**N-acetylglucosamine affects *Cryptococcus neoformans* cell-wall composition and melanin architecture**

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**Abstract**

*Cryptococcus neoformans* is an environmental fungus that belongs to the phylum *Basidiomycetes* and is a major pathogen in immunocompromised patients. The ability of *C. neoformans* to produce melanin pigments represents its second most important virulence factor, after the presence of a polysaccharide capsule. Both the capsule and melanin are closely associated with the fungal cell wall, a complex structure that is essential for maintaining cell morphology and viability under conditions of stress. The amino sugar N-acetylglucosamine (GlcNAc) is a key constituent of the cell-wall chitin and is used for both N-linked glycosylation and GPI anchor synthesis. Recent studies have suggested additional roles for GlcNAc as an activator and mediator of cellular signalling in fungal and plant cells. Furthermore, chitin and chitosan polysaccharides interact with melanin pigments in the cell wall and have been found to be essential for melanization. Despite the importance of melanin, its molecular structure remains unresolved; however, we previously obtained critical insights using advanced nuclear magnetic resonance (NMR) and imaging techniques. In this study, we investigated the effect of GlcNAc supplementation on cryptococcal cell-wall composition and melanization. *C. neoformans* was able to metabolize GlcNAc as a sole source of carbon and nitrogen, indicating a capacity to use a component of a highly abundant polymer in the biospherenutritionally. *C. neoformans* cells grown with GlcNAc manifested changes in the chitosan cell-wall content, cell-wall thickness and capsule size. Supplementing cultures with isotopically labeled GlcNAc demonstrated that the exogenous monomer serves as a building block for chitin/chitosan and is incorporated into the cell wall. The altered chitin-to-chitosan ratio had no negative effects on the mother–daughter cell separation; growth with GlcNAc affected the fungal cell-wall scaffold, resulting in increased melanin deposition and assembly. In summary, GlcNAc supplementation had pleiotropic effects on cell-wall and melanin architectures, and thus established its capacity to perturb these structures, a property that could prove useful for metabolic tracking studies.

**INTRODUCTION**

*Cryptococcus neoformans* is an encapsulated yeast with global distribution that has emerged as a major opportunistic fungal pathogen affecting immunocompromised patients [1]. Cryptococcal infection of the central nervous system (CNS) typically presents as meningoencephalitis, which causes up to 20% of deaths in HIV-infected patients from the developing countries of sub-Saharan Africa [2]. In the United States, the annual incidence of cryptococcosis has decreased to 2–7 cases per 1000 HIV-infected persons; however, up to 89% of those infected will develop a deadly CNS manifestation [3]. This organism has several traits that contribute to virulence, including the ability to grow at 37°C, the presence of a polysaccharide capsule and the production of melanin pigments. The capsule and melanin are intimately associated with the fungal cell wall, and together they are considered to be the two most relevant factors contributing to *C. neoformans* virulence [4, 5]. The polysaccharide capsule is composed primarily of glucuronoxylomannan (GXM) (90%) and galactoxylomannan (GalXM), with small contributions from mannanproteins [6]. The capsule surrounds the exterior of the cell wall and...
is anchored to it by an interaction of GXM with α-1,3-glucan [7, 8], chitin and chitosan [9, 10]. On the other hand, melanin synthesis in *C. neoformans* occurs at the plasma membrane within lipid vesicles, known as fungal melanosomes, which then transit to the cell wall, where melanin pigments are deposited [11, 12].

The fungal cell wall is a flexible and dynamic structure that is critical for viability, and often comprises the interface for interactions between pathogenic fungi and their hosts. Fungal cell walls are composed of glucans, chitin and glycosylated proteins. Many of these constituents are specific to the gal cell walls are composed of glucans, chitin and glycosylated proteins. Many of these constituents are specific to the cell wall [13, 14]. In fact, these biopolymer assemblies can form either long thin microfibrils or short thick rodlets, suggesting that particular morphological variants of chitin could have distinctive structural roles at particular cellular locations [15]. The cryptococcal cell wall also contains significant amounts of chitosan, the deacetylated form of chitin, which is produced by chitin deacetylase enzymes via the removal of acetyl groups from nascent chitin polymers [16, 17]. During *C. neoformans* vegetative growth, the chitin produced by the chitin synthase (Chs3) and the chitin synthase regulator (Csr2) is deacetylated to chitosan by up to three chitin-specific enzymes known as chitin deacetylases (Cda1, Cda2 and Cda3) [18, 19]. Strains of *C. neoformans* lacking CHS3, CSR2, or CDA1, CDA2 and CDA3 have significantly reduced chitosan levels, greater sensitivity to diverse cell-wall stressors and altered mother–daughter cell separation – showing that chitosan is essential for cell-wall integrity. Furthermore, strains such as ΔCHS3, ΔCSR2 and ΔCDA1/ΔCDA2/ΔCDA3 are unable to retain the melanin pigment within the cell wall, instead displaying a ‘leaky melanin’ phenotype [18, 19].

Melanins are natural pigments synthesized by members of all biological kingdoms by polymerizing phenolic and/or indolic compounds, resulting in negatively charged, hydrophobic and acid-resistant materials with remarkable capabilities, including energy transduction and the capture of electromagnetic radiation [20]. As noted above, the ability of *C. neoformans* to produce melanin is strongly associated with virulence and resistance to antifungal agents [21, 22]. This fungus also provides a unique system for the study of melanin biology because pigment synthesis occurs exclusively upon the provision of exogenous catecholamines during growth [12, 23]. Despite the insolubility and structural heterogeneity of the resulting melanin pigments, high-resolution solid-state nuclear magnetic resonance (ssNMR) has proven to be a powerful method that provides insights into the molecular architecture of fungal eumelanins [24–28]. Notably, our ssNMR experiments have demonstrated that a matrix composed of cell-wall components derived from polysaccharides, including chitin, and tightly associated lipid membrane constituents, serves as the scaffold for the layered deposition of the aromatic-based pigments and the formation of a chemically resistant biopolymer complex. Moreover, we obtained two-dimensional ssNMR evidence supporting the hypothesis that covalent linkages were formed between carbons of the melanin pigment and either chitinous or membrane-derived constituents [25].

These links between the formation of a cell-wall scaffold and melanin deposition in *C. neoformans* dovetail with previous studies of *Candida albicans* [29]: the disruption of a chitin synthase inhibited melanin externalization along the cell-wall surface, whereas the addition of N-acetylglcosamine (GlcNAc) to the culture medium boosted melanin production and promoted its extrusion. The generality of intimate associations between chitin and melanin has also been illustrated by diverse organisms in nature, including honeybees [30], damselflies [31] and marine invertebrates [32].

In the current study, we evaluated the effect of the monosaccharide GlcNAc on the cryptococcal cell-wall architecture and composition. Our hypothesis was that supplementation with GlcNAc would alter the synthesis and subsequent deacetylation of the chitin biopolymer, affect the cell-wall structure through changes to the chitin-to-chitosan ratio and impact on the molecular interactions involved in melanin assembly and deposition.

**METHODS**

**Fungal strains and culture conditions**

*C. neoformans* serotype A strain H99 (ATCC 208821) and *C. albicans* (ATCC MYA-2876) were used in this study. Sabouraud dextrose broth medium was used for standard growth of yeast cells at 30 °C with moderate shaking (120 r.p.m.). The yeast cells were kept frozen in glycerol and subcultured at the time of the experiment.

**GlcNAc supplementation**

*C. neoformans* yeast cells were grown from glycerol stocks in Sabouraud dextrose broth for 48 h at 30 °C. The yeast cells were washed three times with phosphate-buffered saline (PBS; pH 7.4), counted using a hemocytometer and adjusted to a cell concentration of 1×10⁷ cell ml⁻¹ in minimal media (MM) (29.4 mM KH₂PO₄, 10 mM MgSO₄, 13 mM glucose, 3 µM thiamine, 15 mM glucose, pH 5.5). For supplementation studies, individual 100 ml cultures were grown in MM supplemented with 1.0 and 5.0 mM GlcNAc (Sigma-Aldrich) conducted at 30 °C and 120 r.p.m. for 96 h. Post-GlcNAc supplementation, 1 ml aliquots of each culture were taken for morphological measurements and antifungal susceptibility testing. Then, powdered L-DOPA (Sigma-Aldrich) was added to each culture at a concentration of 1 mM; further incubation was conducted at 30 °C and 120 r.p.m. for 72 h in darkness. Post-melanization, 1 ml aliquots of each culture were collected for further morphological analyses by transmission electron microscopy (TEM). A
non-supplemented culture served as a control. To verify the monosaccharide incorporation into the cell wall, $^{15}$N-enriched GlcNAc (cat no. GLC-003, Omicron Biochemicals, Inc, USA) was substituted in supplementation cultures. As a control, cultures without the $^{15}$N-enriched supplement were also prepared.

**Cell-wall staining and microscopy**

Post-GlcNAc supplementation in MM, two separate aliquots of each culture were taken for qualitative estimation of cell-wall chitin and chitosan using Uvitex 2B (Polysciences, Inc) and Eosin Y (Sigma-Aldrich), respectively. For Uvitex 2B staining, cells were pelleted and washed three times with 1 ml of PBS (pH 7.4). The pellet was resuspended in 1 ml of PBS and stained with 5 µl of Uvitex 2B (stock 20 mg ml$^{-1}$). Cells were incubated at room temperature in the dark for 10 min. Excess dye was removed by two washes with 1 ml of PBS before resuspension in PBS. The cells were examined with an Olympus AX70 microscope (Olympus America, NY, USA) by using a DAPI (4',6'-diamidino-2-phenyl-indole) filter and a 100× objective with oil immersion. Eosin Y staining was performed following the protocol described by Baker et al. [19]. The cells were examined with an Olympus AX70 microscope by using a fluorescein iso-thiocyanate filter and a 100× objective with oil immersion. Images were taken with a digital camera using QCapture software [33].

**Cell-wall chitin and chitosan content**

To measure the chitin and chitosan cell-wall content, 30 ml aliquots of each culture were taken after 96 h of GlcNAc supplementation. Cell-wall chitin and chitosan content were determined according to the method of Smith and Gilkerson [34]. One aliquot was subjected to deacetylation with $0.5\text{ M }\text{HCl}$ in a heating block at 110 °C for 2 h prior to the colorimetric reaction to account for the total amount of glucosamine stemming from chitin plus chitosan; the second aliquot was not heated in order to measure only chitosan-derived glucosamine residues. The chitin content was calculated by subtracting measurements from these two aliquots. Each aliquot was then subjected to a colorimetric reaction that was highly specific for hexosamines (amino sugars), in which treatment with 3-methyl-2-benzothiazolone hydrazine hydrochloride (MBTH, Sigma-Aldrich) under mildly acidic conditions was used to measure glucosamine. A 200 µl aliquot from each sample was transferred to a 96-well plate and the absorbance at 650 nm was recorded. Concentrations were determined by comparison to a standard curve prepared from a 1 mM GlcNAc stock (Sigma-Aldrich), ranging from 0 to 100 nmol. Because *C. albicans* does not code for the proteins necessary to produce chitosan, a 30 ml culture of yeast cells grown in Sabouraud dextrose broth medium was also analysed as an internal control [35].

**Cell body and capsule size**

A 1 ml aliquot of each *C. neoformans* culture was pelleted to collect the yeast cells, washed three times with PBS (pH 7.4) and suspended in India ink (Remel Bactidrop, Lenexa, KS, USA). Each aliquot was then subjected to a colorimetric reaction according to the method of Smith and Gilkerson [34]. Cells were incubated at room temperature in the dark for 10 min. Excess dye was removed by two washes with 1 ml of PBS before resuspension in PBS. Cells were collected by centrifugation, resuspended in 2 ml of PBS and divided into two equal fractions per sample. As a control, enzymatic determination and protein measurement were performed. To quantify laccase activity, 100 µl of each sample was placed in a 96-well clear flat-bottom plate, followed by the addition of 7 µl of 20 mM L-DOPA. The reaction was incubated at 30 °C for 18 h in a SpectraMax M2 microplate reader (Molecular Devices, USA). Optical density was measured at 450 nm every 15 min; the settings included moderate shaking 15 s before each reading was recorded. Protein concentration of the extracts was determined by comparison to a standard curve prepared from a 1 mM GlcNAc stock (Sigma-Aldrich), ranging from 0 to 100 nmol. Because *C. albicans* does not code for the proteins necessary to produce chitosan, a 30 ml culture of yeast cells grown in Sabouraud dextrose broth medium was also analysed as an internal control [35].

**Laccase activity**

Laccase activity was measured following the protocol previously described by Garcia-Rodas et al. [36]. Briefly, 10 ml aliquots were obtained from GlcNAc-supplemented cultures both prior to and after melanization. Cells were collected by centrifugation, resuspended in 2 ml of PBS and divided into two equal fractions per sample. As a control, enzymatic activity was inactivated in one fraction by incubation with 5% β-mercaptoethanol for 2 h at 37 °C. Both fractions were transferred to 2 ml tubes containing 1 mm silica spheres (Lysing Matrix C, MP Biomedicals, USA) and disrupted using a Fast Prep homogenizer (FP120, Thermo Savant). Cell lysis involved performing four cycles (speed 6 for 30 s) with intervals of 4 min on ice. The cellular mixture was centrifuged at 13 800 r.p.m. for 10 min at 4 °C. The supernatants were transferred into new tubes and stored at 4 °C until enzymatic determination and protein measurement were performed. To quantify laccase activity, 100 µl of each sample was placed in a 96-well clear flat-bottom plate, followed by the addition of 7 µl of 20 mM L-DOPA. The reaction was incubated at 30 °C for 18 h in a SpectraMax M2 microplate reader (Molecular Devices, USA). Optical density was measured at 450 nm every 15 min; the settings included moderate shaking 15 s before each reading was recorded. Protein concentration of the extracts was determined with the bicinchoninic acid method using the micro BCA protein assay kit (cat no. 23235, Thermo Scientific). Specific activity was expressed as mU Abs/min/µg protein.

**Rate of colony darkening**

Cells from GlcNAc-supplemented *C. neoformans* cultures were collected by centrifugation, washed three times with PBS and adjusted to $1 \times 10^7$ cell ml$^{-1}$ in PBS before 10-fold dilutions were made. For each level of GlcNAc supplementation, 3 µl from each of the 10-fold dilutions was spotted...
on solid MM containing l-DOPA. The plates were incubated at 30 °C and pictures were taken daily in order to monitor melanin production.

**Susceptibility to oxidative and nitrosative stress**

The response of melanized *C. neoformans* cells to oxidative and nitrosative stress was also assessed post-GlcNAC supplementation using an adapted protocol [36, 37]. After 72 h of melanization, cells from 2 ml aliquots of each *C. neoformans* culture were collected by centrifugation, washed twice with and resuspended in PBS, and adjusted to a final density of 2×10⁵ cells ml⁻¹. In separate tubes, hydrogen peroxide (H₂O₂) (Sigma cat no. 216763) or sodium nitrite (NaNO₂) were added to each cell suspension to final concentrations of 0.5, 1 or 2 mM and 2, 4 or 8 mM, respectively. The tubes were then incubated at 30 °C for 1 h. Samples without treatment were incubated in parallel as controls. Next, 50 μl from each cell suspension was plated on YPD plates. After incubation at 30 °C for 2 days, the number of colony-forming units (c.f.u.) was counted and viability was calculated as the percentage of colonies obtained in treated samples in comparison to untreated controls. Non-melanized cells with and without treatment were included as internal controls.

**Melanin isolation**

Melanin isolation was performed as described by Chatterjee et al. [26]. This multi-step procedure, which includes the enzymatic digestion of cell-wall components, protein denaturation and subsequent degradation, dilipidation and acid hydrolysis, results in solid yeast-shaped particles lacking all capsule and internal structures, which are customarily designated as 'melanin ghosts'. The dry weight of the ghosts isolated from each GlcNAC-supplemented culture was determined and compared to the wet weight of the pellet showing melanized cells used as the initial material for the isolation protocol. All samples were processed in an identical fashion, and thus the residual water content of the cell pellets was considered to be equivalent.

**In vitro antifungal drug susceptibility of *Cryptococcus neoformans***

*C. neoformans* supplemented with GlcNAC was grown as described above. Stock solutions of four drugs were prepared at 10 000 μg ml⁻¹ in dimethyl sulfoxide (DMSO; Fisher Scientific). Aliquots consisting of 100 μl of the working antifungal solutions were inoculated into 96-well round-bottom microtiter plates, followed by 100 μl of the cellular inoculum suspension. The drugs used were nikkomycin, voriconazole, fluconazole and caspofungin (Sigma-Aldrich). When combined with the inoculum suspension, the final concentrations ranged from 100 to 0.78 μg ml⁻¹. Growth and sterility controls were included for each isolate tested, and we used *C. albicans* SC5314 as a reference quality control strain in every batch. The microlidulation plates were incubated at 37 °C and 180 r.p.m. for 48 h to determine the minimum inhibitory concentration (MIC). The plates were analysed by measuring the absorbance at 492 nm using a spectrophotometer (Genesys 10uv, Spectronic Unicam, Rochester, NY, USA). Antifungal susceptibility testing was performed according to the Clinical and Laboratory Standards Institute guidelines contained in the M38-A2 document and EUCAST protocol.

**Solid-state nuclear magnetic resonance (ssNMR) spectroscopy**

Solid-state NMR spectra were acquired on a Varian (Agilent) DirectDrive2 (DD2) spectrometer operating at a resonance frequency of 600 MHz for ¹H and equipped with a 1.6 mm FastMAS probe (Agilent Technologies, Santa Clara, CA, USA). All measurements were carried out at a magic-angle spinning (MAS) rate of 15.00±0.02 kHz and a nominal spectrometer-set temperature of 25 °C. To determine the fate of exogenous GlcNAC provided in culture media, ¹⁵N cross-polarization (CPMAS) experiments were performed on ~4–6 mg of alkaline-extracted lyophilized cell-wall material isolated from *C. neoformans* cells supplemented with ¹⁵N-enriched GlcNAC. The typical 90° pulse lengths were 1.6 and 2.9 μs for ¹H and ¹⁵N, respectively. Heteronuclear ¹H decoupling with a field strength of 78 kHz was applied using the SPINAL method [38]. As controls, samples of ¹⁵N-GlcNAC standard (starting material) and alkaline-extracted cell-wall material isolated from a culture with no GlcNAC supplementation were also analysed. To estimate the relative amounts of melanized carbon-containing moieties in GlcNAC-supplemented cells, ¹³C direct-polarization (DPMAS) experiments were performed on ~5–8 mg of melanin ghosts. The typical 90° pulse lengths were 1.2 and 1.4 μs for ¹H and ¹³C, respectively. Heteronuclear ¹H decoupling with a field strength of 78 kHz was applied using the SPINAL method. Long recycle delays (50 s) were implemented to obtain quantitatively reliable peak intensities that allowed the integration of defined spectral regions using the GNU image manipulation program (GIMP).

**Transmission electron microscopy (TEM)**

Aliquots of *C. neoformans* supplemented with GlcNAC before and after melanization were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer. The cells were treated with osmium tetroxide and serially dehydrated. Samples were embedded in epoxy resin and ultrathin sections were obtained, stained with uranyl acetate and lead citrate, and observed with a Philips/FEI BioTwin CM120 transmission electron microscope. Average cell wall thickness and capsule size were obtained with ImageJ software (Fiji) [33] by measuring 7–10 cells per condition (five measurements per cell).

**Statistical analyses**

Statistical analyses were performed using GraphPad Prism version 6.00 for Mac OS X (GraphPad Software, San Diego, CA, USA). Chitin and chitosan data from MBTH assays were analysed using a two-way analysis of variance (ANOVA) test with a Bonferroni post hoc test. Morphological measurements with light microscopy were analysed using one-way ANOVA and Tukey’s multiple comparison test, whereas data acquired from TEM micrographs were
analysed using a Kruskal–Wallis non-parametric test. Antifungal susceptibility screens were analysed using a two-way ANOVA and Bonferroni post-test. The 90–95 % confidence interval was determined for each set of results.

**RESULTS**

**C. neoformans** can metabolize GlcNAc as a carbon and nitrogen source

The successful establishment of any unicellular microorganism in a particular environment is highly dependent on its ability to metabolize available nutrients, including sources of carbon, nitrogen and essential elements such as iron. To determine whether **C. neoformans** would tolerate and use the GlcNAc available in culture media, we assessed its growth in MM supplemented with monosaccharide. GlcNAc supplementation at levels between 0.5 and 250 mM had no negative effects upon the fungal growth at 30 °C in this medium (Fig. 1a). This result prompted us to evaluate the ability of GlcNAc to serve as a primary carbon or nitrogen source in MM by omitting the addition of glucose and L-glycine, respectively. Cells grown in glucose-free GlcNAc-supplemented MM reached a lower growth density than in the presence of both carbon sources; additionally, little or no growth was observed below a GlcNAc concentration of 5 mM (Fig. 1b). However, if L-glycine was omitted and GlcNAc represented the sole nitrogen source in the culture media (Fig. 1c), an overall increase in fungal growth density was found, suggesting that **C. neoformans** has a robust tolerance for this monosaccharide nutritional component. These findings led us to test the ability of **C. neoformans** to thrive when GlcNAc served as the exclusive source of both carbon and nitrogen (Fig. 1d). When **C. neoformans** was cultured in glucose- and L-glycine-free MM, cells supplemented with GlcNAc above the 1 mM concentration manifested an abrupt increase in growth density at 36 h. In addition, an overall higher growth density was achieved in comparison to cells cultured with glucose plus GlcNAc (Fig. 1a), indicating enhanced metabolism in the presence of GlcNAc as the sole nutritional fuel. Together, these data demonstrate that **C. neoformans** can respond rapidly to GlcNAc and use it as both a carbon and a nitrogen source.

**GlcNAc supplementation alters the fungal cell wall chitin-to-chitosan ratio**

**C. neoformans** chitin and chitosan levels are known to increase as cultures progress from the late-log to the stationary phase and vary depending on the culture medium [18, 39, 40]. In a nutrient-rich liquid medium such as YPD, **C. neoformans** chitosan levels can be three–five times higher than chitin [18]. To determine the effects of GlcNAc

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Fig. 1. **C. neoformans** can use GlcNAc as a source of carbon and nitrogen. Representative growth curves of **C. neoformans** strain H99 in minimal media (MM) with different concentrations of N-acetylglucosamine (GlcNAc) as supplementation. (a) MM plus GlcNAc. (b) MM glucose-free plus GlcNAc. (c) MM glycine-free plus GlcNAc. (d) MM glucose/glycine-free plus GlcNAc. Fungal cell growth was recorded at an optical density of 600 nm in a Bioscreen reader with continuous shaking at 30 °C for 96 h. Each growth curve was performed in two–three independent experiments with similar results. Data represent mean±SD; each point was analysed in triplicate.
Fig. 2. GlcNAc supplementation alters the C. neoformans cell-wall chitin-to-chitosan ratio. (a) Top panel, India ink staining that allows the capsule and cell body size to be visualized. Bottom panel, Uvitex 2B staining for chitin qualitative detection. (b) Bright field and eosin Y staining for chitosan staining. (c) Bar graph, biochemical determination of chitin, chitosan and the total of chitin plus chitosan using the MBTH colorimetric reaction measured at 650 nm. The data represent an average of four independent cultures for each condition. The error bars indicate standard deviations. An asterisk indicates a significant difference (P<0.05) between the chitosan content of the control and that of the supplemented samples. Two asterisks indicate a significant difference (P<0.005) between the chitin plus chitosan content of the control and the supplemented samples. Line graph, median value of chitin-to-chitosan ratio of three independent cultures for each condition. These experiments were performed after 96 h of supplementation with GlcNAc in MM at 30°C. All pictures were taken with the same exposure time as a control (at a magnification of 100x). Scale bar, 10 µm.
supplementation on the chitin and chitosan (chitinous) content of the cryptococcal cell wall, we grew C. neoformans cultures in MM with various concentrations of this monosaccharide. Post-supplementation, the amounts of cell-wall chitin and chitosan were assessed using both qualitative and quantitative assays.

Cell-wall staining with the fluorescent dyes Uvitex 2B (Fig. 2a) and eosin Y (Fig. 2b), which bind to chitin and chitosan, respectively [19, 41], revealed no changes in the chitinous cell-wall composition upon GlcNAc supplementation at any of the concentrations tested. Biochemical quantification of individual cell-wall chitin and chitosan levels was performed using a modified protocol for the MBTH reaction, a colorimetric assay highly specific for amino sugars [34]. This assay involves deacetylation of chitin to chitosan, allowing the measurement of both sugar components together as glucosamine. Cells cultured in unsupplemented MM cultures had twice the amount of cell-wall chitin content compared to chitosan, which was opposite to the trend observed in a rich medium such as YPD. GlcNAc supplementation was found to significantly increase the amount of total chitinous material (chitin plus chitosan) in the cell wall, which was attributable to an approximately 1.7-fold increase in chitosan content when compared to the non-supplemented cells (Fig. 2c). Although the chitin levels were unaffected, a progressive drop of ~40% in the chitin-to-chitosan ratio was observed when the GlcNAc supplementation was increased to 5 mM. By contrast, C. albicans lacks chitosan; supplementation promotes increased chitin levels and the extrusion of melanin pigments [29].

Exogenous GlcNAc is taken up by C. neoformans cells and utilized as a cell-wall building block

The revelation that GlcNAc-supplemented cells have altered amounts of cell-wall chitin and chitosan prompted us to determine the fate of this exogenous substrate. Although we had already established the ability of C. neoformans to use GlcNAc as a sole carbon and nitrogen source, our supplementation studies were carried out in MM containing glucose, which has the potential to effectively shift nutrient uptake and metabolism. In addition to using the MBTH assay for cell-wall chitin and chitosan quantification, this assay was also used to monitor GlcNAc depletion in supplemented culture media. C. neoformans cells grown for 96 h at 30 °C with 1 or 5 mM GlcNAc supplementation demonstrated ~77% and ~25% uptake of the additional fuel, respectively (Fig. 3a). In a separate study, cell-wall material was isolated from C. neoformans cultures supplemented with 15N-enriched GlcNAc, which allowed us to track this nutrient once internalized by the cells. These samples were analysed using 15N CP MAS NMR experiments and the resulting spectra displayed strong signals at approximately 123 and 30 p.p.m., which are characteristic of chitin and chitosan, respectively [42], and thereby strongly suggest that GlcNAc is indeed readily utilized as a precursor for cell-wall constituents (Fig. 3b).

GlcNAc induced morphological changes in the C. neoformans cell wall and influenced melanin deposition

Fungi synthesize a wide variety of polysaccharides, which constitute the major structural components of the cell wall and comprise up to 90% of its total mass [43]. Polysaccharides are arranged in close proximity to one another and with other cell-wall components, such as glycosylated proteins, lipids, pigments and inorganic salts; together they give rise to the robust architecture of the fungal cell wall [14]. Ultrastructural studies of C. neoformans cell walls have revealed the presence of a two-layered structure: a high electron-density striated inner layer and a low electron-density outer layer with a more particulate appearance [7, 44, 45]. In the current study, transmission electron micrographs showed that GlcNAc supplementation induces significant alterations in the cell-wall thickness and capsule diameter (Fig. 4a, b). These morphological changes appear to be concentration-dependent: 1 mM supplemented cells exhibited diminished capsule diameter and cell-wall thickness in comparison to non-supplemented control cells. Conversely, 5 mM GlcNAc supplementation did not affect the capsule diameter, but the cell walls were observed to be 30% thicker than in control cells, particularly in the region corresponding to the electron-dense inner layer. Chitin, chitosan and β-glucan together form an alkali-insoluble meshwork located on the inner layer of the cryptococcal cell wall that is principally responsible for imparting rigidity [14, 46]. Hence, our data suggest that 5 mM GlcNAc supplementation could be modifying the basal structural layer of the cryptococcal cell wall by augmenting chitosan levels.

Moreover, GlcNAc supplementation yielded cells that displayed significant reductions in both melanized cell-wall thickness and capsule diameter (Fig. 5a, b). The cellular location of melanin throughout the cell wall did not seem to be affected by GlcNAc supplementation, since both conditions resulted in fungal cell walls showing two melanin layers with distinctive electron density; however, the melanin layers in GlcNAc-supplemented cells appeared to become more tightly arranged across the cell wall as GlcNAc concentration was increased. The melanin deposition showed a more heterogeneous distribution in the unsupplemented control cells compared to the cells grown in the presence of GlcNAc; up to six layers could be identified. These results suggest that changes in the cell-wall chitin-to-chitosan ratio stemming from increased chitosan contributions promote a more uniform arrangement of the pigment within the cell wall.

As noted above, we previously performed ssNMR measurements on C. neoformans melanin ‘ghosts’ and demonstrated that an aliphatic framework involving glucans, chitin and ‘lipid-like’ moieties serves as a structural scaffold for deposition of the L-DOPA-derived melanin pigment [24]. In the current work, we again employed this methodology for melanin ghosts isolated from GlcNAc-supplemented cultures, using quantitatively reliable 13C DPMAS spectra to estimate
the relative amounts of indole-based pigment as compared with membrane and cell-wall constituents. The shaded peak area corresponding to the pigment aromatic moieties (110–160 p.p.m.) increased dramatically relative to the total $^{13}$C NMR signal across the spectrum (Fig. 6a), as demonstrated quantitatively by marked increases in the relative aromatic content of both GlcNAc-supplemented samples (Fig. 6b). Concurrent estimates of the melanin ghost dry weight with respect to the wet cell pellets confirmed a higher yield for the 5 mM GlcNAc-supplemented sample (Fig. 6c). The spectra in Fig. 5(a) are noteworthy because they show that the GlcNAc-supplemented samples exhibit prominent aromatic spectral contributions within a very short time frame (3 days) compared with the 14-day melanization monitored in our earlier studies [24]. Taken together, these data demonstrated that GlcNAc supplementation promotes significant changes in the cell-wall structure that favour greater melanin deposition in a condensed format, which could augment isolation of the pigmented cellular materials (melanin ghosts).

**C. neoformans melanin biosynthesis is altered by GlcNAc supplementation**

*C. neoformans* pigment formation is catalyzed via laccase activity upon the provision of exogenous melanin precursors, such as L-DOPA [47]. Thus far our data had indicated that GlcNAc supplementation both augments the chitin-to-chitosan ratio and also promotes the retention of pigment in melanin ghosts. To investigate the causality between changes witnessed in the cell-wall scaffold and melanin deposition, we measured the activity of laccase [48] from non-melanized and melanized GlcNAc-supplemented cells. In addition, we qualitatively monitored the rate of melanin production via colony darkening in GlcNAc-supplemented cells that were plated on solid MM with L-DOPA at 30°C. Prior to melanization, both 1 and 5 mM-supplemented cells were found to have depressed laccase activity in comparison to non-supplemented control cells (Fig. 7a); 5 mM GlcNAc-supplemented cells in particular showed a significant difference in laccase activity, and also displayed a delayed rate of colony darkening at 48 h, which was not evident at a later time point (Fig. 7b). Post-melanization cells from both GlcNAc-supplemented cultures showed higher laccase activity than cells from control cultures (Fig. 7c). These data support the modest reduction in the aromatic pigment content reported for the 5 mM sample by our NMR analysis (Fig. 6b) and suggest that at this level of supplementation, GlcNAc is in excess, such that it functions as a nutrient source and modulates laccase activity as has been reported for glucose [49]. Taken together, our analyses demonstrated that GlcNAc supplementation impacts on *C. neoformans* melanogenesis. This prompted us to establish the physiological relevance of these findings by probing the susceptibility of melanized GlcNAc-supplemented cells to H$_2$O$_2$ and NaNO$_2$. At both levels of GlcNAc-supplementation, the cells showed an overall increase in protection from oxidative and nitrosative damage. No significant differences were seen in comparison to control melanized cells, indicating that melanized cell-wall changes due to the GlcNAc supplementation do not jeopardize fungal viability (Fig. S1, available in the online Supplementary Material).
GlcNAc supplementation affected additional changes in phenotype and susceptibility to antifungal drugs

Recent studies in *C. albicans* demonstrated that growth on alternative carbon sources has a major impact on the fungal cell-wall architecture and drug resistance, thus promoting its adaptation to dynamic changes in the environment [50, 51]. In light of *C. neoformans*’ ability to use GlcNAc as an alternative energy source, and reports that this monosaccharide is involved in fungal and plant cell signalling [52, 53], cells grown in GlcNAc-supplemented media were challenged with diverse stress factors and assessed for *in vitro* phenotypes. Light microscopy with India ink counterstaining, complementing the TEM findings above, was used to monitor changes in cell-body morphology and capsule size (Fig. 8a, b). In the conditions tested, both concentrations of GlcNAc supplementation yielded cells with reduced body size in comparison to unsupplemented control cells, and those grown with 5 mM GlcNAc also manifested larger capsule diameters. However, when GlcNAc supplementation was extended from 4 to 8 days (Fig. S2), the cell-body size of 1 mM-supplemented cells increased, while no differences were seen in the capsule diameter at any GlcNAc concentration in comparison to the control cells.

Phenotypic variations of *C. neoformans* cells, such as augmented cell-wall and capsular polysaccharide thickness, are associated with reduced susceptibility to antifungal drugs, possibly due to insufficient drug access [54–56]. Consequently, we tested whether GlcNAc supplementation affected *C. neoformans* susceptibility to drugs that target cell-wall components (chitin and β-1,3-glucan) and the plasma membrane (ergosterol). Congruent with the increased cell wall and/or capsule thickness previously noted for the supplemented cells, GlcNAc-supplemented cells were less susceptible to fluconazole and voriconazole (Fig. 9). These drugs are major azole-type antifungals that are used in therapy against *C. neoformans* and result in the depletion of ergosterol. However, no changes were evident in the susceptibility to nikkomycin or caspofungin, which target cell wall chitin and β-1,3-glucans, respectively. Finally, we evaluated the sensitivity

![Fig. 4. The *C. neoformans* cell-wall ultrastructural architecture is remodelled by GlcNAc supplementation. (a) Representative transmission electron micrographs of *C. neoformans* post-GlcNAc supplementation, showing electron-dense structures of the cell wall (CW) and capsule (CP). (b) Measurement of cell-wall and capsule thickness. On the cell-wall panel (left), two asterisks indicate a significant difference (*P*<0.005) between the cell-wall thickness of the control and the 5 mM GlcNAc-supplemented sample. On the capsule panel (right), four asterisks indicate a significant difference (*P*<0.0001) between the capsule thickness of the control and the 1 mM GlcNAc-supplemented sample. Scale bar, 200 nm. CY, cytoplasm.](https://www.microbiologyresearch.org)
of GlcNAc-supplemented cells to cell-wall integrity (CWI) stressors (0.5 mg ml$^{-1}$ caffeine, 1.5 mg ml$^{-1}$ calcofluor white, 0.5% Congo red and 1.5 M NaCl) and the ability to produce and retain melanin pigments at 30°C. Caffeine is broadly used to probe signal transduction and CWI phenotypes; calcofluor white binds to chitin and to a lesser extent to glucan, thus disrupting the assembly of chitin microfibrils; Congo red inhibits microfibril assembly in compromised cells; NaCl tests fungal response to osmotic conditions [57]. None of these compounds produced growth defects or cell death. Finally, we tested the sensitivity of GlcNAc-supplemented cells to the development of a leaky melanin phenotype, finding none (data not shown).

DISCUSSION
Fungi can inhabit a wide variety of environments, from terrestrial surroundings such as mine drainages, bird excreta, hydrothermal vents and the Arctic through to isolated locations such as the International Space Station. *C. neoformans* is an environmental organism that requires no human or other metazoan host to survive [58]. Indeed, it is a saprophytic fungus that has evolved to sustain its life cycle within varied ecological niches, including soils and trees. *C. neoformans* is continuously exposed to a wide variety of abiotic and biotic stresses, including fluctuations in temperature, nutrient limitations, pH, osmolarity and exposure to reactive oxygen and nitrogen species [59]. It is anticipated, then, that this fungus can sense and respond to stresses through a complex network of signal transduction pathways, resulting in altered gene expression that enables the *C. neoformans* cell to adapt to and withstand the adverse environment.

An essential structural adaptation that protects the fungus against hostile environments is exemplified by the cell wall, a complex and dynamic composite of various structural polysaccharides, including chitin. Chitin is the second most abundant polymer in nature after cellulose; it serves as the primary constituent of various cellular load-bearing structures and

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Fig. 5. *C. neoformans* melanized cell-wall thickness is reduced by GlcNAc supplementation. (a) Representative transmission electron micrographs of *C. neoformans* melanized cell sections post-GlcNAc supplementation, showing layered (dash lines) melanin deposition on the cell wall (CW) and capsule size (CP). (b) Measurement of melanized cell-wall and capsule thickness. On the cell-wall panel (left), four asterisks indicate a significant difference ($P<0.0001$) between the cell-wall thickness of the control and the GlcNAc-supplemented samples. On the capsule panel (right), an asterisk indicates a significant difference ($P<0.05$) between the capsule thickness of the control and the 5 mM GlcNAc-supplemented sample. Four asterisks indicate a significant difference ($P<0.0001$) between the capsule thickness of the control and the 1 mM GlcNAc-supplemented sample. Scale bar, 200 nm. CY, cytoplasm.
contributes significantly to fungal cell-wall strength and integrity [14, 60]. The key building block of chitin polymers is the amino sugar N-acetylglucosamine (GlcNAc), which can also modify the cell-wall surface via N-linked glycosylation and the synthesis of GPI anchors. Recent studies have revealed an additional role for GlcNAc as an activator and mediator of cellular signalling (reviewed in [61]).

Whereas the current work focused principally on the effects of GlcNAc supplementation on C. neoformans cell-wall structure, we also investigated the role of this amino sugar as an alternative energy source. A precedent for such a role is provided by the uptake of GlcNAc in the model organism C. albicans, which induces a morphological transition to the virulence-associated hyphal state [62]. Moreover, this process is thought to be mediated by the up-regulation of catabolic genes in the ‘NAG regulon’, which includes a GlcNAc-specific deaminase (NAG1), deacetylase (NAG2/DAC1) and kinase (NAG5/HXK1) [63, 64]. Hence, the induction of morphological changes and associated pathogenicity in C. albicans are linked with the use of this amino sugar as an alternative fuel. The current work demonstrates that C. neoformans is also capable of metabolizing GlcNAc: this substrate can serve as both a carbon and nitrogen source in the absence of other nutrients. Since C. neoformans encodes orthologues of GlcNAc deacetylase (NAG2) and kinase (NAG5), it is then plausible that up-regulation of these genes promotes pathogenicity in this organism [65].

We also hypothesized that the presence of the exogenous GlcNAc substrate could be interpreted by the cells as a surplus of chitin polymer ‘building-blocks’, promoting biosynthesis and possibly also encouraging deacetylation to form chitosan. Alternatively, GlcNAc could be viewed as a product of chitin degradation, which has been reported to regulate some steps in chitin catabolism for C. neoformans grown in rich media [65]. The relevant steps are catalyzed...
by four endochitinases (CHI2, CHI21, CHI22 and CHI4), and one exochitinase (HEX1), the last of which is a hexosaminidase responsible for liberating GlcNAc monomers from the non-reducing end of chitin. When cells were cultured in nutrient-rich YP media prepared with 0.9 mM GlcNAc as the sole carbon source, Baker et al. [65] found that endochitinase gene transcription was unaffected while HEX1 expression was increased sevenfold.

Previous work performed in C. albicans using GlcNAc as the sole carbon source revealed that this monosaccharide induces cell death by acting as a signalling molecule that is able to regulate multiple pathways, including its own catabolism [52]. In the current study, C. neoformans grown in MM showed a higher cell-wall content of chitin compared with chitosan, which is opposite to the relationship witnessed for cells cultured in nutrient-rich YPD media. Using chemically defined MM as a base formula for GlcNAc enrichment, we found that incrementally increasing the GlcNAc concentration yielded cells with increased chitosan content but relatively unaltered amounts of chitin. Furthermore, we demonstrated that C. neoformans cells do use exogenous GlcNAc as a building block for the cell wall, namely for the production of chitin and chitosan. However, we did not directly test the potential for GlcNAc to act as a signalling molecule. Thus, we cannot exclude the possibility that the aforementioned cell wall changes are due in part to signalling processes, in addition to being reflective of GlcNAc’s role as a chitin/chitosan precursor.

Baker et al. [65] observed C. neoformans’ retention of cell-wall remodelling capabilities in a panel of chitinase deletion strains, implying that in this fungus the required changes in cell-wall flexibility and fluidity can occur even without degradation of the biopolymer. The remodelling process should allow for localized expansion of the lateral cell wall, accounting for the observed preservation of mother–daughter cell separation during vegetative growth, even when chitinase activity is absent. Thus, these authors proposed an alternative route to reduced chitin content via deacetylation to form chitosan. Our observation of 5 mM GlcNAc-supplemented cells displaying increased enzymatic activity in comparison to non-supplemented melanized control cells. The data represent the mean of results from two independent runs, with each sample analysed in duplicate. The error bars represent standard deviation. One asterisk represents a significant difference (P<0.05) between the laccase activity of the control and that of the 1 mM GlcNAc-supplemented sample.

Fig. 7. C. neoformans melanin biosynthesis is altered by GlcNAc supplementation. (a) Laccase activity of C. neoformans cells post-GlcNAc supplementation, demonstrating inhibition of the enzymatic activity on supplemented cells in comparison to control. The data represent the mean of results from one experimental trial in triplicate. The error bars represent standard deviation. A significant difference (P<0.05) between the laccase activity of the control and the 5 mM GlcNAc-supplemented sample is indicated with one asterisk. (b) Rate of colony darkening after cells were grown in media supplemented with different GlcNAc concentrations or without it, showing that 5 mM GlcNAc-supplemented cells have a noticeable delayed effect on melanin production at 48 h. A 3 µl drop of each culture adjusted to 10^6 cells ml^-1 was spotted on MM with L-DOPA agar at 30 °C. Melanin production was monitored every 24 h. These results are representative of two independent experiments. (c) Laccase activity of C. neoformans cells after melanization for 72 h, showing that both GlcNAc-supplemented cells displayed increased enzymatic activity in comparison to non-supplemented melanized control cells. The data represent the mean of results from two independent runs, with each sample analysed in duplicate. The error bars represent standard deviation. One asterisk represents a significant difference (P<0.05) between the laccase activity of the control and that of the 1 mM GlcNAc-supplemented sample.
that these cells also produced less chitin (a crystalline-like polymer) and significantly more chitosan (an inherently disordered and flexible material). The increased cell-wall thickness exhibited by supplemented cells could then be attributed to the reduced content of those polysaccharides that can form tightly packed structures.

Notably, these supplemented samples demonstrated increased resistance to azole-class drugs, which we can attribute to enlargement of the cell wall and capsular diameter, as proposed previously [54–56]. The azole derivatives voriconazole and fluconazole that were used to challenge the GlcNAc-supplemented cells exert their antifungal effects by inhibiting an intracellularly localized protein essential for sterol metabolism, lanosterol 14α-demethylase [67]. Thus, a thickened cell wall and enlarged capsule polysaccharide could conceivably prevent the drugs from reaching their target. No reduction in susceptibility was found for nikkomycin or caspofungin, which is unsurprising given that their targets are cell wall-localized, and thus only the capsule needs to be traversed for drug interaction to occur. Alternatively, changes in polysaccharide composition associated with GlcNAc supplementation could produce differential antifungal drug susceptibility by altering the fluidity or flexibility of the cell wall and capsule matrix. Taken together, the morphological changes and differential drug susceptibility witnessed for GlcNAc-supplemented C. neoformans cells highlight the ability of this fungus to adapt dynamically to changes in nutrient status by modulating its capsular and cell-wall architectures.

How do the cell-wall properties impact on melanization that is linked to fungal virulence? Prior studies have shown that chitosan deficiency in C. neoformans compromises the ability of the fungal cells to retain melanin pigments, suggesting that this biopolymer is an indispensable element of the cryptococcal cell-wall scaffold that permits melanization [18, 19]. Our work using advanced NMR and imaging techniques supports this hypothesis: (i) a chemically resistant aliphatic matrix serves as a supporting scaffold that fosters eumelanin build-up (reviewed in [68]); (ii) cell walls become thicker in melanized C. neoformans cells [69]; and (iii) the number of melanin layers translates into cell-wall thickness, which increases with the age of the cells [69, 70]. Although a chitosan-poor semicrystalline scaffold could arguably be a suboptimal support structure for melanin pigment

Fig. 8. GlcNAc supplementation leads to changes in C. neoformans cell morphology. (a) Representative images of C. neoformans with India ink counterstaining post-GlcNAc supplementation (at a magnification of x1000). Scale bar, 10 µm. (b) The morphology data show the mean of measurements for 100 cells per condition in three independent experiments for a total of 300 cells. The error bars represent standard deviations. An asterisk indicates a significant difference (P-value <0.05) between the cell-body radius of the control and the 1 mM GlcNAc-supplemented sample. Two asterisks indicate a significant difference (P-value <0.005) between the cell body radius of the control and the 5 mM GlcNAc-supplemented sample. Four asterisks indicate a significant difference (P-value <0.0001) between the capsule radius of the control and the 5 mM GlcNAc-supplemented sample.
deposition, the prior observations leave the role of the chitin-to-chitosan ratio in the regulation of melanin production and deposition open.

Chitin is a neutrally charged, highly hydrophobic, inelastic polysaccharide that is found in the exoskeleton as well as the internal structures of invertebrates [71]. In contrast to chitin and other naturally occurring neutral or acidic polysaccharides such as cellulose or dextran, chitosan is a highly basic polycation (reviewed in [60]); it dissolves in weak acids and displays mucoadhesive properties that make it a potential carrier for diverse therapeutic agents (reviewed in [72]). Chitosan’s highly cohesive nature has been attributed to electrostatic interactions (ionic crosslinking) between its positively charged backbone amine groups and the negative surface moieties displayed by lipids, proteins, or polyanionic polymers [73]. C. neoformans melanin is also negatively charged [74]; moreover it is synthesized by laccase-mediated conversion of catechols to o-quinones (reviewed in [23, 75]) that are capable of covalent cross-linking to chitosan amines [76]. These properties inform the current studies, which revealed significant reductions in cell diameter and a tightly condensed pattern of pigment deposition in melanized cell walls from GlcNAc-supplemented cultures. Our interpretation of these findings is that the increase of cell-wall chitosan content associated with GlcNAc supplementation promotes robust and uniform melanin deposition. The condensed arrangement of the pigment within the cell wall could be a consequence of enhanced chitosan–melanin electrostatic interactions and/or melanin covalent anchoring to chitosan, mediated by laccase enzymatic activity.

Finally, the finding that GlcNAc can be metabolized by C. neoformans and used as a sole nitrogen source makes possible future solid-state NMR studies to dissect melanin’s structure and its molecular interaction with cell-wall components, particularly chitin and chitosan. These polysaccharides can be distinguished by their nitrogen spectroscopic signatures in fungal cell-wall complexes (native or modified). Therefore, using an L-DOPA melanization precursor with nitrogen in

Fig. 9. C. neoformans shows increased azole resistance post-GlcNAc supplementation. C. neoformans cells post-GlcNAc supplementation were tested for antifungal susceptibility (minimum inhibitory concentration) using a microdilution protocol at 37°C, with 180 r.p.m. shaking for 48 h. Growth density was measured at 492 nm. The data represent the mean of results from two to three experimental trials. On the voriconazole panel (top right), two asterisks indicates a significant difference (P-value <0.001) between the MIC 50 of the control and 5 mM supplemented samples, three asterisks indicates a significant difference (P-value ≤0.0005) between the MIC 50 of the control and the 1 mM-supplemented sample. On the fluconazole panel (bottom left), three asterisks indicates a significant difference (P-value ≤0.0005) between the MIC 50 of the control and the 5 mM-supplemented samples.

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natural abundance along with $^{15}$N-enriched GlcNAc could reveal the chitin amide and chitosan amine contributions in C. neoformans melanin ghosts. Needless to say, the usefulness of this approach will require rigorous validation, because GlcNAc supplementation has protein concentration-dependent effects on the cell wall and melanin: a direct precursor for chinin and chitosan, a possible effector of cellular signaling, a nutritional feedstock that alters the metabolic state of the cell. Given the difficulties inherent in studying complex amorphous structures such as melanin and cell walls, and their interactions, an approach via GlcNAc supplementation that allows their perturbation and modification is welcome as a potential new tool in the very limited analytical kit that is currently available.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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