

**Methylation of glycosylated sphingolipid modulates membrane lipid topography and pathogenicity
of *Cryptococcus neoformans***

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Supplementary materials and methods

Radiolabelled isotopes. [³H]- palmitic acid and [³H]-myo-inositol are from American Radiolabelled Chemicals Inc. [³²P]- CTP is from Perkin Elmer.

Reconstitution of *SMT1* gene. A 3269 bp long fragment containing a 1699 Kb putative *SMT1* open reading frame (ORF), a 1294 bp fragment corresponding to the upstream 5' untranslated (5'UTR) region and a 276 bp fragment corresponding to the downstream 3' untranslated (3'UTR) region was amplified by PCR using genomic H99 DNA as a template. The 3269 fragment was then cloned in pSC-A-amp/kan (Stratagene) and sequenced. This fragment was then digested with *Xba*I, gel purified, eluted and subcloned in *Xba*I-digested and dephosphorylated pSK-HYG vector containing the hygromycin B gene (HYGB conferring resistance to hygromycin B) cassette. HYGB gene is under control of *C. neoformans* actin promoter. Hygromycin was purchased from Calbiochem (#400051).

***In-vivo* [³H]-palmitic acid and [³H]-myo-inositol labeling studies.** 15 ml culture, each of WT, Δ *smt1* in YNB supplemented with 25 mM HEPES and 2% glucose, pH 7.2 were grown for 20 hrs in a shaker-incubator at 37°C in presence of 5% CO₂. Approximately 2 hours prior to cell collection, 5x10⁷ cells/ml were collected and incubated with 2 μCi /ml of [³H]-palmitic acid for 2 hours at the same conditions in a shaker incubator. For [³H]-myo-inositol labeling, 4 μCi/5x10⁷ cells were used. The cells were then pelleted, washed in PBS and the lipids were extracted by Mandala and Bligh and Dyer. A quarter of the sample was aliquoted before drying for the determination of inorganic phosphate. The dried lipid were dissolved in Chloroform:Methanol (2:1 by volume) before spotting on a TLC and analyzed by radio scanning. The complex sphingolipid *viz* IPC, MIPC and M(IP)₂C were quantified by scraping the radiolabelled spots from the TLC. Five μg of soy GlcCer (Avanti Polar Lipids Inc) was loaded as a control.

***In vitro* growth studies.** A 15 ml starter culture in YPD of WT, Δ *smt1* and Δ *gcs1* from freshly streaked YPD plates was grown for 24 hrs at 30°C. Cells were pelleted and washed in sterile PBS and counted in a haemocytometer and resuspended in DMEM supplemented with 50 mM Hepes in pH 7.2 and pH 4.0 to

have a density of 2.75×10^5 cells/ml. 200 μ l from this suspension were inoculated into a 24 well plate to have a final cell density of 5.5×10^4 cells/ well and incubated on a shaker at 37°C in presence of 5% CO_2 . Aliquots were taken at the designated time points and serial dilutions were plated on YPD plates for assessment of colony forming units (CFU).

Tissue burden culture. For tissue burden, organs were homogenized in 10 ml PBS using Stomacher 80 (Lab System Fisher Scientific, Pittsburgh, Pennsylvania, USA) for 2 min at high speed. Serial dilutions were then plated on YPD plates and incubated for 72 hrs at 30°C . CFU/organ was counted and recorded.

Spot-dilution Assay. Cells from a log phase culture were harvested, washed and resuspended in sterile PBS at a density of 5×10^8 cells/ml. 5 μ l this cell suspension was spotted in serial dilutions on YPD-agar plates containing either 0.05% SDS, or 0.025% Triton X-100 or 0.025% Triton X-114 and incubated at $30^\circ\text{C}/37^\circ\text{C}$ for 48 hrs or 72 hours in presence of 0.04%/5% CO_2 . Cell growth was examined and photographed.

Supplementary Figure Legends

Figure S1. Illustration of the deletion and the reconstitution scheme of the *SMT1* gene (A) The scheme for the deletion of the *SMT1* gene with the deletion cassette p Δ *smt1*. The cassette has the *NATI* gene ORF under *C. neoformans* actin promoter flanked upstream by 5'UTR and downstream by 3'UTR. The genomic DNA of the homologous recombinants was digested with *Pst*I. Southern Blot analysis of the transformants with a fragment of 5'UTR, *SMT1* ORF and *NATI* as probe showed that the transformant # 1.2 was formed by a double cross over event with insertion of the *NATI* fragment. It was designated as *C. neoformans* Δ *smt1*. **(B)** Reconstitution scheme of the *SMT1* gene showing the plasmid cassette with the *SMT1* ORF flanked upstream by 5'UTR and downstream by 3'UTR and the *HYGB* as a selection marker upstream of 5'UTR. A single cross-over event at the 5' end resulted in the re-introduction of the *SMT1* gene by the insertion of the plasmid loop and a second copy of the *SMT1* gene.

Figure S2. Quantification of the radiolabelled image. The ^{14}C –labelled spot from the *in vitro* enzyme assay of the sphingolipid C9 methyltransferase in *C. neoformans* was scraped off from lane 2 and quantitated by liquid scintillation counter. Corresponding unlabelled spots from all the other lanes in the TLC plate were also scraped and quantified. A spot from a blank lane was scraped for individual background correction. The radio signal was normalized to the corresponding Pi value.

Figure S3. Quantification of complex sphingolipids by *in vivo* labeling studies with [^3H]-Palmitic acid. WT and Δ *smt1* were grown for 20 hours in YNB pH 7.2, 37°C and 5% CO₂. Then, 2 μCi of [^3H]-Palmitic acid was added to 5×10^7 cells for 2 hours prior to cell collection. Cells were harvested by pelleting and washing in sterile PBS. Lipids were extracted and loaded on a TLC. The radioactive spot were scraped off, counted in a scintillation counter and the values were normalized with inorganic phosphate. Δ *smt1* shows similar level of complex sphingolipid comprising of IPC, MIPC and M(IP)2C as compared to the WT. Similar results were obtained using [^3H]-myoinositol (data not shown).

Figure S4. *In vitro* growth of WT, Δ *smt1*, Δ *smt1*+*SMT1*, Δ *gcs1*, and Δ *gcs1*+*GCSI* at pH 7.2 and pH 4.0, 37°C, 5% CO₂. Cells from starter culture in YPD were washed and inoculated in DMEM media supplemented with 50 mM Hepes (either pH 7.2 or pH 4.0) at a concentration of 2.75×10^5 cells/ml. 200 μ l from this suspension were inoculated in a 24 well plate to have a final cell density of 5.5×10^4 cells/ well and incubated in a shaker at 37°C, 5% CO₂. At different time points, cells in different dilutions were plated and Colony Forming Units (CFU)/ml were counted after 48-72 h. The Δ *smt1* shows a growth arrest after 36-48 hours at pH 7.2 (A) but not at pH 4.0 (B). (C) A change to acidic medium at pH 4.0 restores growth of both Δ *smt1* and Δ *gcs1* strains.

Figure S5. Tissue burden culture in mice. Brain, spleen, liver, and kidney were collected from CBA/J mice 90 days post infection with Δ *smt1* strain, homogenized in PBS for 2 minutes. Serial dilutions were plated on YPD plates and incubated for 72 hours at 30°C and CFU/organ was counted.

Figure S6. Histopathology of lung infected with Δ *smt1*. Lung was excised from CBA/J mice infected with Δ *smt1* cells after 90 days and stained with MOVAT. The picture depicts the ring of foamy macrophages (white arrows) surrounding necrotic tissue with *C. neoformans* Δ *smt1* cells stained in blue. Scale white bar: 100 μ m.

Figure S7. Histopathology of lung infected with Δ *gcs1*. A typical granuloma formation in mice lung infected with Δ *gcs1*. The necrotic tissues with several *C. neoformans* Δ *gcs1* cells is indicated by the double arrow. The ring of foamy macrophages is indicated by the white arrow and normal tissue by the green arrow. Scale white bar: 500 μ m.

Figure S8. Spot-dilution assay in presence of SDS. The panels show *C. neoformans* wild-type (WT) Δ *smt1*, Δ *smt1*+*SMT1*, Δ *gcs1*, and Δ *gcs1*+*GCSI* spotted in serial dilutions on YPD with 0.05% SDS. The plates were incubated for 72 hours in presence of 0.04%, 5% CO₂, at 30°C.

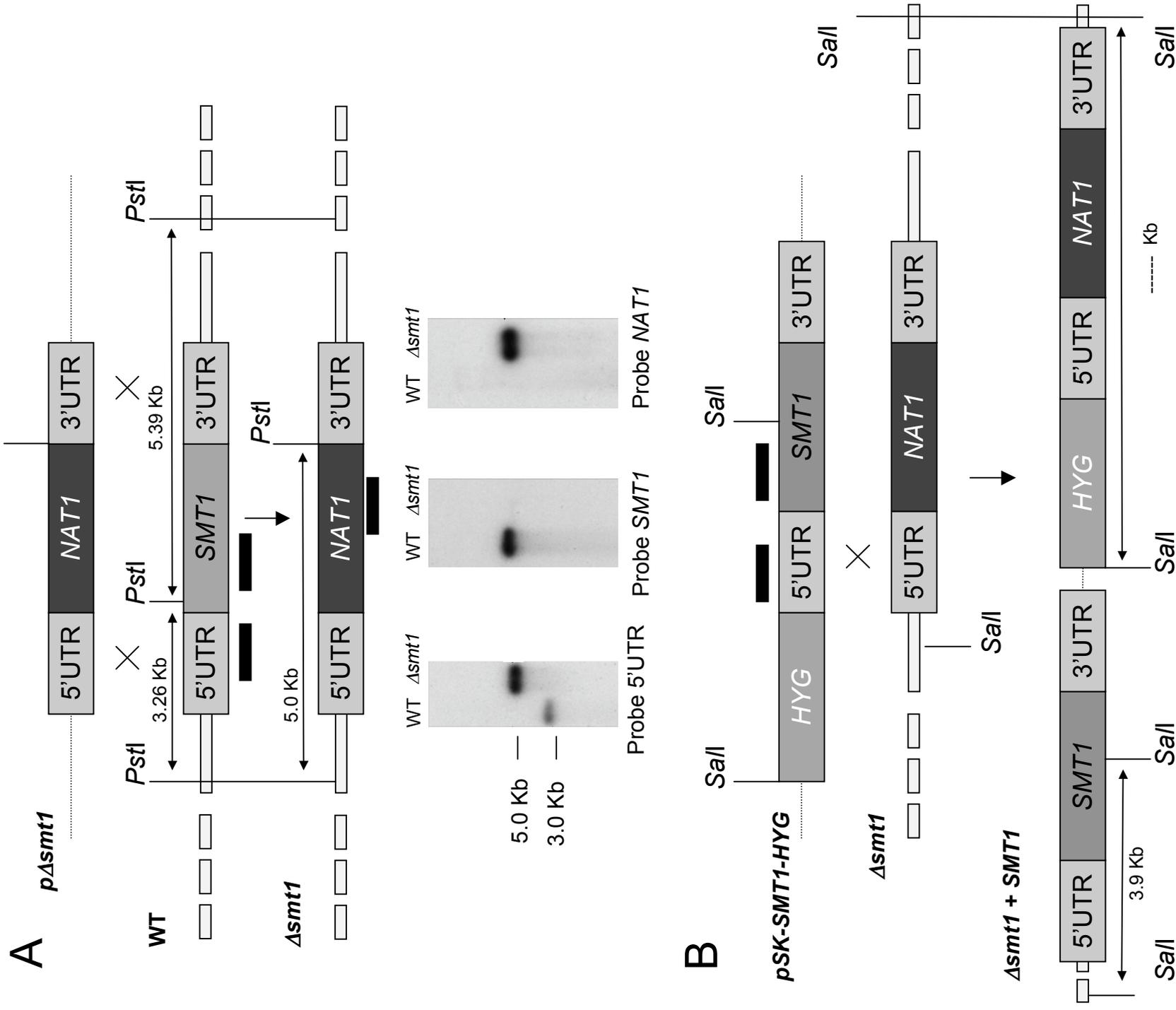
Figure S9. A time course of SYTOX GREEN (SG) uptake in untreated cells. *C. neoformans* WT and Δ *smt1* cells grown in YNB, pH 7.2, 37°C and 5% CO₂ were pelleted, washed and resuspended in PBS at a cell density of 10⁸ cell/ml in 24 well plate. 100 μ l of PBS + 2 μ M SG were added into the wells and incubated at 37°C in presence of 5% CO₂ in dark. Fluorescence was recorded after 20 and 60 minutes of SG uptake. Δ *smt1* cells shows the highest uptake which tends to saturate after 20 minutes. The results are the mean \pm St. Dev. of three separate experiments. Only $P < 0.05$ are shown. [¶] $P < 0.05$, Δ *smt1* versus WT.

Figure S10. Illustration of the glucosylceramide pathway in *C. neoformans*. The de-novo biochemical pathway of glucosylceramide is shaded. In wild-type cells, only α -OH- Δ 4- Δ 8, 9methyl-glucosylceramide is produced. The dotted arrow shows the alternate pathway that Gcs1 takes when the Smt1 or the sphingolipid desaturase 8 (Sld8) is deleted.

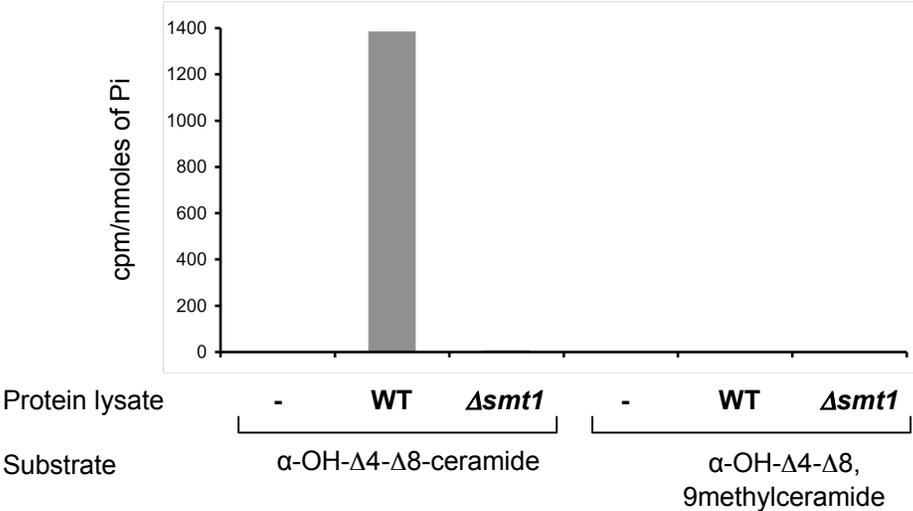
Table S1 List of restriction enzymes used in this study.

	Primer name	Sequence (5'-3')	Restriction sites	Purpose
1	Frag 1F	CAGGGATCCGAACATATCATCA TGGAGCACTTC	<i>Bam</i> H1	PCR amplification of 5'-UTR
2	Frag 1R	CAGAAGCTTCCACGAAATTGAA ATGTTGGAAGT	<i>Hind</i> III	PCR amplification of 5'-UTR
3	Frag 3F	CGAGATATCGTATGTGCATGTG AATATGATATG	<i>Eco</i> RV	PCR amplification of 5'-UTR
4	Frag 3R	CAGTCTAGACTAATCTATAAGC AGGCCATCGGT	<i>Xba</i> 1	PCR amplification of 5'-UTR
5	R2F	CAGTCTAGAGAACATATCATCA TGATGGAGCACTTCATC	<i>Xba</i> 1	PCR amplification of the <i>C9M</i> gene including the 5'-UTR, ORF and 276 bp of 3'-UTR
6	R2R	CAGTCTAGAAGTATCTCCTCCC CGCCAAAGATA	<i>Xba</i> 1	PCR amplification of the <i>C9M</i> gene including the 5'-UTR, ORF and 276 bp of 3'-UTR

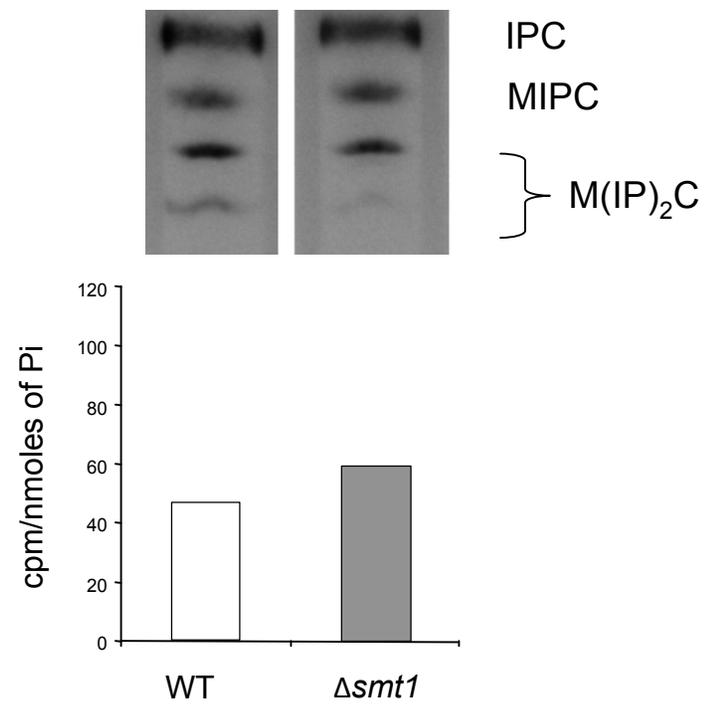
Supplementary Figure 1. Singh et al



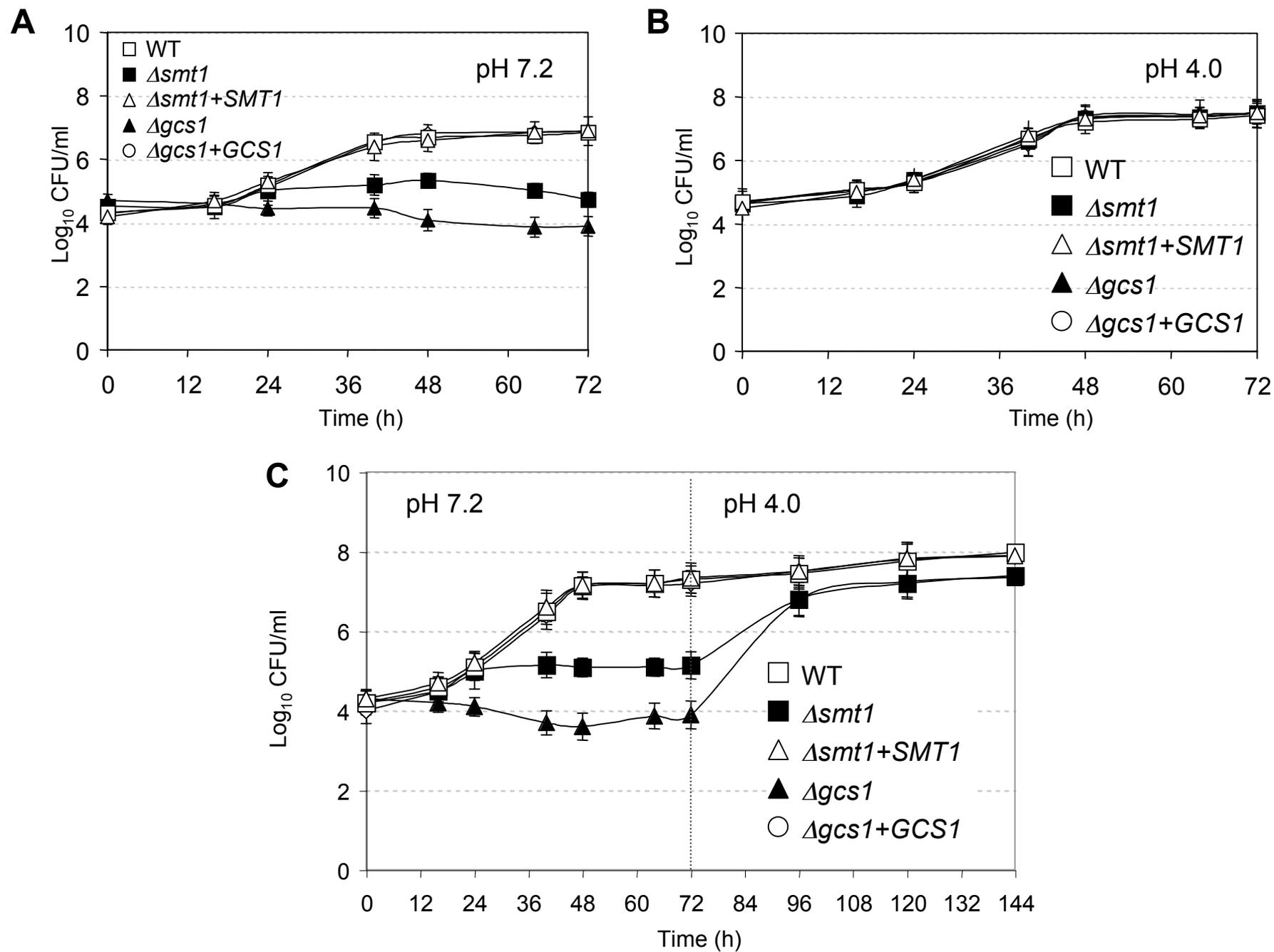
Supplementary Figure 2. Singh et al



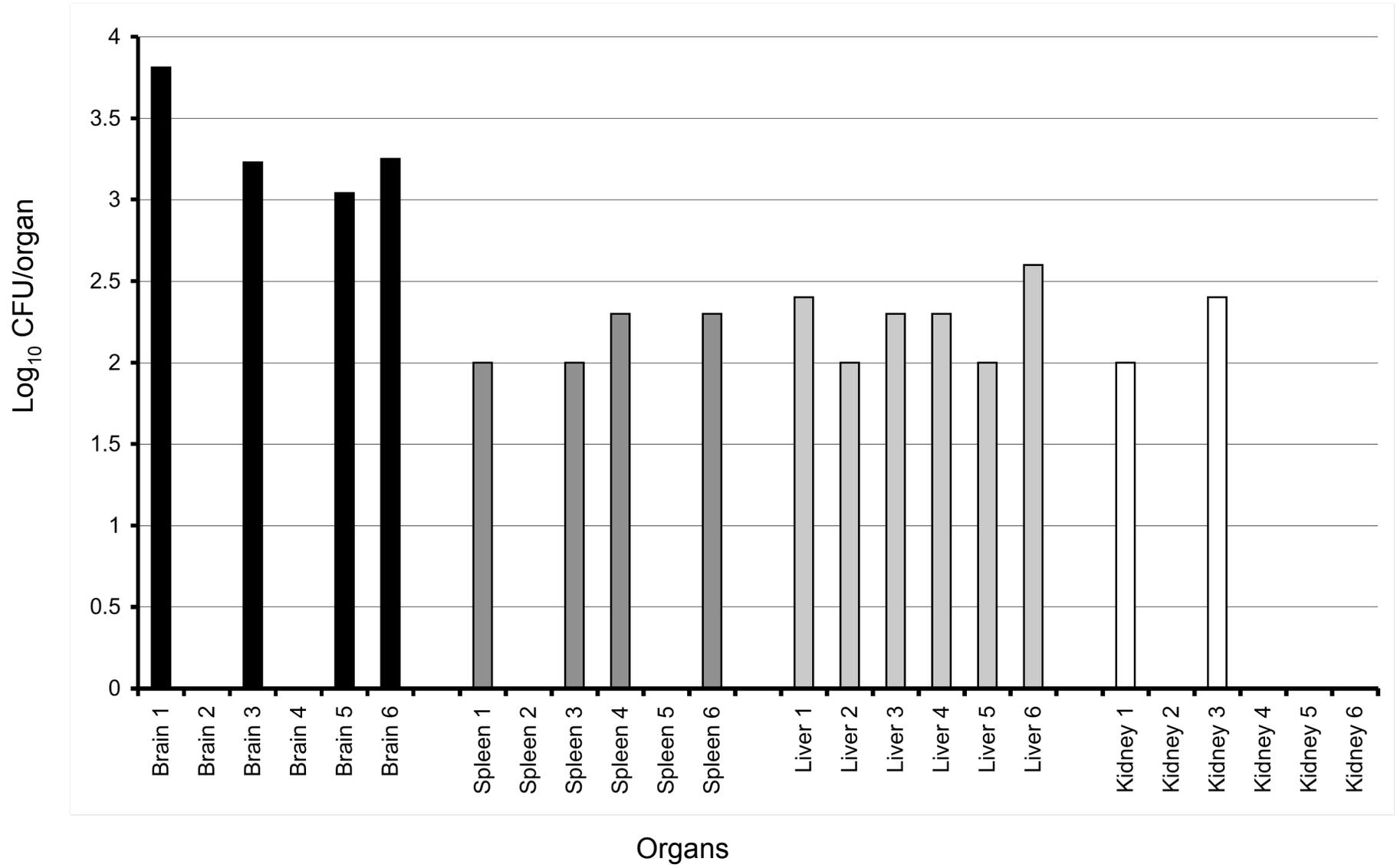
Supplementary Figure 3. Singh et al



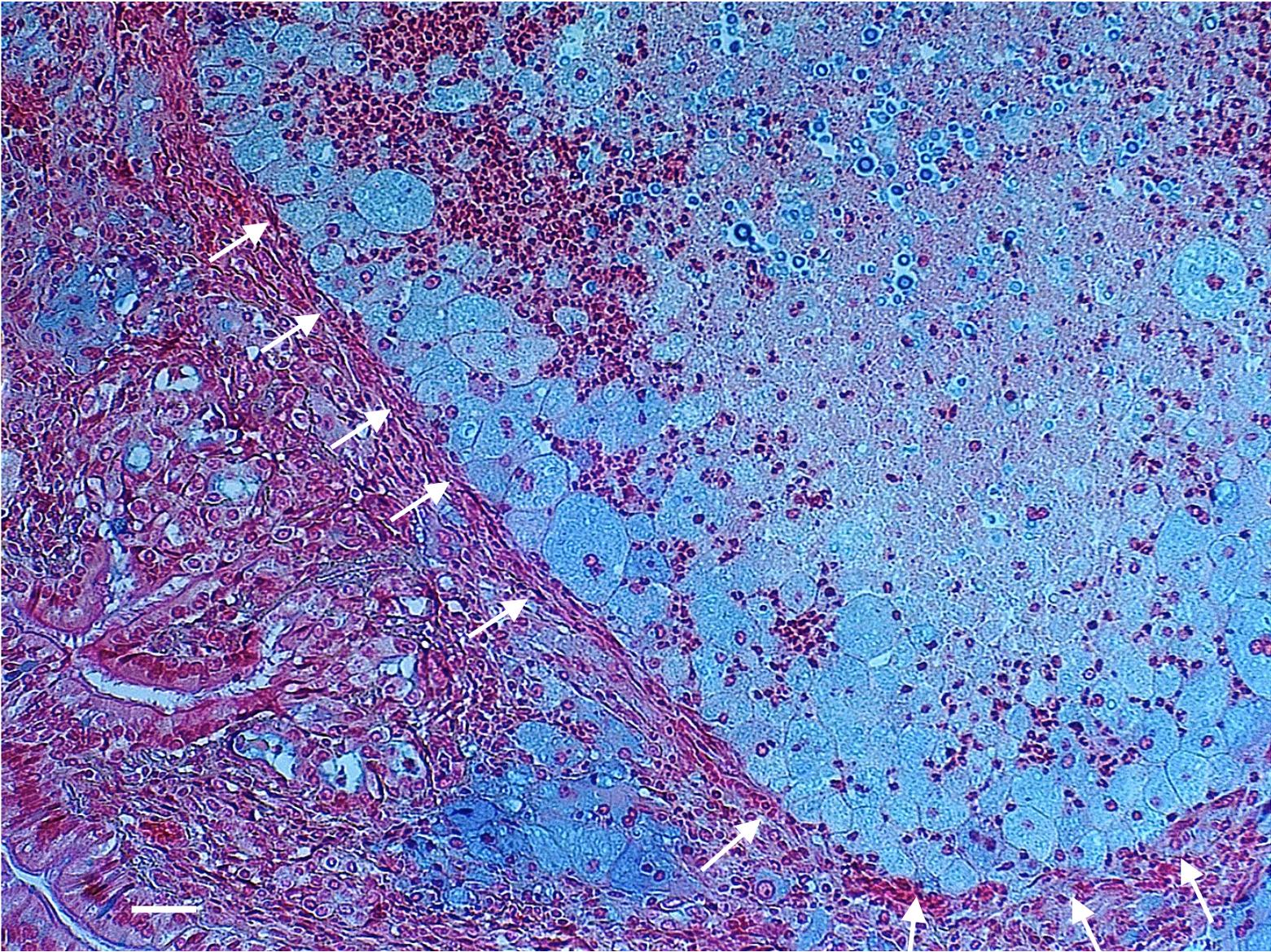
Supplementary Figure 4. Singh et al



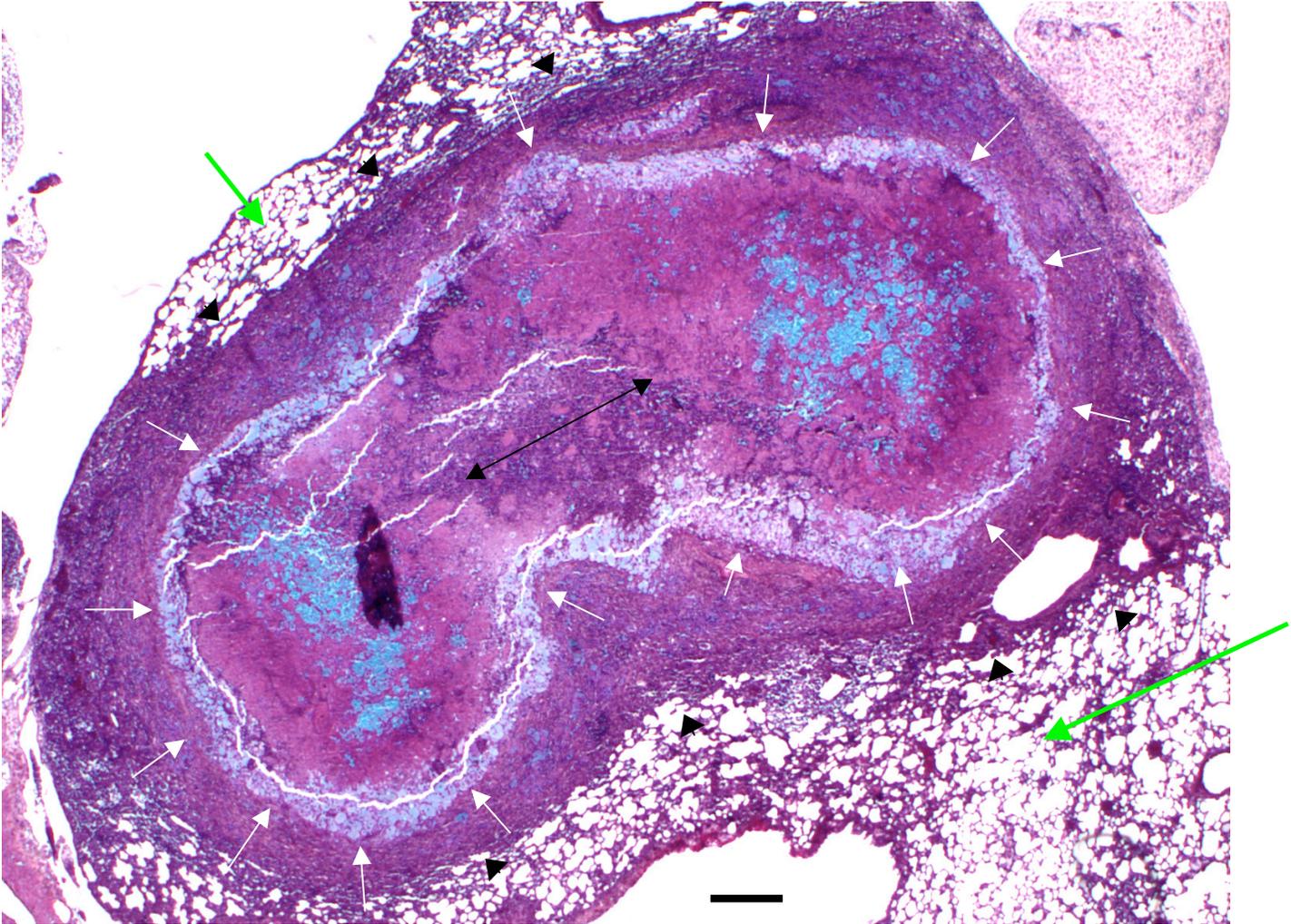
Supplementary Figure 5. Singh et al



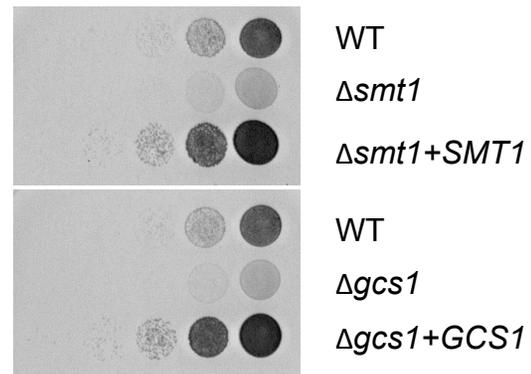
Supplementary Figure 6. Singh et al



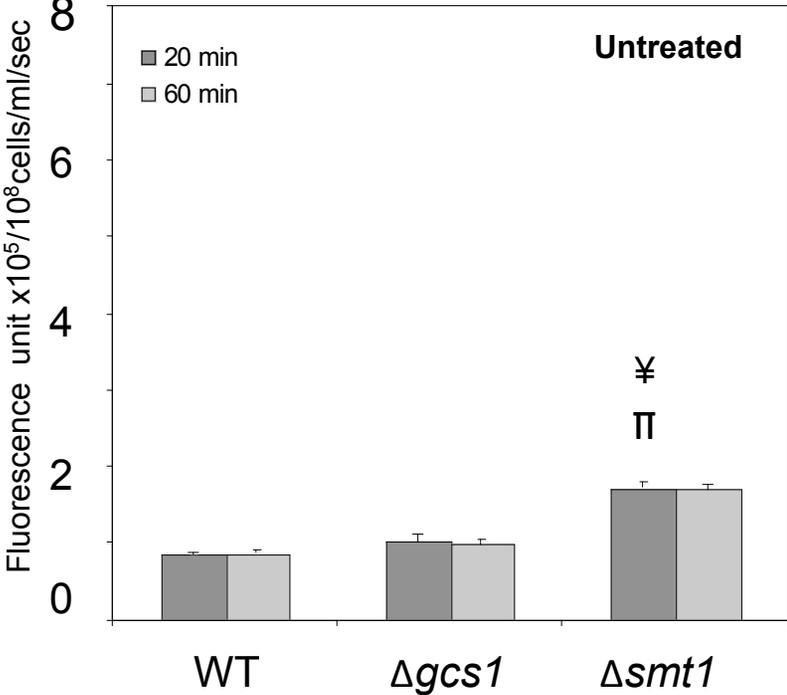
Supplementary Figure 7. Singh et al



Supplementary Figure 8. Singh et al



Supplementary Figure 9. Singh et al



Supplementary Figure 10 Singh et al

