SUPPLEMENT TO:

Glucosylceramide synthase is an essential regulator of pathogenicity of Cryptococcus neoformans

Philipp C. Rittershaus¹,⁵, Talar B. Kechichian¹,⁵, Jeremy C. Allegood³, Alfred H. Merrill Jr.³, Mirko Hennig⁴, Chiara Luberto¹, & Maurizio Del Poeta¹,²*

¹Departments of Biochemistry and Molecular Biology and ²Microbiology and Immunology, Medical University of South Carolina, Charleston, SC 29425; ³School of Biology, Georgia Institute of Technology, Atlanta, GA 30332; and ⁴Department of Molecular Biology, Scripps Research Institute, La Jolla, CA 92037, USA. ⁵These authors contributed equally to this work.

* Corresponding author: Maurizio Del Poeta, M.D., Department of Biochemistry and Molecular Biology, Medical University of South Carolina, 173 Ashley Avenue, BSB 503, Charleston, South Carolina, 29425. Tel: (843) 792-8381, Fax: (843) 792-8565, delpoeta@musc.edu

Supplemental Figure 1. (A) A diagram illustrating the deletion of Cn GCS1 gene using pΔgcs1 plasmid cassette. (B) Southern analysis of genomic DNA digested with HindIII (H3) of wild-type (WT) and 5 transformants, using the indicated probes. Transformant #42 showed homologous recombination with double cross over event without any ectopic or loop integration. This strain was designated Cn Δgcs1. (C) Reintroduction of GCS1 gene into the Cn Δgcs1 strain using pCR-GCS1-HYG plasmid cassette. (D) Southern analysis of genomic DNA digested with EcoRI (RI) extracted from wild-type (WT) Δgcs1 and transformant #14, which shows a single crossover event at the 5’-UTR plus insert of plasmid loop with consequent insertion of a second
This strain was designated Δgcs1 + GCS1.

**Supplemental Figure 2.** Lung tissue burden culture of CBA/J mice infected intranasally with Cn wild-type (WT) or Δgcs1 strain during the course of infection. Lungs infected with Cn wild-type show a progressive increase number of yeast cells and, eventually, mice will succumb by day 20-28 of infection (please see Figure 3B). Lungs infected with Δgcs1 strain show a ~500-fold decrease in the number of yeast cells by day 7 and ~100-fold decrease during the remaining time of infection compared to the initial inoculum.

**Supplemental Figure 3.** Histopathology of 2 different lungs (A and B and C and D) obtained from CBA/J mice infected intranasally with Cn Δgsc1 strain, and 2 different lungs (E and G) and 2 different brains (F and H) obtained from CBA/J mice infected intravenously. A, B, E and G, hematoxylin and eosin; C, D, F and H, mucicarmine. A, C, and E–H, 10X; B and D represents 40X magnification of squared areas in A and C, respectively. In A, white arrow indicates lymphocyte infiltration in proximity of a macrophage aggregation (square). Green arrow indicates normal lung. White bar = 200 μm. In B (hematoxylin and eosin), Cn cells (white arrowhead) are readily recognized within a large macrophage. Other macrophages contain “ghosts” or degenerated yeast cells (yellow arrowhead). White bar = 50 μm. In C, lymphocyte and macrophage (white arrows) infiltration in proximity of Cn engulfed within a giant macrophage, readily appreciated in D. Green arrow indicates normal lung tissue. In C, black bar = 200 μm. In D, white bar = 50 μm. In E and G, Cn cells are localized in small nodules with a host cellular infiltration comprised by lymphocytes and neutrophils (black arrows). Green arrows indicate normal lung. In F and H, Cn Δgsc1 cells are almost exclusively contained within brain abscess (black arrowheads) with the absence of a granulomatous response. In E–H, black bar = 200 μm.
**Supplemental Figure 4. Intracellular growth.** Five hours of post-incubation of J774.16 macrophage-like cells with Cn WT, ΔgcsI or ΔgcsI + GCS1, the number of macrophages containing yeast cells was determined (phagocytic index) and buds were counted only in yeast cells inside macrophages (% budding/phagocytic index). Counts are geometric means ± standard deviation of at least 8 different fields.

**Supplemental Figure 5. Virulence factors.** The ability to grow at 37 °C and ambient CO₂ (A), production of polysaccharide capsule (B) and melanin formation (C) are not affected by lack of Gcs1/GlcCer. D) Cn ΔgcsI mutant exerts a growth defect on 0.05% SDS agar plate compared to WT or reconstituted strains but not in 1M NaCl, or in presence of nitric oxide or hydrogen peroxide.

**Supplemental Table 1. NMR analysis.** The $^1$H and $^{13}$C chemical shifts (ppm) from $^1$H,$^{13}$C-heteronuclear single quantum correlation (HSQC) experiment and measured vicinal $^3$J$_{HH}$ coupling constants from Double Quantum Filtered-COrrelation SpectroscopY (DQF-COSY) experiment of hexose attached to ceramide correspond to glucose (46, 47).

**Supplemental Methods**

**Deletion of Cn GCS1 gene and reconstitution**

To delete the GCS1 gene from the genome of Cn a plasmid construct was created that contained 1.5 kilo bases (kb) of the 5’ untranslated region (5’-UTR) upstream the open reading frame (ORF) as well as 1.5 kb of 3’-UTR flanking the nourseothricin acetyltransferase gene (NAT1) gene, whose expression is under the control of actin promoter producing yeast cells resistant to the antibiotic nourseothricin (Werner BioAgents). The 5’-UTR was generated by PCR.
using genomic DNA as a template and primers: PRUTR1Sac 5'-CTG GAGCTC CGA AGT
AAA GGC TGG CTT AGC TGA-3’ and PRUTR1Spe 5’-GAG ACTAGT ACC TAT GAA GGG
AAT GAA TAT TGC-3’, which contain SacI and SpeI sites (bold and underlined), respectively.
The 3’-UTR region was generated by PCR using H99 genomic DNA as a template and primers
PRUTR2Fw 5'-GAG AGATCT TTT GGT TTT CAA AGG CTC TGC ATG-3’ and
PRUTR2Rev 5’-GAG GGTACC TAT ATC ACC GCT CAA TAA TAG CTG-3’, which contain
BglII and KpnI sites (bold and underlined), respectively. The resulting fragments were cloned into
the pCR2.1-TOPO vector generating plasmids pCR-5UTR and pCR-3UTR and sequenced. The
plasmid pCR-5UTR was digested with SacI and SpeI and the resulting 1.5 kb fragment was
The pCR-NAT1 was created as previously described (48, 49). Plasmid pCR-3UTR was digested
with KpnI and XhoI and the 1.5 kb 3’-UTR fragment was subcloned into a KpnI-XhoI restricted
pBluescript SK vector generating plasmid pSK-3UTR. Finally, plasmid pCR-5UTR:NAT1 was
digested and the 3.2 kb fragment corresponding to the 5UTR-NAT1 was subcloned into pSK-
3UTR vector, generating plasmid pSK-5UTR-NAT1-3UTR which was re-named pΔgcs1
(Supplemental Figure 1A).

The Cn wild-type strain H99 was transformed with plasmid pΔgcs1 using biolistic
delivery of DNA, according to (50). Transformants were grown on YPD plates containing 100
µg/ml of nourseothricin. Colonies were chosen randomly and purified. Genomic DNA preparation
for Southern blot analysis was performed according to (51). Five transformants showing deletion
of the GCS1 gene and insertion of the plasmid cassette were obtained and transformant #42 was
chosen and designated Cn Δgcs1 strain (Supplemental Figure 1B).

To reintroduce the GCS1 gene back in the Δgcs1 mutant we generated the pCR-GCS1-
HYG plasmid construct as follow (Supplemental Figure 1C): 1) Fragment A (4.7 kb) containing
the entire GCS1 ORF and 1.5 kb of the upstream (5’UTR) and downstream (3’UTR) regions were
generated by PCR using H99 genomic DNA as a template and primers PRUTR1Sac 5’-CTG
GAGCTC CGA AGT AAA GGC TGG CTT AGC TGA-3’ and PRUTR2Rev 5’-GAG
GGTACA TAT ATC ACC GCT CAA TAA TAG CTT-3’, containing a SacI and KpnI site,
respectively (bold and underlined). This 4.7 kb fragment was cloned into the pCR2.1-TOPO
vector generating plasmid pCR-GCS1. 2) Fragment B (2.5 kb) containing the hygromycin B gene
(HYGB) conferring resistance to hygromycin B (Calbiochem #400051) was obtained by digesting
the pCnTel vector (52) using XbaI and HindIII. This fragment was blunt ended and cloned into SpeI
restricted-blunted pCR-GCS1 vector, generating pCR-GCS1-HYG construct. The Cn Δgcs1
mutant was transformed with pCR-GCS1-HYG plasmid using biolistic delivery of DNA.
Transformants were grown on YPD plates containing 100 µg/ml of hygromycin B. Stable
transformants were selected, grown on YPD, DNA extracted and three transformants showed re-
itroduction of wild-type GCS1 gene and the introduction of a second GCS1 copy by the insertion
of the plasmid loop. Transformant #14 was chosen and designated Cn Δgcs1 + GCS1
(Supplemental Figure 1D).

Purification of GlcCer

GlcCer was purified from Cn cells following the protocol described in (10). Briefly, Cn
wild-type, Δgcs1 and Δgcs1 + GCS1 strains were grown on YPD media in a shaker incubator for
48 h at 30 °C. The cells were washed twice with SDW and 6 pellets of 5x10⁶ cells per strain were
resuspended in 1 ml total lipid extraction (TLE) buffer (95%
ethanol:SDW:diethylether:pyridine:14.8N NH₄OH -15:15:5:1:0.018) and incubated for 30 min at
60 °C with brief vortexing. After centrifugation for 10 min at 2000 x g the supernatants of two
samples were combined and dried down. Dried pellets were suspended in 2 ml methanol, 1 ml
chloroform was added and samples were incubated for 30 min at 37 °C with brief vortexing. After
centrifugation for 10 min at 2000 x g, the supernatant was transferred to another tube, 1 ml
chloroform and 1 ml H₂O were added, and the phases were homogenized by vortexing twice for 30 sec. Then, phases were separated by centrifugation for 5 min at 2500 x g and the lower phase was dried down. The pellet was suspended in 1 ml chloroform:acetic acid (99:1) and loaded into a Sep-Pak® Cartridge (Waters) previously equilibrated with 15 ml chloroform. Neutral lipids fraction were eluted with 15 ml chloroform:acetic acid (99:1) and discarded. Glycolipid-fraction was eluted with 10 ml acetone and dried down. Pellets were suspended in 0.5 ml chloroform and 0.5 ml of 0.6 M KOH in methanol, incubated for 1 h at room temperature, neutralized with 0.325 ml of 1 M HCl, and phases were separated by adding 0.125 ml of H₂O. The organic phase was transferred into a new tube, dried down and resuspended in 1 ml chloroform:acetic acid (99:1). The mixture was then re-loaded into a Sep-Pak® Cartridge (Waters) previously equilibrated with 15 ml chloroform. The column was sequentially eluted with 15 ml chloroform:acetic acid (99:1), 10 ml chloroform:methanol (95:5), 15 ml chloroform:methanol (9:1), and 10 ml chloroform:methanol (8:2), 5 ml chloroform:methanol (1:1) and 5 ml methanol. The fractions eluted with the chloroform:methanol ratio of 9:1 would contain GlcCer and were dried down for high performance thin layer chromatography (HPTLC) analysis. These lipid fractions were suspended in chloroform:methanol (2:1) and spotted on a Kieselgel 60 (HPTLC-Merck) and developed in the solvent chloroform:methanol/H₂O (65:25:4). Plates were dried at room temperature and the sugar residues were stained by repeated spraying of the plate with 0.2 mg/l orcinol/70% H₂SO₄ and heating for 15 min in an oven at 100 °C. To make sure that equal amount of lipid extracts were loaded in each lane, plates were also exposed to iodine, which revealed that approximately an equal amount of lipids were loaded (arrowhead in Figure 2B).

**Histology analysis**

Lung, brain, liver, kidney and spleen of CBA/J mice infected with the above strains were collected. Organs were fixed in 37% formaldehyde (Sigma), embedded in paraffin, and stained
with hematoxylin and eosin to visualize the host inflammatory response, mucicarmine as specific staining for Cn capsule (53), Russell’s modification of Movat’s pentachrome stain (54), and Verhoeff-van Gieson (VVG) staining (55). Movat is a pentachrome dye which stains mucin in alcian-blue, fibrous tissue in intense red, and elastic tissue in black. VVG stain is used to identify connective tissue, such as elastic fibers, which are stained in black, and collagen, which is stained in red. Also, organs were homogenized for tissue burden culture analysis in 10 ml PBS using the Stomacher 80 (Lab System, Fisher Scientific, Pittsburgh, Pennsylvania, USA) for 2 min at high speed. Serial dilutions were then plated onto YPD plates and incubated at 30 °C for 72 h and yeast colonies were counted and recorded as Colony Forming Unit (CFU) per organ. Data were recorded as the average ± standard deviation of Log10 CFU/organ.

REFERENCES


Supplemental Figure 1
Log CFU/lung

Time of infection (days)

Wild-type
Δgcs1

Supplemental Figure 2
Supplemental Figure 3
Supplemental Figure 4
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Supplemental Table 1