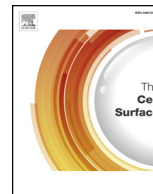




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Lack of chitin synthase genes impacts capsular architecture and cellular physiology in *Cryptococcus neoformans*

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ABSTRACT

Cryptococcus neoformans mutants lacking each of the eight putative chitin synthase genes (*CHS*) have been previously generated. However, it is still unclear how deletion of chitin synthase genes affects the cryptococcal capsule. Since the connections between chitin metabolism and capsular polysaccharides in *C. neoformans* are numerous, we analyzed the effects of deletion of *CHS* genes on capsular and capsule-related structures of *C. neoformans*. *CHS* deletion affected capsular morphology in multiple ways, as determined by scanning electron microscopy and immunofluorescence analysis. Molecular diameter, serological reactivity and export of capsular polysaccharide were also affected in most of the *chsΔ* mutants, but the most prominent alterations were observed in the *chs3Δ* strain. *C. neoformans* cells lacking *CHS* genes also had altered formation of extracellular vesicles and variable chitinase activity under stress conditions. These results reveal previously unknown functions of *CHS* genes that greatly impact the physiology of *C. neoformans*.

Introduction

Cryptococcus neoformans and its sibling species *C. gattii* are responsible for approximately 200,000 human deaths per year (Rajasingham et al., 2017). During infection, *Cryptococcus* species release massive amounts of polysaccharides to the extracellular milieu (Zaragoza et al., 2009). Cryptococcal extracellular polysaccharides, which are the building blocks of a thick capsule, are directly linked to the ability of the fungus to cause damage to host cells (Taylor-Smith and May, 2016).

The fungal cell wall is mainly composed of polysaccharides, including glucans, mannans and chitin (Nimrichter et al., 2005). The latter is a linear polymer composed of β 1,4-linked units of *N*-acetylglucosamine that plays essential roles for cell wall integrity (Munro, 2013). Chitin metabolism in fungi is highly redundant (Banks et al., 2005; Munro et al., 2003). In *C. neoformans*, chitin synthesis involves eight putative chitin synthases (Banks et al., 2005). *Cryptococcus* species are extremely resistant to inhibitors of chitin synthases (Munro, 2012).

Besides its structural role in cell wall architecture, chitin plays additional functions that directly impact the interaction of fungi with the

host. For instance, chitin is an efficient activator of innate immunity (Da Silva et al., 2009). In *C. neoformans*, chitin and chitin-derived molecules interact with glucuronoxylomannan (GXM), its major capsular polysaccharide and principal virulence regulator, to form hybrid, immunologically active glycan complexes (Ramos et al., 2012). Pharmacological inhibition of *N*-acetylglucosamine synthesis resulted in distorted cell wall and capsules (Fonseca et al., 2009). Chitosan, the deacetylated derivative of chitin, was necessary for maintaining normal capsule width and the lack of chitosan resulted in *C. neoformans* cells with low efficacy in retaining cell wall pigments (Baker et al., 2007; Banks et al., 2005).

C. neoformans mutants lacking each of the eight putative chitin synthase genes have been previously generated (Banks et al., 2005). None of the chitin synthase genes were essential for viability, but *CHS3* was important for fungal growth (Banks et al., 2005). Although the connections between chitin metabolism and capsular polysaccharides are numerous (Fonseca et al., 2013; Fonseca et al., 2009; Ramos et al., 2012; Rodrigues et al., 2008; Rodrigues et al., 2015b), it remained unclear how deletion of chitin synthases affected the capsule of *C. neoformans*. In this study, we evaluated the properties of cryptococcal

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capsular structures in mutants lacking chitin synthase genes (*CHS*) 1–8. Our results reveal that *CHS* genes and capsular architecture are functionally connected. Cryptococcal components related to capsule assembly, including extracellular vesicles and chitinase, were affected too. These results demonstrate that *CHS* genes have multiple and previously unknown functions that affect the cellular physiology of *C. neoformans*.

Material and methods

Strains and growth conditions

C. neoformans strain H99 was the background for production of the *chs1-8Δ* mutants (Banks et al., 2005), which were obtained from the Fungal Genetics Stock Center (Department of Plant Pathology 4024, Thorckmorton Plant Sciences Center, Kansas State University, Manhattan, KS 66506). All strains were maintained in Sabouraud agar at room temperature. Unless specified, for all experiments *C. neoformans* cells were pre-inoculated in liquid Sabouraud medium and incubated at 30 °C with shaking for 24 h. The cells were washed with PBS and then cultivated for 48 h in minimal medium (Nimrichter et al., 2007) at 30 °C.

Cell surface labeling

Fungal cells (10^7) were fixed in 4% paraformaldehyde for 1 h and then incubated in PBS supplemented with 1% bovine serum albumin for 1 h at 37 °C. For flow cytometry, the samples were incubated with monoclonal antibodies (mAbs) 18B7 (IgG1), 13F1 (IgM) or 2D10 (IgM) at 10 µg/ml for 1 h at 37 °C (Casadevall et al., 1998). The antibodies were a kind gift of Dr. Arturo Casadevall (Johns Hopkins University, Baltimore, USA). After washing with PBS, the cells were incubated with an anti murine IgG Alexa Fluor™ 488-conjugated (Invitrogen) at 10 µg/ml. The cells were again washed, suspended in PBS and analyzed by flow cytometry using a FACScan apparatus (Becton Dickinson). Data was analyzed using the FlowJo7 software (FlowJo© Tree Star) (Rodrigues et al., 2015b). For fluorescence microscopy, the cells were sequentially stained with calcofluor white (CFW; cell wall staining of chitin; 5 µg/ml for 30 min; 37 °C) and tetramethylrhodamine (TRITC)-labeled wheat germ agglutinin (WGA; cell wall staining of chitooligomers; 5 µg/ml for 30 min; 37 °C) before exposure to the mAbs (Rodrigues et al., 2008). The cells were observed under a fluorescence optical microscope Axioplan 2 (Zeiss, Germany). Images were processed using the analysis software (Zen 2.0) and Adobe Photoshop CS5 was used for preparing figure panels.

Scanning electron microscopy

Yeast cells (10^7 cells/ml) were washed three times in PBS and fixed in 0.1 M sodium cacodylate buffer (pH7.2) containing 2.5% glutaraldehyde for 1 h at room temperature. After fixation, the samples were washed three times in a buffer (pH7.2) containing 0.1 M sodium cacodylate, 0.2 M sucrose and 2 mM MgCl₂. Samples were placed on poly-L-lysine-coated (0.01%) coverslips for 20 min at room temperature. Then preparations were serially dehydrated in ethanol (30%, 50%, 70% for 5 min and 95%, 100%; 10 min), followed by critical point drying and metallization. Cells were finally observed on a JEOL-JSM-6390-LV scanning electron microscope.

Measurement of capsule size

C. neoformans cells were pre-inoculated in liquid Sabouraud medium and incubated at 30 °C with shaking for 24 h. The cells were washed with PBS and then cultivated for 48 h in minimal medium (Nimrichter et al., 2007). WT and *chsΔ* cells were suspended in India ink and placed onto glass slides. The cells were photographed using an

Axioplan 2 microscope (Carl Zeiss). Capsular and cellular body diameter were measured *in silico* using ImageJ 1.48v software (National Institute of Health NIH, Bethesda, MD, USA). At least 100 cells were used for each strain studied.

Measurement of polysaccharide diameter

Capsular polysaccharides were extracted with dimethyl sulfoxide (DMSO) as described previously (Bryan et al., 2005). Polysaccharide dimensions were determined by dynamic light scattering (DLS) on a NanoBrook Omni Multi Angle Particle Sizing analyzer (Brookhaven Instruments Corp., Holtsville, NY), as previously described by our group (Frasces et al., 2009). Since diameter distribution of polysaccharides in the size range of 1 to 1000 nm was similar for all samples (not shown), DLS analysis in this manuscript was concentrated in molecules ranging from 1,000 to 10,000 nm.

Extracellular vesicle analysis

Fungal cells were pre-cultivated overnight in liquid Sabouraud medium at 30 °C under shaking (200 rpm). The cells were then washed twice with PBS, counted, and inoculated at a final density of 5×10^6 cell/ml in 10 ml of yeast nitrogen base (YNB). After incubation for 48 h under the same conditions described above, the cultures were diluted to 25 ml with YNB and cultivated for another 48 h. The cells were harvested and supernatants were filtered through 0.45 µm membranes. Aliquots (100 µl) of the filtered supernatants were collected for GXM determination by ELISA (Casadevall et al., 1992) and the remaining volumes were ultracentrifuged for 1 h at 100,000 × g at 4 °C. The pellets were washed with PBS under the same conditions. The vesicle-containing pellets were then diluted 1,000 times in PBS and quantified by particle tracking analysis using a Zetaview equipment (Particle Metrix GmbH, Meerbusch, Germany) following the manufacturer's instructions. The equipment was set to detect and quantify particles ranging from 30 to 100 nm.

Chitinase activity

Enzyme activity was measured after fungal growth in YPD or under the stress conditions used by Alanio et al. (2015). *C. neoformans* cells were grown for 24 h in YPD, washed with PBS three times, counted, and adjusted to the cell density of 10^7 yeast cells/ml in i) Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, ii) sterile water or iii) 1 µM NaNO₂ in PBS. The cells were incubated for 24 h in DMEM or water or for 2 h in NaNO₂. The suspensions were centrifuged for supernatant collection and the remaining cells were suspended in 300 µl of a lysis buffer (5 mM Tris-HCl, pH 7.5; 150 mM NaCl; 5 mM EDTA; 5 mM EGTA; 1 mM phenylmethylsulfonyl fluoride; 2 µM E-64; 0.1 mM pepstatin A; 2 mM phenantroline) and supplemented with a similar volume of 600 µm glass beads. The cells were submitted to 30 cycles alternating 2 min vortexing and 2 min on an ice bath. The fluorimetric Chitinase Assay Kit (CS1030 – Sigma Aldrich) was used to measure exochitinase activity according to the manufacturer instructions. Briefly, 5 µl of each supernatant or 1 µg of lysates (protein concentration) were diluted to 100 µl of the kit's working substrate and incubated for 1 h at 37 °C. Activity was measured in a fluorimeter (wave length 360 – 450 nm, Spectramax – Molecular Devices).

Statistical analysis

All statistical comparisons were performed with GraphPad Prism 6.0.

Results

We have previously demonstrated that distribution of chitin-related

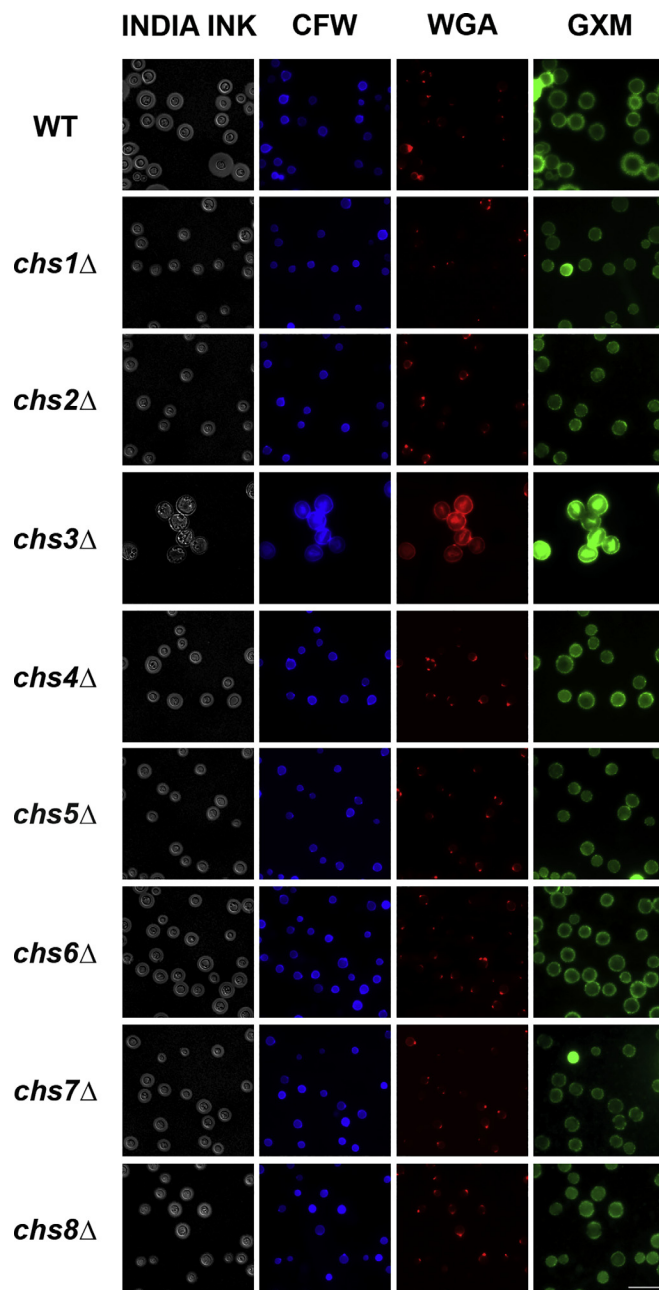


Fig. 1. Fluorescence microscopy analysis of wild type (WT) *C. neoformans* cells and chitin synthase mutants (*chs1-8Δ*). Fungal cells were counter-stained with India ink for capsule visualization. Alternatively, the cells were stained with calcofluor white (CFW), wheat germ agglutinin (WGA) or mAb 18B7 for visualization of surface chitin (blue), chitooligomers (red) and GXM (green), respectively. Scale bar, 20 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

structures on the cell wall of *C. neoformans* directly affects capsular architecture (Fonseca et al., 2013; Fonseca et al., 2009; Ramos et al., 2012; Rodrigues et al., 2008). On the basis of this observation, we analyzed capsular morphology in the eight *C. neoformans* chitin synthase mutants (*chs1-8Δ*) by light microscopy and compared their morphologies with that of wild type cells. As previously demonstrated (Banks et al., 2005), all *chsΔ* mutants still produced chitin, as concluded from positive staining with calcofluor white (Fig. 1). Chitooligomer distribution was also similar in WT and mutant cells, with the exception of the *chs3Δ* strain. In these cells, an annular pattern of cell wall staining with WGA was observed, in contrast to the typical focal

staining observed for *C. neoformans* (Rodrigues et al., 2008). All strains reacted with mAb 18B7 and had capsules that were detectable by India ink counterstaining. Since light microscopy has resolution limitations in the analysis of capsular architecture, we also analyzed the cells by SEM.

With the exception of *chs7Δ* cells, deletion of *CHS* genes profoundly affected capsular morphology (Fig. 2A). The changes in capsular morphology included lower fiber density (*chs1Δ*, *chs3Δ*, *chs5Δ*, *chs6Δ*, and *chs8Δ*), polarized distribution of surface fibers with enrichment of capsular structures in some regions of the cell surface (*chs2Δ*, *chs5Δ*, and *chs6Δ*) and clear reduction in capsule size (*chs3Δ*). Most noticeably, the *chs3Δ* mutant showed very short capsular fibers in association with defective cell separation, which impacted cell morphology (Fig. 2B). Quantitative determination of capsular dimensions confirmed the visual observations (Fig. 3A) and demonstrated that deletion of each *CHS* gene significantly impacted capsule size. However, the most prominent reduction was in fact observed after deletion of *CHS3*. Since polysaccharide diameter correlates with capsular dimensions (Frasces et al., 2009), we determined GXM diameter by dynamic light scattering (Fig. 3B). This analysis demonstrated that the average diameter of polysaccharides obtained from the *chs3Δ* mutant was significantly smaller than those analyzed in all other samples. Together, these results indicate that interference with chitin metabolism directly affect the architecture of capsular structures in *C. neoformans*.

Capsular architecture relies on the structural aspects of GXM (Nimrichter et al., 2007). We therefore hypothesized that deletion of *CHS* genes could impact GXM structure and, consequently, its serological properties. We analyzed the reactivity of each of the nine strains used in the study with three mAbs raised to GXM (Fig. 4). The three antibodies recognized all strains, but different profiles of reactivity were observed. In comparison with WT cells, subpopulations that were over reactive with mAb 18B7 (IgG1) were detected in strains *chs1Δ*, *chs6Δ*, *chs7Δ* and *chs8Δ*. Strain *chs3Δ* was generally more reactive than WT cells with mAb 18B7. MAb 13F1, an IgM, reacted similarly with all strains, with the exceptions of *chs2Δ* and *chs3Δ*, which were poorly recognized by the mAb. All strains were similarly recognized by a second IgM, mAb 2D10, with the exception of a subpopulation of over reactive cells in strain *chs8Δ*. These results indicate that the serological epitopes of GXM change depending on the expression of *CHS* genes.

Alterations in the *CHS* genes were previously linked to a leaky melanin phenotype, suggesting that regulation of chitin synthesis in *C. neoformans* affected cell wall permeability (Banks et al., 2005). Export of GXM in *C. neoformans* requires trans-cell wall passage of polysaccharide-containing fungal vesicles (Rodrigues et al., 2007). We then hypothesized that deletion of *CHS* genes could affect extracellular vesicle detection in *Cryptococcus*. In fact, clear differences between WT and mutant strains were observed (Fig. 5). Extracellular vesicle detection was similar in WT, *chs1Δ* and *chs8Δ* strains. However, strains *chs4Δ* and mainly *chs5Δ* overproduced extracellular vesicles. On the other hand, strains *chs2Δ*, *chs3Δ*, *chs6Δ* and *chs7Δ* showed decreased extracellular vesicle production. The lowest levels of extracellular vesicle detection were observed in strain *chs3Δ*.

Since extracellular vesicles are the vehicles required for GXM transport (Rodrigues et al., 2007), the polysaccharide was also quantified in supernatant samples (Fig. 6). All mutant strains had reduced GXM secretion, which was consistent with the reduced capsular dimensions. Once again, strain *chs3Δ* was the one producing the smaller values of GXM detection, which was consistent with its strongly reduced capsule size and possibly related to the decreased detection of extracellular vesicles. However, although extracellular GXM detection was generally decreased in all mutants, there was no apparent correlation with the changes observed during analysis of extracellular vesicles.

It has been speculated that members of the chitin synthase and chitinase gene families operate under coordinated regulation for cell wall synthesis and hydrolysis during fungal growth (Cabib et al., 1992).

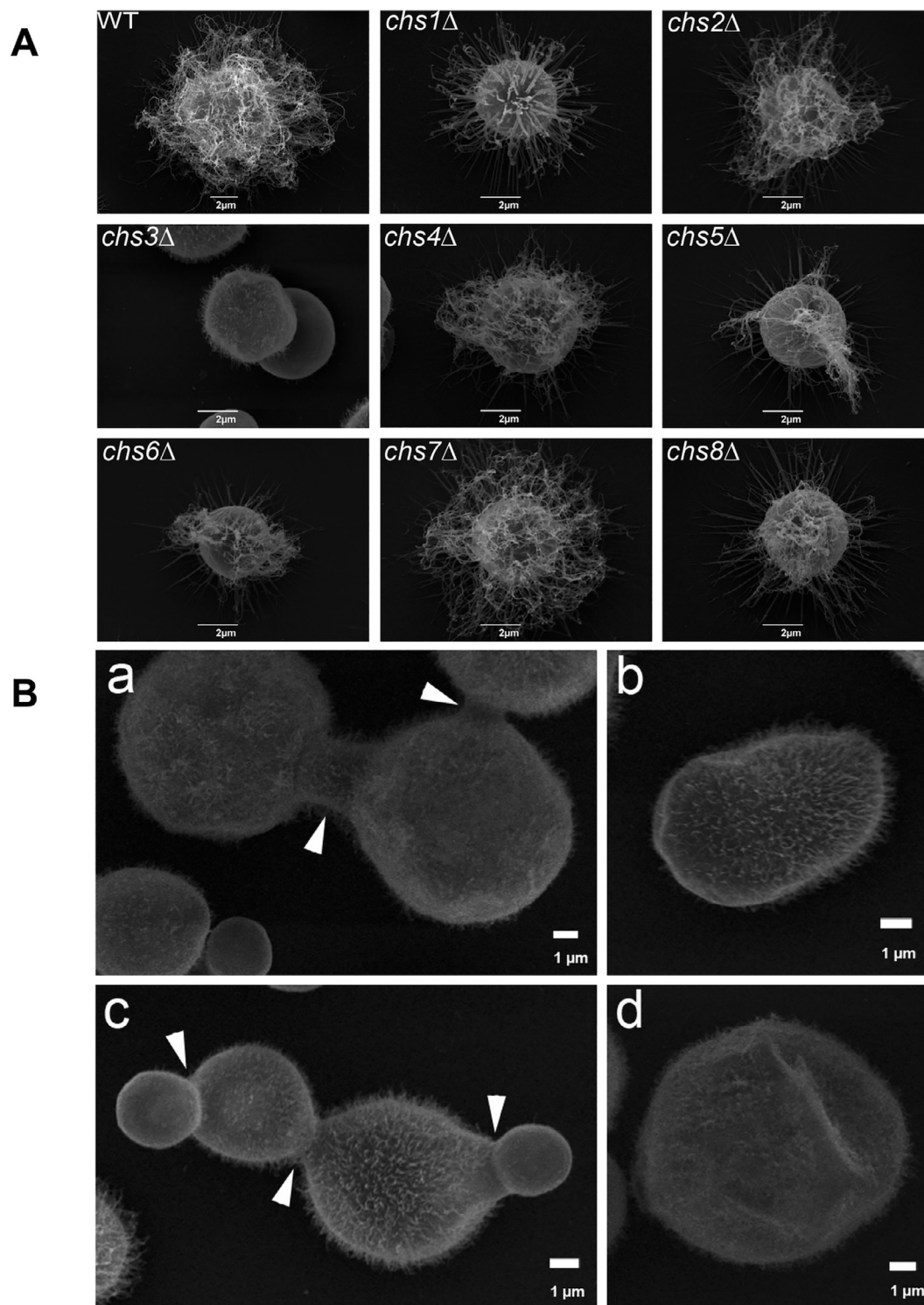


Fig. 2. SEM analysis of wild type (WT) cells and chitin synthase mutants (*chs1*-8Δ). (A) Morphological evidence of capsule alterations in mutant cells in comparison to the parental strain. Changes in capsular morphology included lower fiber density (*chs1*Δ, *chs5*Δ, *chs6*Δ, and *chs8*Δ), altered distribution of surface fibers (*chs2*Δ, *chs5*Δ, and *chs6*Δ) and clear reduction of capsule size (*chs3*Δ). (B) Detailed analysis of the *chs3*Δ mutant, showing impaired cell separation (a, arrows), capsule reduction (b), defective budding (c, arrows) and irregular morphology (d). Images are representative of the most consistent phenotypes observed for each strain.

Chitinase activity affects cell wall and capsule architecture in *C. neoformans* (Mayer and Kronstad, 2017; Rodrigues et al., 2008). On the basis of our current observations of altered capsular structure in *chs*Δ mutants, we asked whether deletion of *CHS* genes also affected chitinase activity in *C. neoformans*. Cell-associated and extracellular chitinase activities were measured in all nine strains (Fig. 7). Under standard conditions (growth in YPD), chitinase levels were similar in all

strains. Capsule formation is induced under stressing conditions (Zaragoza and Casadevall, 2004). One example of such conditions is the incubation of *C. neoformans* in DMEM, which induces capsule enlargement (Zaragoza and Casadevall, 2004). We therefore asked whether chitinase activity was affected when *C. neoformans* was incubated in the presence of DMEM. Under this condition, an increased detection of chitin hydrolysis was observed in all strains, excepting *chs3*Δ.

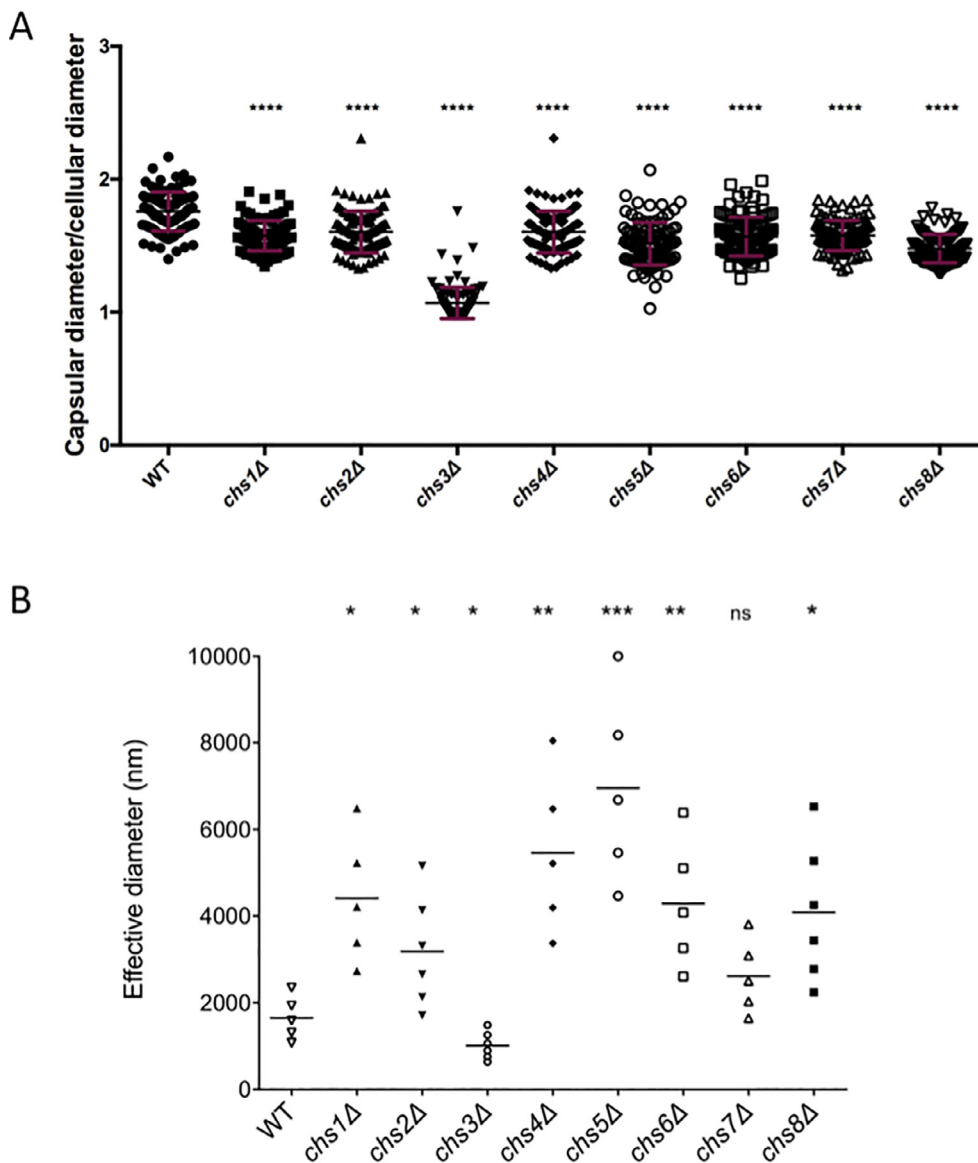


Fig. 3. Quantitative analysis of capsular components of wild type (WT) cells and chitin synthase mutants (*chs1-8Δ*). (A) Measurement of capsular dimensions. All mutant strains had capsular dimensions significantly reduced in comparison to WT cells, but the *chs3Δ* was clearly less efficient than all strains in forming a capsule. Asterisks denote $P < 0.0001$ in comparison with WT cells. (B) Ranges of polysaccharide dimensions obtained by DLS. Once again, the lowest values were obtained for the *chs3Δ* mutant, but no correlation with capsular dimensions was observed. Results are representative of three independent experiments. Asterisks denote P values obtained from comparison between mutant and WT cells as follows: * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$. Experiments were performed in triplicates and data represent average values. ns, not significant.

Intracellular chitinase activity of DMEM grown cells was similarly enhanced in strains H99, *chs1Δ*, *chs3Δ*, *chs5Δ*, *chs7Δ* and *chs8Δ*, but not in strains *chs2Δ*, *chs4Δ* and *chs6Δ*. These results indicated that deletion of *CHS* genes differentially affected the chitinase response of *C. neoformans* under conditions of stress. To evaluate whether these responses were specific to DMEM, we included two previously reported stressing conditions (Alanio et al., 2015) and measured chitinase activity. In the presence of H₂O, extracellular chitinase activity was similarly enhanced in all strains. However, intracellular enzyme activity was not affected by incubation in water for strains *chs7Δ* and *chs8Δ*. NaNO₂ did not affect either extracellular or intracellular chitinase activity of strain H99. However, incubation in NaNO₂ resulted in significantly increased chitinase activity for all *chsΔ* mutants, with the exception of the cell-associated chitin hydrolysis by the *chs1Δ* mutant. These results suggest a complex and still obscure regulation of chitinase and chitin synthase activities under stressing conditions. The overall effects of *CHS* deletion on the physiology of *C. neoformans* are summarized in Table 1.

Discussion

The classical understanding of chitin as a structural component responsible for the rigidity of cell walls of most fungi has changed

dramatically during the last decade. In *C. neoformans*, it has been suggested that chitin participates in capsular architecture (Fonseca et al., 2013; Fonseca et al., 2009; Ramos et al., 2012; Rodrigues et al., 2008) and morphological changes that are important for fungal survival within the host (Wiesner et al., 2015). *C. neoformans* forms large titan cells during infection and increased chitin content in these cells signals the host immune system to generate a detrimental response that promotes disease progression in mice (Wiesner et al., 2015). In *Candida albicans*, chitin influences immune recognition by blocking dectin-1-mediated engagement with fungal cell walls (Mora-Montes et al., 2011). In the same pathogen, elevated cell wall chitin conferred echinocandin resistance in vivo (Lee et al., 2012). *C. neoformans* is particularly resistant to echinocandins (Munro, 2012), which might be related to the metabolic redundancy used by this fungus to synthesize chitin (Banks et al., 2005). On the other hand, recent reports on *C. neoformans* and *H. capsulatum* suggest that external interventions that disrupt chitin structure instead of inhibiting its synthesis are beneficial to the host during fungal infection (Fonseca et al., 2013; Liedke et al., 2017). The clear roles of fungal chitin in surface architecture, immune recognition and antifungal susceptibility demonstrate the need of a better understanding of the impact of chitin synthesis on fungal physiology.

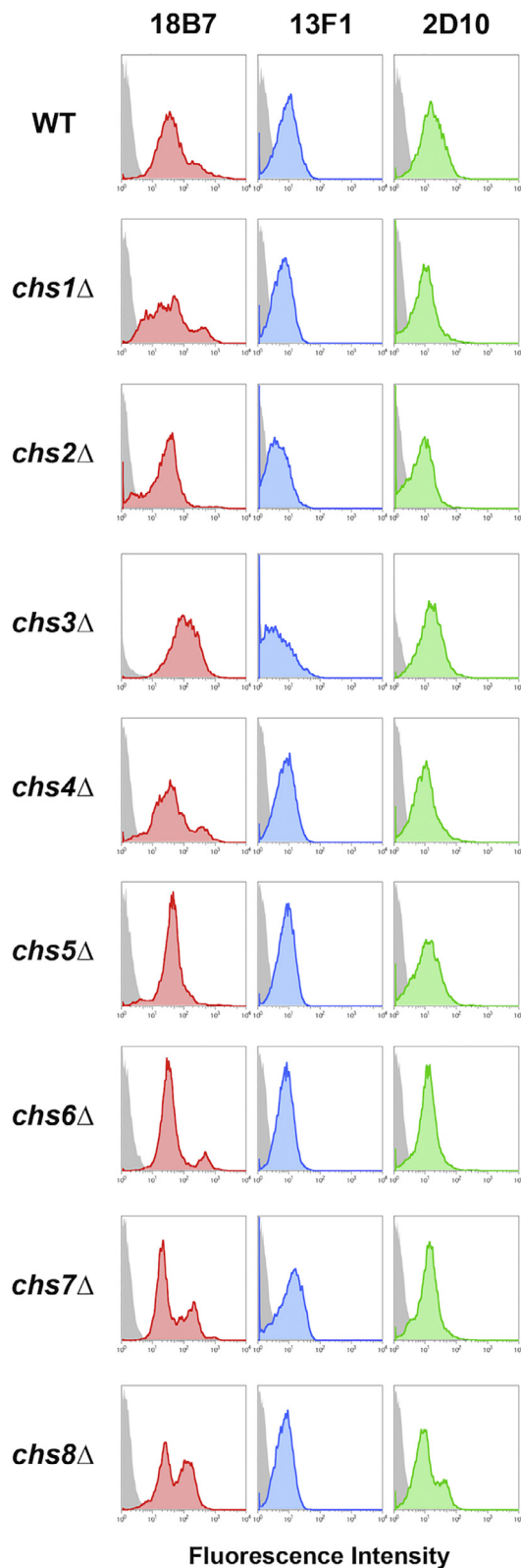


Fig. 4. Antibody labeling of the cell surface of wild type (WT) and mutant strains of *C. neoformans*. Subpopulations that were over reactive with mAb 18B7 were detected in strains *chs1Δ*, *chs6Δ*, *chs7Δ* and *chs8Δ*. Strain *chs3Δ* was generally more reactive than WT cells with the mAb 18B7. MAb 13F1, an IgM, reacted similarly with all strains, with the exceptions of *chs2Δ* and *chs3Δ*, which were poorly recognized by the mAb. All strains were similarly recognized by mAb 2D10, with the exception of a subpopulation of over reactive cells in strain *chs8Δ*. Results are representative of one experiment producing results that were similar to those obtained in three independent experiments.

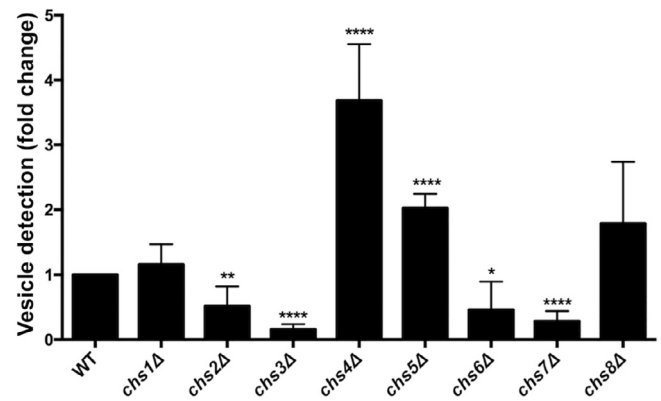


Fig. 5. Quantitative analysis of extracellular vesicles produced by wild type (WT) cells and chitin synthase mutants (*chs1-8Δ*). EV production was similar in WT, *chs1Δ* and *chs8Δ* cells ($P > 0.05$). Strains *chs4Δ* and *chs5Δ* produced significantly more vesicles than WT cells ($P < 0.0001$). Strains *chs2Δ*, *chs3Δ*, *chs6Δ* and *chs7Δ* were less efficient in producing extracellular vesicles than WT cells ($*P = 0.0135$; $**P = 0.0039$; $****P < 0.0001$). Results are presented as average values of four independent experiments.

None of the chitin synthase genes or their regulators was essential for viability of *C. neoformans* (Banks et al., 2005). However, the *chs3Δ* mutant showed dramatic phenotypic alterations in comparison to wild type cells, including increased size, irregular shape and incomplete budding. In addition, the *chs3Δ* strain lost the ability to retain melanin in the cell wall (Banks et al., 2005). In our study, these alterations were confirmed, but additional features were clearly detectable. The *chs3Δ* strain showed defective extracellular vesicle formation and altered reactivity with monoclonal antibodies to GXM, in addition to a markedly reduced capsule and smaller polysaccharide fibers. However, previously unknown phenotypic characteristics of the other *chsΔ* mutants were also detected, suggesting that not only *CHS3* but also the other *CHS* genes impact the physiology of *C. neoformans*. Overall, the variability in extracellular polysaccharide dimensions produced by the chitin synthase mutants was higher than in capsule size. This divergence is likely linked to the high efficacy of GXM to self-aggregate in its soluble state, a processes that is influenced by chitin-related structures (Fonseca et al., 2009) and usually results in broad ranges of polysaccharide dimensions (Nimrichter et al., 2007). It is important to highlight that factors unrelated to chitin synthesis can also affect capsular structures, including microevolution-derived changes during laboratory maintenance of *C. neoformans* cells (Franzot et al., 1998). However, the present study and others associating chitin synthesis with capsule formation (Fonseca et al., 2013; Fonseca et al., 2009; Ramos et al., 2012; Rodrigues et al., 2015a; Rodrigues et al., 2008) reinforces the notion that these two events are functionally connected. On the basis of the analysis of capsular morphology, polysaccharide dimensions, serological reactivity, GXM release, extracellular vesicle formation and chitinase responses to stress conditions, each of the *chsΔ* mutants differed from wild type cells in at least four phenotypic traits (Table 1). These results reveal multiple roles for *CHS* genes in *C. neoformans* and reinforce the idea that, although pharmacological inhibition of chitin synthesis in *C. neoformans* is hard to accomplish (Munro, 2012), targeting the products of chitin synthase genes may be a promising strategy to control cryptococcosis.

The pattern of extracellular vesicle detection in the *chsΔ* mutants was highly variable. It has been recently demonstrated that liposomes ranging from 60 to 80 nm in diameter remained intact during transit through the cell wall of both *C. neoformans* and *C. albicans* (Walker et al., 2018). However, the predicted size of cell wall pores is approximately 6 nm (De Nobel et al., 1990), suggesting that the wall is capable of rapid remodeling to allow vesicular transit. This observation can provide a mechanism for release of extracellular vesicles and is in

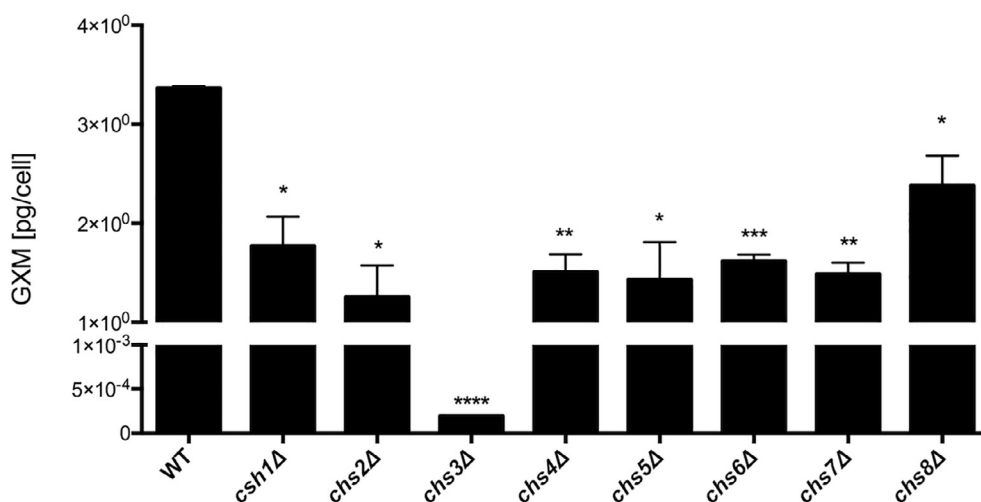


Fig. 6. Extracellular GXM determination in cultures of wild type (WT) cells and chitin synthase mutants (*chs1-8Δ*). All strains produced reduced amounts of extracellular GXM. Extracellular polysaccharide production by the *chs3Δ* mutant was strongly reduced in comparison with WT cells. Results are representative of three independent experiments producing similar results. Asterisks denote P values obtained from comparison between mutant and WT cells as follows: *P < 0.05; **P < 0.005; ***P < 0.001; ****P < 0.0001.

agreement with the fact that *C. albicans* mutants with altered cell wall thickness and composition were similar in both their in vitro susceptibility to liposomal amphotericin B and the ability of liposomes to penetrate the cell wall (Walker et al., 2018). In our model, we initially expected the *chs3Δ* strain to be more efficient in releasing vesicles, on the basis of its previously suggested enhanced cell wall porosity (Banks et al., 2005). However, this mutant was the one producing the lowest values of extracellular vesicle detection. Other mutants with no apparent cell wall alterations (*chs4Δ* and *chs5Δ*) produced high values of extracellular vesicle detection. We therefore concluded that the differences observed in our analysis of extracellular vesicles were not a consequence of altered cell wall porosity, but rather of an altered efficacy to produce vesicles to be exported. The efficacy of extracellular vesicle release in each strain might be influenced by a number of still poorly known phenotypic traits, including metabolic rates and pattern of expression of secretion-related genes. The influence of these factors on the formation of extracellular vesicles in the chitin synthase mutants is still unpredictable on the basis of our current knowledge on the relationship between polysaccharide synthesis and extracellular vesicle export. The lack of an expected correlation between GXM secretion and extracellular vesicle release (Rodrigues et al., 2007) in the *chs4Δ* and *chs5Δ* mutants efficiently illustrates this situation.

The connections between extracellular vesicle formation and chitin synthesis are unclear. However, the major reservoir of chitin synthase in fungi is the chitosome, an endosome-like membrane compartment (Bartnicki-Garcia, 2006). Endosomes are the organelles originating exosomes, the most well studied extracellular vesicles (van Niel et al., 2018). In *S. cerevisiae*, chitin synthase III activity demands the expression of additional chitin synthase genes, including *CHS4*, *CHS5*, and *CHS6* (Ziman et al., 1998). *Chs6p* is required for anterograde transport of *Chs3p* from the chitosome to the plasma membrane (Ziman et al., 1998). In summary, these results indicate a direct correlation between chitin synthesis and secretory activity, which might be related to the diverse effects of *CHS* deletion on extracellular vesicle formation in *C. neoformans*. Considering that chitin interacts with GXM through *N*-acetyl groups forming immunologically active glycan hybrids (Ramos et al., 2012), *CHS* deletion was also expected to affect the capsule of *C. neoformans*. It is still unclear why *CHS* deletion affected the serological properties of GXM, but we speculate that the unbalanced carbohydrate synthesis caused by altered chitin metabolism could result in altered structures of GXM fibers. In fact, it has been recently demonstrated that *C. neoformans* cells grown with *N*-acetylglucosamine, the building unit of chitin, manifested changes in capsular properties (Camacho et al., 2017).

In *C. albicans*, synthesis and hydrolysis of chitin are uncoupled, but both are regulated during yeast-hypha morphogenesis (Selvaggini et al.,

2004). Chitin synthase and chitinase gene families are in fact expected to operate under coordinated regulation for cell wall synthesis (Cabib et al., 1992). In a recent report, Mayer and Kronstad demonstrated that exogenous chitinases target and destabilize the cell surface of *C. neoformans* (Mayer and Kronstad, 2017). In our study, *chsΔ* mutants had altered capsules, which led us to ask whether extracellular and cell-associated chitinase activities were affected in each of the mutants. Specifically, under stressing conditions (Alanio et al., 2015), including those stimulating capsule production (Zaragoza and Casadevall, 2004), the profiles of both extracellular and cell-associated chitinase activity were highly variable. These results suggest that alterations in chitin synthesis are linked to the levels of chitin hydrolysis. Quantitative correlations, however, cannot be established at this moment since the levels of chitin synthesis in the *chsΔ* mutants are variable. In fact, chitin contents can be higher in *chsΔ* mutants than in WT cells (Banks et al., 2005), probably because of compensatory mechanisms. In any case, these results in combination reveal that alterations in chitin synthesis impact multiple events in *C. neoformans* cells.

The comprehension of the connections between chitin synthesis and capsular structures may reveal novel antifungal targets. Our data clearly demonstrate that, besides the previously known role of *CHS3* in cryptococcal physiology (Banks et al., 2005), *CHS1-8* also play key roles with many effects on *C. neoformans*, with emphasis to the novel functions proposed for *CHS3*. These results reinforce the potential of chitin synthases as promising targets for antifungal development, but also reveal a huge complexity derived from chitin metabolism in *C. neoformans* cells.

Conflict of interest

None.

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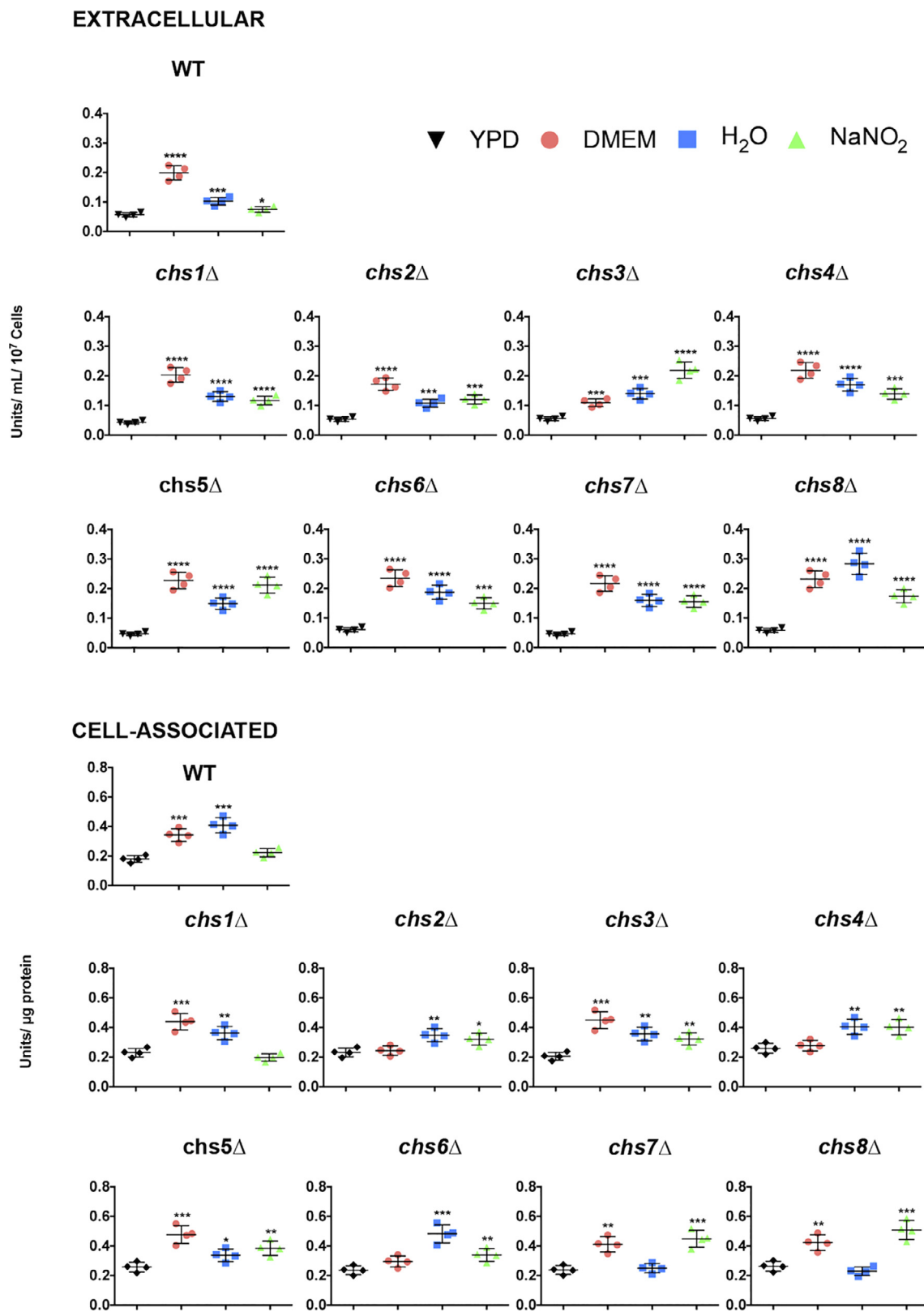


Fig. 7. Extracellular and cell-associated chitinase activities in wild type (WT) cells and chitin synthase mutants (*chs1-8Δ*). Fungal cells were incubated under standard conditions (YPD) or under stress (DMEM, H₂O, NaNO₂). Chitinase activity was similar in all strains incubated in YPD. Stress induction resulted in clearly altered patterns of enzyme activity. Statistical comparisons between WT and mutant cells produced P values as follows: *P < 0.05; **P < 0.005; ***P < 0.001; ****P < 0.0001. Results represent average values of two independent experiments.

Table 1

Effects of *CHS* deletion on capsular morphology, polysaccharide dimensions, serological reactivity, GXM release, extracellular vesicle formation and chitinase responses to stress conditions.

Mutant strain								
Phenotypic trait ¹	<i>chs1Δ</i>	<i>chs2Δ</i>	<i>chs3Δ</i>	<i>chs4Δ</i>	<i>chs5Δ</i>	<i>chs6Δ</i>	<i>chs7Δ</i>	<i>chs8Δ</i>
Capsular morphology	Altered	Altered	Altered	Altered	Altered	Altered	Similar	Altered
Polysaccharide diameter	Increased	Increased	Reduced	Increased	Increased	Increased	Similar	Increased
Serological reactivity with GXM mAbs	Altered	Altered	Altered	Similar	Similar	Similar	Altered	Altered
Efficacy to release GXM	Partially reduced	Partially reduced	Strongly reduced	Partially reduced	Partially reduced	Partially reduced	Partially reduced	Partially reduced
Extracellular vesicle formation	Similar	Partially reduced	Strongly reduced	Strongly increased	Increased	Partially reduced	Partially reduced	Similar
Chitinase response to stress conditions	Similar	Altered (i)*	Altered (e)*	Altered (i)	Altered (e)	Similar	Altered (i)	Altered (i and e)

* (i), intracellular; (e), extracellular.

¹ Comparison with wild type cells.

who was responsible for the generation of the chitin synthase mutants and granted us access to the mutant strains from the Fungal Genetics Stock Center.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.tcs.2018.05.002>.

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