The vacuolar-sorting protein Snf7 is required for export of virulence determinants in members of the *Cryptococcus neoformans* complex.

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Fungal pathogenesis requires a number of extracellularly released virulence factors. Recent studies demonstrating that most fungal extracellular molecules lack secretory tags suggest that unconventional secretion mechanisms and fungal virulence are strictly connected. Proteins of the endosomal sorting complex required for transport (ESCRT) have been recently associated with polysaccharide export in the yeast-like human pathogen *Cryptococcus neoformans*. Snf7 is a key ESCRT operator required for unconventional secretion in Eukaryotes. In this study we generated *snf7* deletion mutant strains of *C. neoformans* and its sibling species *C. gattii*. Lack of Snf7 resulted in important alterations in polysaccharide secretion, capsule formation and pigmentation. This phenotype culminated with loss of virulence in an intranasal model of murine infection in both species. Our data support the notion that Snf7 expression regulates virulence in *C. neoformans* and *C. gattii* by ablating polysaccharide and melanin traffic. These results are in agreement with the observation that unconventional secretion is essential for cryptococcal pathogenesis and strongly suggest the occurrence of still obscure mechanisms of exportation of non-protein molecules in Eukaryotes.

Cryptococcosis is the leading cause of human deaths related to fungal infections in Africa. This disease is caused by the yeast-like pathogens *Cryptococcus neoformans* and *C. gattii*. The former is an opportunistic fungal pathogen that causes highly lethal meningitis in immunocompromised individuals. *C. gattii*, on the other hand, can infect both immunocompromised and immunocompetent individuals.

The progress of cryptococcal disease relies on several extracellular virulence factors, implying that secretion is fundamental for cryptococcal pathogenesis. Eukaryotic molecules are exported through different cellular pathways, including conventional and unconventional secretory routes. Conventionally secreted proteins follow the classical endoplasmic reticulum (ER)/Golgi-dependent secretory pathway. Protein secretion through conventional mechanisms is dependent on an N-terminus-linked signal peptide, which is responsible for translocation of polypeptides into the lumen of the ER. Proteins that lack a typical signal peptide are transported independently of this classical ER-Golgi route and engage the so-called unconventional secretion mechanisms.

Most of the pathogenic determinants of *C. neoformans* lack secretory tags, supporting the notion that unconventional pathways of secretion are required for cryptococcal virulence. *C. neoformans* and *C. gattii* export massive amounts of polysaccharides to the extracellular milieu. Polysaccharide export is required for capsule formation, which is essential for the survival of cryptococci in host tissues. The main component of the polysaccharide capsule is glucuronoxylomannan (GXM). GXM is co-transported to the extracellular space with other virulence factors inside vesicles that traverse the cell wall. The morphological aspects of these vesicles suggest that they are similar to mammalian exosomes, their cellular origin remains undetermined (reviewed by Oliveira and colleagues).
The eukaryotic sucrose non-fermenting protein 7 (Snf7) is a key operator in the endosomal sorting complex required for transport (ESCRT)-III19-20. This molecular complex, in association with other ESCRT components, is involved in multivesicular body (MVB) formation and cargo. MVB formation requires Snf7 oligomerization into a membrane-associated filament, which is nucleated by its association with the vacuolar protein sorting regulator Vps201. MVBs, which derive from late endosomes, can be directed for fusion with the plasma membrane, resulting in extracellular release of their luminal vesicles, the so-called exosomes21,22. The ESCRT machinery has also been implicated in environmental pH sensing and adaptation in various fungal species through mechanisms that require the RIM101 pathway, a signaling mechanism necessary for the regulation of alkaline environment-related genes24,25. Recently, the RIM101-mediated stress response has been demonstrated to be up regulated during human meningitis caused by C. neoformans26. The supposition that MVBs are associated with the export of virulence factors in C. neoformans27 and the fact that adaptation to alkaline pHs is required for fungal survival in both alveolar spaces and the bloodstream28 suggests that Snf7 is involved in cryptococcal pathogenicity.

In this work we generated C. neoformans and C. gattii snf7Δ mutant strains and characterized their biological and pathogenic properties. Lack of Snf7 affected key virulence determinants in both C. neoformans and C. gattii. This phenotype culminated with loss of virulence in an intranasal model of murine infection in both species. These results strongly support the hypothesis that unconventional secretion pathways are essential for the pathogenesis of C. neoformans and C. gattii.

Results

Snf7-related sequences in C. neoformans and C. gattii. A database search of the C. gattii genome (http://www.broadinstitute.org/annotation-genome/cryptococcus_neoformans_b/MultiHome.html-August 5, 2009, strain R265) revealed that the snf7 (accession number CNBG_3856) coding region comprises 1,087 bp, includes five introns, and encodes a protein of 221 amino acids. A similar search in the C. neoformans genome (http://www.broadinstitute.org/annotation-genome/cryptococcus_neoformans/MultiHome.html) identified the putative SNF7 sequence (accession number CNAG_015832.2) based on the similarity to the corresponding C. gattii sequence (accession number CNBG_3856). The C. neoformans SNF7 ortholog is 1,413 bp long, contains five introns, and also encodes a putative 221 amino-acid protein. Phylogenetic analysis of Snf7 was performed with orthologues from distinct eukaryotic organisms (Figure 1A). The putative sequence of the C. gattii protein was highly similar to its C. neoformans counterpart (98% identity) and both display similarities to plant proteins (A. thaliana ~35% identity; O. sativa ~24% identity). In comparison to other fungi, the Snf7 sequences of both species studied here showed 45% identity with the S. cerevisiae protein and 43% identity with a C. albicans homologue.

Disruption and complementation of SNF7 genes in both C. neoformans and C. gattii. To analyze the functions of SNF7, we generated snf7Δ disruption mutants in the backgrounds of the standard strains H99 (serotype A isolate of C. neoformans) and R265 (serotype B isolate of C. gattii). These mutant strains were called CN-snf7Δ and CG-snf7Δ, respectively. To ensure that the eventual phenotypes observed in the mutant strains were due to the knockout of the SNF7 gene, we constructed the complemented strains (CN-snf7Δ::SNF7 for C. neoformans and CG-snf7Δ::SNF7 for C. gattii) by integrating a wild-type copy of the SNF7 gene into the genome of the mutant strains. Deletion and complementation of SNF7 in both species were confirmed by Southern blot analysis (Figure 1B) and RT-PCR (Figure 1C).

Growth rates of the snf7Δ mutants under different conditions. We first evaluated whether the growth rates of the snf7Δ strains varied under different conditions. C. neoformans and C. gattii mutant strains showed normal growth at both 30 and 37°C, in comparison to parental and reconstituted strains (Figure 2A). Since copper acquisition is important for C. neoformans melanization and capsule formation26,28 we also evaluated the ability of the snf7Δ mutants to grow under copper deprivation conditions. Once again, the mutants showed normal growth rates, in comparison to WT and complemented cells (Figure 2B).

The ESCRT machinery, including Snf7, plays a central role in resistance to higher pHs and tolerance to ionic lithium29,30. Therefore, we analyzed the ability of the snf7Δ mutants to survive under these conditions. Analysis of fungal growth in pHs ranging from 7 to 9 suggested lower growth rates of both CN-snf7Δ and CG-snf7Δ in pHs above 7.5, in comparison to parental and reconstituted cells (Figure 2C; p < 0.05 for all comparisons between cells expressing Snf7 and snf7Δ strains). A similar result was obtained when the C. neoformans and C. gattii strains were cultivated in the presence of 150 mM LiCl2 (Figure 2D).

To analyze the functions of snf7Δ, we first evaluated whether the growth rates of the snf7Δ mutants were impaired in the presence of copper. The growth rates of the snf7Δ mutants were significantly lower than that of the wild type in both C. neoformans and C. gattii (Figure 2D).

SNF7 disruption alters pigmentation but not urease and phospholipase activities. Extracellularly released enzymes and exported pigments are fundamental for cryptococcal pathogenesis32,33,36–39. Therefore, we analyzed whether SNF7 disruption would impact melanization and the extracellular activities of urease and phospholipase. Pigmentation was evaluated visually after growth of C. neoformans and C. gattii on both Niger seed and L-DOPA-containing solid media (Figure 4A). Under both conditions, the CN-snf7Δ mutant strain was similar to parental (WT) and complemented (reconstituted) strains in its ability to melanize at 30°C. However, when cultivated at 37°C, the mutant CN-snf7Δ showed reduced pigmentation. Differently, the CG-snf7Δ mutant showed subtle melanization defects when cultivated on L-DOPA agar at 30°C. At 37°C, the pigmentation defects were more evident. On Niger seed agar, snf7Δ deletion in C. gattii affected pigmentation at 30°C, but not at 37°C. These results revealed an unexpected interspecies diversity in the relationship between Snf7 and pigmentation.

Crude phospholipase activity was also assessed in the SNF7 disruption model. Although the rate of phospholipid hydrolysis showed some tendency to be higher in the CN-snf7Δ mutant, there were no statistical differences between any of the C. neoformans strains (Figure 4B). The C. gattii snf7Δ mutant showed levels of phospholipase activity that tended to be lower than those observed for parental and complemented strains (Figure 4B). Urease activity was similar in all strains analyzed in this study (Figure 4C).

Lack of Snf7 severely impairs GXM secretion and capsule formation. GXM secretion and capsule enlargement are essential for cryptococcal pathogenesis (reviewed in40). Since it has been suggested that GXM is exported by unconventional secretion mechanisms (reviewed elsewhere41-43), we evaluated whether SNF7 deletion affects capsule size and extracellular polysaccharide secretion.

Quantification of secreted GXM in all strains by ELISA revealed that deletion of SNF7 nearly extinguished polysaccharide export in both C. neoformans and C. gattii (Figure 5A). Morphological analysis of the capsule by India ink counterstaining, immunostaining of GXM...
and scanning electron microscopy revealed a clear reduction in capsular dimensions in the \textit{snf7} \textit{D} mutants (Figure 5B), implying defects in capsular assembly. Determination of the capsule/cell diameter ratio confirmed a significant reduction (\(p < 0.0001\)) in capsular dimensions in both CN-\textit{snf7} \textit{D} and CG-\textit{snf7} \textit{D} cells (Figure 5C).

\textbf{snf7} \textit{D} mutants are avirulent in a murine infection model. The involvement of Snf7 in melanization, polysaccharide secretion and capsule assembly was in agreement with the possibility that \textit{SNF7} deletion could affect pathogenesis. To address this question, mice were infected intranasally with each strain used in this work for mortality assessment. The totality of animals infected with wild type and CN-\textit{snf7} \textit{D}::\textit{SNF7} reconstituted strains died by day 14 post infection (Figure 6A). Infection of mice with the CN-\textit{snf7} \textit{D} strain, however, resulted in nearly 63% survival at day 40-post infection, when animals were sacrificed. Animals infected with the \textit{C. gattii} \textit{snf7} \textit{D} mutant had a survival rate of 87.5% by day 40 (Figure 6B). Mice infected with parental and complemented strains of \textit{C. gattii} died by days 18 and 29, respectively. Statistical analysis (Mantel-Cox test) confirmed that \textit{C. neoformans} (\(p = 0.0008\)) cells lacking Snf7 were less efficient than parental and complemented strains in killing mice.

To assess GXM secretion in vivo, mice were infected with \textit{C. neoformans} for determination of fungal loads and polysaccharide concentration in lung tissues (Figure 6C). In pulmonary samples from infected mice with the CN-\textit{snf7} \textit{D} mutant, the concentration of extracellular GXM was nearly half the values obtained when mice were infected with parental cells (\(p = 0.0017\)). Fungal loads were also greatly reduced when animals were infected with the CN-\textit{snf7} \textit{D} mutant, in comparison to animals infected with the wild type strain (\(p < 0.0001\)).

\textbf{Discussion}

Secretory activity is mandatory for cryptococcal pathogenesis. The vast majority of the well-characterized molecular determinants of cryptococci are extracellular, including GXM, urease and phospholipase \(B^{37,42-44}\). In addition, \textit{C. neoformans} melanin, which is also fundamental for virulence, is exported to the cell surface by vesicle-dependent secretory mechanisms\(^{17,45}\). Except for phospholipase B, all the above-mentioned virulence factors lack the leader peptide...

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**Figure 1** | Analysis of cryptococcal SNF7. (A). Phylogenetic analysis was performed by applying the neighbor-joining method with Snf7 amino acid sequences from distinct organisms as follows: \textit{S. cerevisiae}, \textit{C. albicans}, \textit{A. nidulans}, \textit{P. brasiliensis}, \textit{S. pombe}, \textit{N. crassa}, \textit{M. oryzae}, \textit{A. thaliana}, \textit{O. sativa}, \textit{C. neoformans} and \textit{C. gattii}. The bar marker indicates the genetic distance, which is proportional to the number of amino acid substitutions. Bootstrap values obtained with 1,000 re-samplings are displayed at the nodes. (B). Analysis of SNF7 disruption in \textit{C. neoformans} and \textit{C. gattii} by Southern blot. Genomic DNA (10 \(\mu\)g) from WT, \textit{snf7} \textit{D} and \textit{snf7} \textit{D}::\textit{SNF7} strains were digested with the XbaI restriction enzyme. The \(3’\) gene flank was used as probe in Southern hybridization. (C). For RT-PCR, cDNA from \textit{C. neoformans} and \textit{C. gattii} (WT, \textit{snf7} \textit{D} and \textit{snf7} \textit{D}::\textit{SNF7} strains) were used as template and genomic DNA of both fungi (WT cells) was used as positive control. Actin gene (\textit{ACT1}) was used as reference gene for normalization of RT-PCR.
Figure 2 | Effects of SNF7 disruption on fungal growth under different conditions. (A). Wild type (WT), mutant (snf7Δ) and complemented (snf7Δ::SNF7) strains of C. neoformans and C. gattii were grown in YPD broth with shaking at 30°C or 37°C. Growth measurements (OD₆₀₀) were performed at 4 h, 6 h, 8 h, 10 h, 12 h and 24 h. (B). Ten-fold serial dilutions of all strains were plated on LCM + Cu and LCM agar plates. The plates were incubated for 2 days at 30°C or 37°C. Fungal growth in LCM + Cu represented the control condition. (C). Growth rates of WT, mutant and complemented strains at pHs 7, 7.5, 8, 8.5 and 9. (D). Growth of WT, snf7Δ and snf7Δ::SNF7 strains on YPD agar plates supplemented with 150 mM LiCl₂. Experiments were performed in triplicate and representative examples are shown.
required for conventional secretion\textsuperscript{46}. This observation and the recent notion that most fungal extracellular molecules lack secretory tags\textsuperscript{17} strongly suggest that unconventional secretion mechanisms and fungal virulence are strictly connected. Due to high efficacy of both \textit{C. neoformans} and \textit{C. gattii} in exporting massive amounts of polysaccharides, we used this model to investigate connections between unconventional export of molecules and fungal pathogenesis.

The ESCRT machinery participates in MVB formation (reviewed in\textsuperscript{23,47}), which is required for exosome release to the extracellular space. Snf7, one of the components of the ESCRTIII protein complex, is responsible for the genesis of intraluminal vesicles of MVBs\textsuperscript{47}. Therefore, considering that fungal cells might use exosome-like vesicles for the transfer of intracellularly synthesized polysaccharides to the extracellular milieu\textsuperscript{17}, it seems reasonable to suppose that Snf7 is required for unconventional export of GXM in fungal cells. This hypothesis is supported by the fact that \textit{C. neoformans} sec mutants, which have defects in post-Golgi, conventional secretory mechanisms, have normal capsules\textsuperscript{48,49}. On the other hand, \textit{C. neoformans} ESCRT-I mutants lacking expression of Vps23 manifested defective capsule formation and reduced virulence in a mouse model of cryptococcosis\textsuperscript{50}.

Most of the studies on secretory mechanisms in eukaryotic cells were focused on protein trafficking\textsuperscript{17,48,51–55}. The mechanism for export of molecules of a non-protein nature, including pigments and polysaccharides, are poorly known. In mammalian cells, MVBs have been correlated with the initial biogenesis of melanosomes\textsuperscript{56}, implying connections between unconventional secretion and pigment traffic. In plant cells, endomembranes participate in polysaccharide traffic\textsuperscript{57}, but the identity of the components necessary for the transport of cell wall enzymes and polysaccharides is not known. In this study, deletion of \textit{SNF7} resulted in reduced efficacies of pigmentation, GXM secretion and capsule formation in \textit{C. neoformans} and \textit{C. gattii}. These observations suggest that MVB formation and unconventional secretion mechanisms participate directly in the export of surface and/or extracellular molecules of non-protein nature. Although this hypothesis still needs experimental confirmation, it agrees with the observation that in \textit{C. neoformans} Golgi reassembly and stacking protein (GRASP) and the Apt1 flippase, which are both unconventional secretion regulators, were required for polysaccharide, but not protein export\textsuperscript{58–60}.

Melanin is essential for cryptococcal virulence\textsuperscript{61}. In our study, the effects of \textit{SNF7} deletion on melanin formation varied depending on the species analyzed, temperature of growth and source of substrates for pigment synthesis. This observation efficiently illustrates the physiological diversity between the sibling species \textit{C. neoformans} and \textit{C. gattii}, which is likely linked to their well-known differences in pathogenic potential\textsuperscript{3}. These results also exemplify the functional diversity of Snf7 in eukaryotes. In our model, fungal growth was only
slightly affected in mutants cultivated at 37°C, but a previous study with *S. cerevisiae* lacking Snf7 demonstrated a clear correlation between carbon source and temperature-sensitive growth\(^6\), suggesting an important metabolic variation that is affected by both nutrient availability and temperature of growth. The mechanisms explaining the differential ability of *C. neoformans* and *C. gattii* Snf7 mutants to produce pigments at different temperatures in the presence of distinct melanization substrates are still unknown, but our results and the seminal observations in the *S. cerevisiae* model\(^2\),\(^3\),\(^2\) clearly support the notion that the physiological functions of Snf7 are regulated at multiple levels and result in variable fungal phenotypes. It is noteworthy that the enzymatic activity required for melanization can be also influenced by pH. Due to the putative roles of Snf7 in pH sensing\(^2\),\(^3\),\(^2\) we cannot rule out the hypothesis that defective melanin formation resulted from altered laccase activity in the two species analyzed in our study.

**SNF7** deletion also interfered with tolerance to alkaline pH and high lithium chloride concentrations. This data is in agreement with previous literature on *S. cerevisiae*\(^6\), *C. albicans*\(^2\),\(^4\) and *A. nidulans*\(^8\) suggesting the participation of Snf7 in the RIM101 signaling cascade. The RIM family includes a sensor protein (RIM21p) and a catalytic complex (RIM20p in association with RIM13p) capable of inducing the proteolytic activation of RIM101p. The lack of Snf7 has been correlated with inability to activate the RIM101 transcription factor independently of the presence of RIM20p and RIM13p\(^2\),\(^3\),\(^4\),\(^8\). In fact, the active form of Rim101p supposedly modulates the expression of

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**Figure 4 | Disruption of SNF7 affects pigmentation but not extracellular enzyme activity.** (A). Fungal cells were grown on Niger seed agar and L-DOPA plates to evaluate melanin production by wild-type (WT), mutant (*snf7Δ*) and complemented (*snf7Δ::SNF7*) strains of *C. neoformans* and *C. gattii*. The cultures were incubated at 30°C and 37°C and monitored for 2 (Niger), 3 (L-DOPA 30°C) or 4 (L-DOPA 37°C) days of cultivation. (B). Phospholipase activity in egg yolk agar. All strains were inoculated on plates containing egg yolk agar and incubated at 30°C for 4 days, and *Pz* values were then determined. (C). Urease activity levels of WT, *snf7Δ*, *snf7Δ::SNF7* cells. Fungal cells were incubated in urea broth with shaking (200 rpm) at 37°C for 24 hours for colorimetric determination. Statistical differences were not observed when parental, mutant and complemented strains were compared.
several genes involved in the adaptation to different environments and its own expression, which is in agreement with the reduction of RIM101 mRNA observed in our study.

O’Meara and collaborators have recently shown that Rim101p is also essential for capsule enlargement in C. neoformans. Although a rim101Δ mutant had normal GXM secretion, several genes involved in cell wall-biosynthesis were affected by RIM101 deletion, which was likely related to a hypcapsular phenotype. The mutant retained virulence in a murine intranasal infection model, suggesting that secretion of GXM could sufficiently impact host cells in favor of the pathogenic process. The results obtained in our study are in agreement with this supposition. It is noteworthy, however, that the SNF7 disruption mutants generated in this work additionally had reduced capsules and low efficacy to melanize, which likely renders these cells more susceptible to the antimicrobial arsenal produced by the host. Future studies on how SNF7 and RIM101 impact immunomodulation are necessary for a broader understanding on how these cellular pathways interfere with the pathogenic process.

Figure 5 | Surface architecture and GXM release are affected by SNF7 deletion. (A). Determination of GXM in C. neoformans and C. gattii culture supernatants. Asterisks denote statistically significant differences (**, p < 0.005; *** p < 0.001) between the values obtained for the mutant strains and those obtained for wild type (WT) and complemented (snf7Δ::SNF7) cells. (B). Microscopic analysis of WT, snf7Δ and snf7Δ::SNF7 strains of C. neoformans and C. gattii. India ink counterstaining and immunostaining (upper and middle panels, respectively) revealed that SNF7 disruption profoundly affects capsule formation. GXM and chitin are stained in green and blue respectively (middle panels). Scale bars represent 5 μm. Scanning electron microscopy of the C. neoformans and C. gattii strains (Lower panels). Scale bar represents 1 μm. (C). Quantification of capsular dimensions based on in silico measurements of the results illustrated in B (*** p < 0.001).

Figure 6 | Cryptococcal virulence is affected by SNF7 disruption. Mortality rates of mice lethally infected with wild type (WT), mutant (snf7Δ) and complemented (snf7Δ::SNF7) strains of C. neoformans (A) and C. gattii (B) were monitored daily. SNF7 deletion was associated with a non-virulent phenotype in both cryptococcal species. Determination of GXM concentration (black bars) and lung CFU counts (grey bars) in the lungs of mice infected with wild type (CN-WT) and mutant (CN-snf7Δ) cells (C) revealed defective polysaccharide production and reduced survival of the mutant (**, p = 0.0017; *** p < 0.0001).
Our observations support the notion that Snf7 expression regulates virulence in both C. neoformans and C. gattii by reducing poly-saccharide and melanin traffic. These results further support the observation that unconventional secretion is essential for cryptococcal pathogenesis\(^a,b,5-6\) and strongly suggests the occurrence of still obscure mechanisms of exportation of molecules of a non-protein nature in Eukaryotes.

### Methods

**Fungal strains, plasmids and media.** The C. neoformans strain H99 (serotype A) and the C. gattii strain R265 (serotype B) were the recipients for the constructs of the mutant strains. Cells were maintained in YPD medium (yeast extract 1%, peptone 2%, glucose 2% and 1.5% agar). To evaluate the effects of Snf7 deletion on the growth of C. neoformans and C. gattii, the cells were grown at 37°C in YPD medium (BD Difco, Sparks, MD, USA). This reaction mixture was transformed into E. coli DH5α (pir) and the deletion was confirmed by Southern blot analysis and PCR. This was repeated with each strain.

The generation of the mutant strain. Genomic insertion was confirmed by Southern blot and PCR. The possible snf7Δ mutant strain was screened by PCR, and the deletion was confirmed by Southern blot analysis and reverse transcription-PCR (RT-PCR). For complementation of the snf7Δ mutant strain, a genomic PCR fragment containing a 300 ng of each) were submitted to BP cloning reaction, according to manufacturer’s instructions (Invitrogen, Carlsbad, CA). This reaction mixture was transformed into Escherichia coli OmniMAX 2-T1. The deletion construct was linearized by I-SceI enzymatic digestion and submitted to blastic transformation in 9 C. neoformans (H99 strain). The possible snf7Δ mutant colonies were screened by PCR, and the deletion was confirmed by Southern blot analysis and reverse transcription-PCR (RT-PCR). For complementation of the snf7Δ mutant strain, a genomic PCR fragment containing a wild type C. neoformans SNF7 gene was cloned into the SmaI site of the pAI4 plasmid. Biologistic transformation was performed to introduce the resultant vector into the mutant strain. Genetic insertion was confirmed by Southern blot and RT-PCR.

**Disruption and complementation of SNF7.** Degeliate methodology\(^a,5,7\) was used to construct the snf7Δ mutant strains. For the C. neoformans mutant, pDONR N- vector was constructed as previously described\(^b,5,7\). The 5‘ and 3‘ SNF7 flanks (–700 bp) were PCR amplified and gel purified using the Illustra GFX PCR DNA and gel band purification kit (GE Healthcare, Fairfield, USA). This reaction mixture was transformed into E. coli DH5α (pir) and the deletion was confirmed by Southern blot analysis and reverse transcription-PCR (RT-PCR). For complementation of the snf7Δ mutant strain, a genomic PCR fragment containing a wild type C. neoformans SNF7 gene was cloned into the SmaI site of the pAI4 plasmid. Biologistic transformation was performed to introduce the resultant vector into the mutant strain. Genetic insertion was confirmed by Southern blot and RT-PCR.

**Phenotypic assays.** For spot assays, wild type C. neoformans (C. neoformans (CN-CT and CG-WT), C. gattii SNF7 Δ and C. gattii SNF7 Δ mutants and complemented (CN-...
snf7Δ::SNF7 and CG-snf7::SNF7 strains were cultivated in YPD medium and serially diluted. Diluted cells were spotted on LCM agar, LCM + Cu agar and 150 mM LiCl,4 (Stigma, St. Louis, MO). Melanin production was visualized in cells spotted on Niger seed agar plates and on defined media containing 1 mM of L-DOPA.4 For capsule analysis, WT, snf7Δ and complemented strains of C. neoformans and C. gattii (5 × 10^5 cells/plate) were incubated using six well culture plates in DMEM (Invitrogen, Carlsbad, CA) at 37˚C and 5% CO₂ for 48 hours. Capsule measurements were performed as previously described.4 Staining of surface components (chitin and GXM) was performed as described elsewhere.4 Briefly, fungal cells were fixed in 4% paraformaldehyde (Stigma, St. Louis, MO) for 1 hour followed by a incubation with phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (Stigma, St. Louis, MO). Cell structures were then stained using calciofluor white (25 μM) (Stigma, St. Louis, MO) and 18βG anti GXM monoclonal antibody (1 μg/mL) (kindly provided by Dr. Arturo Casadevall) followed by anti murine IgG Alexa Fluor 488 conjugated (Invitrogen, Carlsbad, CA). Images were acquired using an Axyoplan 2 microscope (Carl Zeiss, Germany). Cell surface structures were also observed by scanning electron microscopy (SEM) as described elsewhere.4 Analysis of extracellular GXM was performed according to the method described by Casadevall and colleagues,4 with minor modifications.4 Phospholipase activity was determined by using egg yolk agar.4 Approximately 10^6 cells of WT, snf7Δ and complemented strains of both C. neoformans and C. gattii were spotted onto the center of petri dishes containing egg yolk agar, and incubated at 30˚C. After 4 days, the phospholipase activity (Pε values) was determined as described by Price and colleagues.4 To test growth in alkaline pH, YPD medium was buffered with 150 mM HEPES to pH 7, 7.5, 8, 8.5 and 9 with NaOH. Cultures were serially diluted 75. Diluted cells were spotted on LCM agar, LCM plates in DMEM (Invitrogen, Carlsbad, CA) at 37˚C and 5% CO₂ for 48 hours. The Universidade Federal do Rio Grande do Sul Ethics Committee.4 Estimation of the current global burden of cryptococcal meningitis and their relationship to the RIM101 pathway.4 Statistical analysis. Statistical comparisons were performed with the Graphpad 5.0 software. Paired comparisons between different groups were performed using the Student’s t-test. Survival curves were statistically analyzed using the Mantel-Cox test.
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