco2* mutants when exposed to 1 M NaCl and when *Δtco1Δtco2* mutants are exposed to 1 M NaCl, MG, and FDX [30]. This may be the reason *Δtco2* and *Δtco1Δtco2* mutants

showed comparable defects to *Δssk1* and *Δhog1* mutants when exposed to MG and H_2O_2 and that Tco1 and Tco2 independently conjoined against FDX stress.

By inference, Tco1 and Tco2 may be redundant sensors modulating the activation of Hog1p. However, late dephosphorylation of Hog1p in *Δtco1*, *Δtco2*, and *Δtco1Δtco2* mutant could have brought about the *wt* resistance against NaCl and 15 mM MG. In the actual sense, however, the *Δhog1* mutants are hypersensitive to 1 M NaCl but moderately sensitive to 15 mM MG (check **Supplementary 3**). The Tco2 and probably along with other sensors, are solely responsible sensors against MG levels >15 mM because the *Δtco2* and *Δtco1Δtco2* are the more sensitive mutants with 20 mM MG after *Δhog1* and *Δssk1* mutants [30].

Furthermore, *Δmbs1* mutant showed the *wt* phenotypic characteristics to FDX, $CdSO_4$, and *t*-BOOH, but *Δssk1*, *Δssk2*, *Δpbs2*, and *Δhog1* mutants exhibited cadmium-tolerance by inducing cadmium-responsive genes higher than the *Δskn7* mutant and the *wt* [81]. In addition, Bahn et al. observed that *Δssk1*, *Δpbs2*, and *Δhog1* are completely resistant to FDX, but the *Δskn7* mutant with its Hog1 MAPK activation showed moderately high tolerance to FDX compared to the *wt* [30] (other phenotypic responses of mutants are in **Supplementary 3**), which means Hog1 pathway negatively influences resistance to FDX and that the function of Skn7 kinase response is parallel and independent of Hog1 pathway. Further analysis showed that all genes involved in the posttranslational modification, protein turnover, lipid transport, and secondary metabolite biosynthesis and transport are upregulated in the *wt* strain exposed to FDX. However, genes involved in the transport and metabolism of carbohydrates, nucleotides, lipids, and other metabolites are downregulated [81]. Contrary to the Hog1 pathway components, all *Δtco* mutants except *Δtco2* showed only a *wt* cadmium tolerance, similar to AmpB response [81]. This is a reminiscence that Hog1p and Tco2p control the regulation of Mbs1, and cadmium toxicity is motivated by their presence. There are extensive works on the phenotypic responses of *C. neoformans* mutants to various environmental stress, and these are summarised in **Supplementary 3**.

C. neoformans detoxify xenobiotics by expressing ABD-type multidrug homologues transporters such as Pdr5, Pdr5-2, Pdr5-3, Yor1, and Snq1. The result from Ko et al. showed a several-fold increase in Pdr5 in the *Δhog1* mutant, which explained the resistance of this mutant against FDX [81]. FDX induces specific response genes, which might explain why deletion of specific drug efflux transporter showed no significant growth defect compared to the *wt* with respect to the general stress response. Specifically, the deletion of *Pdr5* and *Yor1* increases sensitivity to FDX and FCZ (check **Supplementary 3**). There is speculation that both genes may play a redundant role regarding drug efflux; after all, *C. neoformans* has been identified to contain several copies of ABC efflux pump-related genes [81]. The *Afr1* is another ABC pump encoding transporter in *C. neoformans* that is upregulated in the *wt* when exposed to FCZ to facilitate resistance, virulence, and macrophage survival in an animal model without altering the thermotolerance and virulence-associated genes such as *Cap10*, *Cap59*, *Cap60*, *Cap64*, *Lac1*, *Plb1*, *Ure1*, *Sod1*, and *Vph1* [218]. Therefore, multiple copies of these *Pdr*-like genes might have conferred resistance against various antifungal drugs. Serendipitously, Ko et al. discovered that the cAMP-Pka signalling event is co-induced in a Hog1-dependent manner against FDX stress; however, only *Aca1* mutation yielded significant growth defect compared to the *wt* [81] (check **Supplementary 3**). This means that the deletion of *Hog1* gene must have induced the expression of *Aca1* gene component of cAMP-Pka cascade unit.

Cell-to-cell communication via the release of adhesin protein is important for cell aggregation and population density. In addition, the physiological state of the cell determines the rate of growth and cell density. Studies showed that quorum sensing-like molecules regulate not only the cell density-dependent growth in *C. neoformans* but also sexual reproduction. Quorum sensing-like molecules are biomolecules that generally arouse cell growth to higher density. Lee et al. discovered that inoculum of *MATα* serotype D *Δtup1*

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mutant at 5 x 10^6 and *MAT**a*** serotype D *Δtup1* mutant at 5 x 10^5 at 25°C and 30°C, respectively, grew as much as the *wt* followed by the isolation of an 11-*mer* peptide that was proposed as a growth factor. This *mer* autoregulated the density-dependent growth of these mutants irrespective of the media used, and any inoculum of fivefold less than this specific density stated above will thwart *Δtup1* growth [219]. This quorum sensing-like peptide 1(Qsp1) was later identified to match a putative *cqs1* gene product.

Double deletion mutant *Δtup1Δcqs1* culture was found with minimal production of Qsp1, which could barely support *Δtup1* growth, but on the contrary, the culture filtrate from *Δtup1* can support *Δtup1Δcqs1* mutant better than the self-culture filtrate [219]. The lower expression rate of Tup1 in hypoxic and other environmental conditions during exponential growth phase shines a light on the master controlling effect of Tup1p as cells transition to the stationary growth phase where *Cqs1* expression may not have a direct correlation with *Tup1* expression. In addition, Cqs1p has been implicated in the pheromone-controlling effect of bisexual reproduction and the initiation of unisexual differentiation, but these modulatory effects can be attenuated in the absence of the second Qsp2 encoded by the *cqs2* gene [220].

The extracellular release of Qsp1 and Qsp2 is catalysed by the cell-associated protease Pqp from their corresponding pro-Qsp. The mature Qsp is re-absorbed into the cytoplasm via an oligopeptide transporter, Opt, to orchestrate differential transcriptional responses such as virulence factors, sexual reproduction, cell wall biogenesis, and extracellular proteomes, which are coordinated for higher cell density. This Qsp is described as an autoregulatory peptide that gets matured extracellularly but functions in the cytoplasm [221]. Simultaneously, the production of Qsp1p is under the concerted regulation of *Gat201, Gat204,* and *Liv3* to ensure the virulence of *C. neoformans* (check the mutants in **Supplementary 1**). Apart from the attenuated infection and lower tissue burden, *Δqsp1* mutant displayed dried and wrinkled cell morphology between 25 – 30°C, and this was restored to smooth and mucoid colonies when confronted with the *wt* patch. Morphologically, the *Δliv3* mutant appeared as *Δqsp1* mutant, but it can neither be rescued by confrontation assay nor by synthetic Qsp1 supplementation; however, it can assume a *wt* appearance at 37°C. This indicates that Liv3p is probably a downstream functional protein to Qsp1p and that overexpression of liv3 bypassed all the phenotypic deformations associated with the lack of quorum peptide secretion [221].

Mutants such as *Δarf1, Δpka2, Δndh1, Δdrp1, Δcbk1, Δkic1, Δtao3, Δpgi1, Δcap10, Δcap60, Cap64, Δpbx1, Δpqp1,* and *Δshe4* have been identified with dried, hyperpolarised, actin delocalisation/mislocalisation due to perturbation and wrinkled colony-forming mutants [62]. However, mutants deficient in serine protease, an aspartyl protease, metalloprotease, carboxypeptidase, *Tco1, Tco2, Tco3, Tco4, Tco5, Tco7, Gpr1-7*, and *Cpr2* failed to produce dried and wrinkled colonies like *Δqsp1* mutant [221]. Besides, deleting any of these morphologically related genes usually increases susceptibility to immunosuppressive drugs (check the mutant responses to immunosuppressive drugs in **Supplementary 3**). Therefore, mutants compromised for Cna1- and RAM-signalling pathways will likely be inviable at >30°C in the presence of immunosuppressive drugs. A proper investigation eventually showed that RAM genes are necessary for proliferation at 37°C but not required for viability at 37°C. In the same way, dormancy at 37°C is fungistatic, but the presence of immunosuppressive drugs killed the mutants at this temperature [62].

Further analysis showed that *Qsp1* and *Opt1* are nested to each other and probably share the same promoter associated with *Gat201*, *Gat204*, and *Liv3*; therefore, *Δqsp1* and *Δopt1* mutants displayed similar phenotypic features [221]. Logically, this hypercapsulated *Δqsp1* mutant can be complemented with *Δopt1* mutant for quorum-sensing and cell aggregation because Qsp1p is produced in the *Δopt1* mutant, which means that only the *Δqsp1* mutant can respond to exogenous Qsp1p, mutant expressing Qsp1p or be complemented with the *wt* patches. The *Δqsp1* mutant is yet attenuated for infection in the pulmonary tissue but not in the CNS. Though this mutant is phenotypically hypomelanised yet, this may not justify its attenuation in the pulmonary tissue; after all, this mutant was

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hypercapsulated [221]. Thus, the attenuated infection may be due to the impacts of the deletion on various proteolytic enzymes, such as virulence-promoting aspartyl proteinase, whose secretion is significantly reduced in the *Δqsp1* mutant.

Ko et al. compared the regulatory genes that are either upregulated or downregulated among two pathogenic fungi (*C. neoformans* and *C. albicans*) with two non-pathogenic fungi (*S. cerevisiae* and *S. pombe*) to delineate the evolutionary relationship in terms of the gene-relatedness but then there were no exact common genes among these fungi that were equally regulated against osmotic stress except *Ald5, Ena1, Prm10,* and *Stl1*, which were common but differentially regulated among them [81]. Further comparison showed that *C. neoformans* deployed more genes against oxidative stress among the fungi. Again, there are no common-stress regulatory genes (osmotic and oxidative stress) common to them as well [81].

Convergently, different types of stress can eventually result in ER stress with the accumulation of unfolded or misfolded proteins around the ER, which can trigger unfolded protein response (UPR) signalling pathways. This well-conserved eukaryotic ER stress-protective pathway has been characterised in *C. neoformans.* One major transcription factor identified is Ire1, an ER stress sensor kinase similar to Tco in the Hog1 pathway. The Ire1 receives stress information to regulate other downstream transcription factors such as Hxl1/Bzp1, which is structurally and conventionally conserved within cryptococcal species but unique and diverged from other yeast Hac1 and human Xbp1 [222,223]. The Hxl1p is a **H**ac1 and **X**bp1-**l**ike bZIP transcription factor that is UPR-induced via Ire1-dependent unconventional splicing, and its absence is more devastating in *C. neoformans* than the Ire1 gene. The unconventional intron splicing of *Hxl1 m*RNA is the regulatory rate-limiting step toward UPR activation. It has been shown to be governed by Puf4, which regulates the splicing rate of *Hxl1* but can also initiate the attenuation of the UPR signalling pathway, not through Kar4 but via the *hxl1 m*RNA decay process orchestrated by Ccr4p relative to thermotolerance adaptation [223,224]. The *wt* virulence observed in *Δpuf4* mutant in murine cryptococcosis is attributed to late activation of Hxl1p to compensate for the early induction of UPR signalling and considerable growth of this mutant at physiological temperature, though a temperature of ≥ 37°C seems to attenuate the growth of this mutant [223]. On the other hand, *Δccr4* mutant has been characterised by elevated constitutive activation of UPR-related transcription factors such as Ost2, Sss1, Kar2, Per1 and many others (check **Supplementary 2** for relevant transcription factors to UPR signalling pathway). This constitutive activation, together with stabilised ER stress transcripts, may probably be the reason for thermotolerance and moderate resistance observed with *Δccr4* mutant in the presence of tunicamycin (TCM) (check **Supplementary 3**) [224].

Among the functions associated with the Ire1-Hxl1-dependent UPR pathway are the response to ER stress, cell wall integrity, thermotolerance, drug resistance, and virulence [222]. Obviously, the avirulence *Δire2* and *Δhxl1* mutants are highly susceptible to physiological temperature, ER stress agents, and cell wall destabilising agents; however, because most of the defects in the *ire1* mutant can be suppressed by the active form of Hxl1p from spliced *m*RNA, then Hxl1 is a downstream functional protein to Ire1p. Also, Ire1p activity can be Hxl1-dependent or -independent because both mutants showed differential responses to temperature, capsule synthesis, and diamide resistance. The *Δire1* mutants are defective in capsule formation, but *Δhxl1* mutants are not. Again, both mutants showed normal melanin production and resistance to H_2O_2 (check **Supplementary 3**). Non-radical forming diamide affects *Δire1* mutants only, which predicted *Ire1* gene expression to control capsule formation and diamide-resistance in an Hxl1-independent route [222].

Essentially, UPR regulates the expression of genes in response to the stress signal received from the ER, and any impairment in this tandem stress response makes the yeast inviable [179]. Among the UPR-dependent transcription factors, which are related to heat-

 doi:10.20944/preprints202301.0461.v1

and osmotic-induced ubiquitin- or proteasomal-tagged ER-mediated protein degradation, are Mga2, Kar2, Alg7, Pps1, and Sod2; however, Erv29, Ost1, Pmt1, Pmt2, Pmt4, and Wbp1, which are involved in glycosylation processes are Hxl1-dependent genes and are repressed in *Δhxl1* mutant but not in *Δire1* mutant. The Pmt4 and Chs2, which are involved in glycan formation, are significantly induced in *Δire1* mutants [222]. This means that cell wall stressors, such as Congo red and Calcofluor white repress *Ire1* expression to induce cell wall integrity and promote relevant transcription factors. Kar2 is an ER luminal Hsp70 molecular chaperone, which is needed for cell viability, and it is induced by heat shock or TCM treatment to promote and regulate UPR signalling event via Ire-Kar2 interaction [225-227]. In addition, it works together with protein disulphide isomerase (encoded by the *Pdi* gene) to bring about the folding of non-glycoproteins, while the same Pdip in the Calnexin cycle brings about the folding of glycoproteins [228]. Overexpression of kar2 partially suppressed the growth defect associated with *Δire1* and *Δhxl1* mutants in low TCM and DTT (dithiothreitol) concentrations; however, Kar2p appeared not to suppress the growth defect at higher concentrations of these ER stress-inducing agents. Apart from the Hxl1 transcription factor, Kar2p is a sub-level downstream effector protein to Ire1 sensor kinase [225].

Further investigation showed that the restoration effect of Kar2 overexpression in *Δire1* mutant seems more significant in the presence of DDT (a disulphide bridge disruptor) and diamide (thiol oxidant that induces abnormal disulphide bridge) than TCM because the tendency of forming unfolded/misfolded proteins is higher in the former two agents [225]. Furthermore, the *Δire1* mutants are more sensitive to diamide than *Δhxl1* mutants; therefore, the restoration effect of Kar2 appeared better in the *Δhxl1* mutant than the *Δire1* mutant, just like in the presence of TCM. However, the opposite is the case in the presence of DTT, where *Δire1* mutant appeared better restored. Also, because *Δhxl1* mutants are more sensitive to temperature, the restoration is better in the *Δire1* mutants, but no restoration of growth defects above 37°C. Though both mutants are hypersensitive to cell wall disruption agents; however, restoration appeared better with the *Δire1* mutant than *Δhxl1*. Collectively, Kar2 can be induced in the presence of azole and phenylpyrrole antifungals to regulate susceptibility in an *Erg*-independent manner. Though not as much as the reconstituted mutants, the overexpression of *Kar2* can partially restore growth defects in *Δire1* and *Δhxl1* mutants at 37°C as well as support the cell wall integrity, genotoxic and antifungal responses (exclusively in Ire1-dependent manner at a low concentration of each antifungal). However, Kar2 appeared not to have any significant effect on the Ire1-mediated capsule production [225].

3.7. Osmotic Shock

Every eukaryotic cell has designed various mechanisms to ensure osmoregulation within the cytoplasm. An imbalance between the solute concentration and water content within the cell results in osmotic shock/stress. *C. neoformans* has evolved a unique mechanism to address solute fluctuation in the cytoplasm. This function is majorly centred on the phosphorylation-dephosphorylation relay activities of Pbs MAPKK and phosphotyrosine phosphatase (Ptp)/phosphoserine or phosphothreonine phosphatase (Ptc) associated with Hog1 protein. The Hog1 activity is primarily under the negative feedback repression of distinctive but redundant Ptp1 and Ptp2 and the downstream Aft1 regulator [149]. The Ptp generally promotes Hog1 dephosphorylation, thereby reducing the hyperphosphorylation of this p38-MAPK transcription factor. This phosphatase also promotes thermotolerance, osmotic, and oxidative stress resistance in *C. neoformans* via the Atf1p downstream regulator. Further evidence showed that both Ptp1p and Ptp2p localised more to the nucleus than the cytoplasm to anchor the nuclear transient movement of Hog1p in a stressed cellular state [229].

Hog1 MAPK is primarily responsible for the osmotic-stress response, and this becomes more effective as the level of constitutive phosphorylation of the Hog1p increases

 doi:10.20944/preprints202301.0461.v1

[81]. The expression of glycerol-3-phosphatase encoded by *Gpp1* and glycerol-3-phosphate dehydrogenase encoded by *Gpd1* is significantly linked to osmotic-stress response, and this expression has been shown to be 2-fold reduced in *Δhog1* and *Δssk1* mutants [81]. With the evidence of overlapping gene regulation, the Hog1 and Ssk1 expressions significantly controlled *C. neoformans* genes under stressed and unstressed conditions more than the Skn7 – a response regulator kinase, like Ssk1, activated by Tco system [81]. Ample of these genes are environmental stress regulatory (*ESR*) genes, common stress regulatory (*CSR*) genes, and stress-specific regulatory (*SSR*) genes. Apart from this, Ssk1p and Hog1p can independently regulate other cellular responses.

According to Bahn et al., the Hog1p of serotype A is constantly in the phosphorylated state (Hog1-P) in a normal cellular state; however, phosphorylation of the Hog1 in serotype D is induced by osmotic stress [32]. With respect to Tco sensor kinases, Hog1p, in the absence of *Tco1* expression, is generally under dephosphorylation, but activation of Hog1 by dephosphorylation is delayed in the *Δtco1Δtco2* or *Δtco2* mutants, which possibly means that regulation of Hog1p may partly rely on the distal Tco2 sensor kinase via the Ssk1-Ssk2 MAPKKK signalling [149]. Generally, stress response is an energy-consuming process due to fashionably coordinated MAPK. Therefore, less attention is given to other high-energy consuming metabolisms, which leads to their genes being downregulated during the osmotic-stress response by *C. neoformans*.

The transient translocation of Hog1-P into serotype A nucleus ensures that the transcription initiation for virulence genes and mating is perpetually repressed. At the same time, osmotic stress induces dephosphorylation of the Hog1-P to promote adaptation to the osmotic shock via a concomitant activation of *Ena1* (encoding a putative P-type ATPase Na^+ pump), *Nha1* (encoding a Na^+/H^+ antiporter), and *Aqp1* (encoding aquaporin) genes for osmoregulation and ionic balance. Osmotically, 1 M sorbitol could only and transiently induce Ena1 but no effect on Nha1; likewise, 1 M NaCl or KCl is enough to induce Ena1 and Nha1 with higher induction in the formal than the latter [193]. This shows that under the control of the Hog1 pathway, the two transporters are involved in the Na^+/K^+ efflux in *C. neoformans* to maintain ionic homeostasis, but only Ena1 is involved in osmotic shock response. Specifically, both Ena1 and Nha1 are essential for the less toxic K^+ homeostasis, but only Ena1 is majorly involved in toxic Na^+ and Li^+ cation homeostasis. Also, none of them is involved in Ca^{2+} homeostasis [193]. Regarding the environmental influence, Ena1 is induced in an alkaline medium where the concentration of the H^+ is low to control Na^+ or K^+ stress. Nha1 is otherwise induced in an acidic environment to control K^+ stress, while *Ena1* expression is dispensable under this condition. Surprisingly, Jung et al. actually discovered that though Nha1p seemed to be active in the acidic medium, its induction was not strongly induced in the acidic medium *per se* under K^+ stress [193].

Furthermore, apart from maintaining the intracellular pH (H^+ homeostasis across plasma membrane), these membrane-located cation transporters, though not really involved in cell wall integrity but are required to maintain membrane potential, integrity, and stability for an effective antifungal counterattack under a moderate to low level production of capsule and melanin. Like *Δhog1* mutants, deletion of *Ena1* and *Nha1* cumulatively improved capsule and melanin production at 37°C and 30°C, respectively. From Jung et al. observation, capsule diameter/volume marginally increased from *Δnha1*, *Δena1* to *Δena1Δnha1* mutants, with the double mutants having as much as a 2.8-fold increase in the *Lac1* transcripts after 2 hours of incubation in YNB medium at 30°C. Nevertheless, none of these cationic transporters played any significant role in cell differentiation and mating, unlike Hog1 MAPK [193].

In terms of infection, *C. neoformans* seems to shut down completely the expression of *Nha1* but not *Ena1* for virulence factors. The expression of *Nha1* is dispensable for virulence probably because the activating condition of *Nha1* is scarcely experienced *in vivo*, but *Ena1* expression is highly needed partly because of the alkaline systemic condition and micro-alkaline environment created by the *C. neoformans* urease activity [192,193,230].

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This observation seems very subtle with *C. neoformans* during infection to reduce the cumulative effect of the two redundant cationic transporters on the virulence expression while maintaining ionic homeostasis with Ena1p and other essential ionic transporters such as Cft1, Pho84, Vph1, Ccc1, and Ctr1. Thus, the avirulence features of *Δena1* and *Δena1Δnha1* mutants in an animal model of cryptococcosis seem to come from impaired tissue dissemination and persistence but not systemic clearance.

The *Δhog1* mutants are known to resist azole because of the increased expression of the *Erg* genes but are sensitive to polyene. One could have expected the same for the *Δena1* and *Δnha1* mutants because both are under the regulation of the Hog1 pathway; however, the *Δena1Δnha1* mutant appeared highly sensitive to azole and polyene antifungal drugs more than the *Δhog1* mutants in a manner independent of ergosterol biosynthesis [193]. It is not surprising, therefore, to observe hypersensitivity to Hygromycin B in *Δhog1* mutants but hyper-resistance in *Δena1*, *Δnha1*, and *Δena1Δnha1* mutants because the presence of cationic transporter allows for the hyper-polarisation caused by Hygromycin B in the *Δhog1* mutants [193].

To avoid the redundancy of transcription factors and the bioeconomical loss of metabolites, any environmental cue that induces osmotic stress may likely repress the formation of virulence factors in *C. neoformans*. No wonder the mutation of any Hog1 pathway-associated transcription factors promotes oxidative stress and reduces osmo-tolerance, yet such mutations enormously enhance mating and the formation of virulence factors. The *skn7* mutation, however, has no significant contribution to capsule production but melanin [30]. Contrarily, Hog1-P is continuously retained in the nucleus of serotype D after osmotic stress to initiate adaptation [32]. This means that the phosphorylation-dephosphorylation relay activity of Hog1p is differentially regulated in different serotypes to achieve the same goal. Interestingly, the upstream activity of Pbs2-MAPKK to phosphorylate Hog1 protein is highly conserved and inevitable in the functioning of the Hog1 cascade event. Therefore, any mutation in the *Pbs2* gene will produce the same phenotypic defect as the *Δhog1* mutant strain.

The *Δena1* mutants are highly sensitive to osmotic stress caused by KCl and NaCl, especially in the absence of glucose, but in the presence of glucose, *Δena1* mutants showed a *wt* resistance to osmotic stress caused by KCl and oxidative stress caused by H_2O_2 [81]. This indicates that Ena1p may not be needed against oxidative stress under normal conditions. Similar to *Δcna1*, *Δena1* mutants are susceptible to alkaline pH and are upregulated at high pH in the *wt* [192]. Hyperosmolarity also upregulates the expression of *Hnm1* (encoding putative high-affinity choline/ethanolamine transporter/permease) to cope with common environmental stress [81]. The upregulation of *Hnm1*, as observed in *S. cerevisiae*, may lead to a high turnover rate of membrane phosphatidylcholine to glycerophosphocholine in the presence of choline [231]. This glycerophosphocholine is an osmoprotective accumulated methylamine, earlier identified in Madin-Darby Canine Kidney renal (MDCK) cell line in the presence of high salt content and urea [232]. Perhaps, *C. neoformans* may be using a similar mechanism to defend against the high salinity and the urea content of the bird droppings.

In the *wt* strain, osmotic stress downregulates the expression of genes involved in carbohydrate metabolism, as evidenced in the reduced transcript level of *Gal2*, *Hxt5*, *Hxt13*, and *Hxt17* and every other gene involved in actin/cytoskeleton formation, signal transduction, intracellular trafficking/secretion, and vesicular transport but because of the proximity of ionic homeostasis to osmotic stress, *Cfo1* and *Fre2* are concurrently upregulated [81]. In addition, diverse transporters and permeases such as Dur3, Mep2/Amt2, Stl1, Aqy1, Aqp1, Pho84, and Qdr1 are all activated to allow for the movement of osmolytes in a mechanism to promote cellular homeostasis during osmotic stress [81]. Therefore, the expression of all these specific osmotic stress regulatory genes is automatically let down in *Δhog1* and *Δssk1* mutants exposed to osmotic stress caused by NaCl and KCl. Unfortunately, but strategically, *C. neoformans* failed to deploy Pdr-like ABC efflux pumps against

 doi:10.20944/preprints202301.0461.v1

general osmotic stress in the presence or absence of glucose, oxidative stress, UV, and metal-induced stress, except against high concentrations of FDX and FCZ.

Lastly, the *Δtco1* and *Δtco2* mutants showed a *wt* resistance to osmotic stress caused by 1.5 M KCl. While *Δtco1* was resistant to 0.05% SDS, *Δtco2* mutant was relatively susceptible [126]. Though there was no significant effect of H_2O_2, UV exposure, high temperature, MG, and 1 – 1.5 M KCl osmotic stress on the *Δskn7* mutant yet, the mutant showed extreme sensitivity to as low as 1 M NaCl in YPD media despite a similar *wt* Hog1p activation in this mutant [30]. This re-emphasises the scrupulous involvement of *Skn7* expression in Na^+-tolerance. On the contrary, Ko et al. observed a *wt* growth of *Δskn7* to KCl only in the glucose-deprived media but a complete hypersensitivity to KCl and NaCl in YPD and glucose-deprived media, respectively [81] (check **Supplementary 3** mutant responses to osmolytes). In view of this, *C. neoformans* has shown an intricately networking yet highly regulating transcription factors in response to stresses.

6. Conclusions and Perspectives

Signalling cascades, wired by intracellular proteins, as induced by various environmental factors are the basis for expressing multiple transcription factors that facilitate responses to immediate inconveniences. This review identified different clusters of transcription factors and extensively discussed their biological functions and associated phenotypic deficiencies. Furthermore, we identified cascades of events that showed the interconnection of these transcription factors and how they coordinate and regulate phenotypic traits and cellular responses to nutrients, light, oxidative and nitrosative stressors, disruptive membrane dyes/detergent, antifungals, CO_2, pH, temperature, genotoxic, and cytotoxic compounds. These phenotypic characteristics manifest in virulent expressions, such as capsule, melanin, secretory, and hydrolytic enzymes. Besides, with the recruitment of these transcription factors, pathogenic *Cryptococcus* species invariably develop resistance to temperature, pH, nutrients depletion, oxidative and osmotic stress, phagocytosis, irradiation, and antifungals.

Strategically, *C. neoformans* cautiously repress some genes for others to function, while some are co-expressed for survival, adaptation, and infection. Being dominantly haploid, the mating of different serotypes facilitates survival, adaptation, aneuploidy, and genetic variation among the basidiospores. Natural diploid isolate also exists after the unisexual activity of the haploids. This provides the basis for gene segregation, redundancy, and ploidy. All these are carefully controlled under the regulation of different transcription factors related to mating, filamentation, and monokaryotic fruiting.

Targeting these key transcription factors by gene disruption or deletion has enabled an understanding of how this pathogenic/opportunistic fungus switches the arrays of genes to augment the deficiencies or give in to the impeding factors in some instances where such deletion is critical to cellular activity. It seems much is known yet there are transcription factors whose functions are unknown and their relevance to human infection remains folded. With this extensive review work, the importance of numerous but specific transcription factors is further expatiated, grouped , and linked together based on their functional expression regarding survival, adaptation, and human infection. This article provides adequate information to understand the cellular framework of *C. neoformans* relative to other fungi and offers insight into attractive transcription factors suitable for a therapeutic approach in drug targeting for more effective management and control of cryptococcal infection, especially in immunocompromised and organ-recipient individuals. With this, the antifungal chemotherapy will go beyond the classical ergosterol and translational process blockers.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, File: Genes, Transcription Factors, Protein Kinases, Intracellular Proteins, and Other Abbreviations as Used in the Article.

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

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

Institutional Review Board Statement: "Not applicable."

Informed Consent Statement: "Not applicable."

Data Availability Statement: "Not applicable."

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

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

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