SUPPLEMENT TO:

Glucosylceramide synthase is an essential regulator of pathogenicity of *Cryptococcus neoformans*

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Supplemental Figure 1. (A) A diagram illustrating the deletion of *Cn GCS1* gene using p*Δgcs1* plasmid cassette. **(B)** Southern analysis of genomic DNA digested with *Hind*III (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* Δ*gcs1*. **(C)** Reintroduction of *GCS1* gene into the *Cn* Δ*gcs1* strain using pCR-GCS1-HYG plasmid cassette. **(D)** Southern analysis of genomic DNA digested with *Eco*RI (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

GCS1 copy. 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, Δ*gcs1* or Δ*gcs1* + *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 CO_2 (A), production of polysaccharide capsule (B) and melanin formation (C) are not affected by lack of Gcs1/GlcCer. D) *Cn* Δ*gcs1* 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 *Sac*I and *Spe*I 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 CTT-3', which contain *Bgl*II and *Kpn*I 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 *Sac*I and *Spe*I and the resulting 1.5 kb fragment was subcloned into the *Sac*I/*Spe*I-restricted pCR-NAT1 vector, generating plasmid pCR-5UTR:NAT1. The pCR-NAT1 was created as previously described (48, 49). Plasmid pCR-3UTR was digested with *Kpn*I and *Xho*I and the 1.5 kb 3'-UTR fragment was subcloned into a *Kpn*I-*Xho*I 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 p*Δgcs1* (Supplemental Figure 1A).

The *Cn* wild-type strain H99 was transformed with plasmid pΔ*gcs1* 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* Δ*gcs1* strain (Supplemental Figure 1B).

To reintroduce the *GCS1* gene back in the Δ*gcs1* 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 **GGTACC** TAT ATC ACC GCT CAA TAA TAG CTT-3', containing a *Sac*I and *Kpn*I 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 *Xba*I and *Hind*III. This fragment was blunted and cloned into *Spe*I restricted-blunted pCR-GCS1 vector, generating pCR-GCS1-HYG construct. The *Cn* Δ*gcs1* 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-introduction 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 Δ*gcs1 + 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 $5x10^8$ cells per strain were resuspended in 1 ml total lipid extraction (TLE) buffer (95% ethanol:SDW:diethylether:pyridine:14.8N NH_4OH -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 H_2O 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 H_2O. 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/H_2O (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% H_2SO_4 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 ºC 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.

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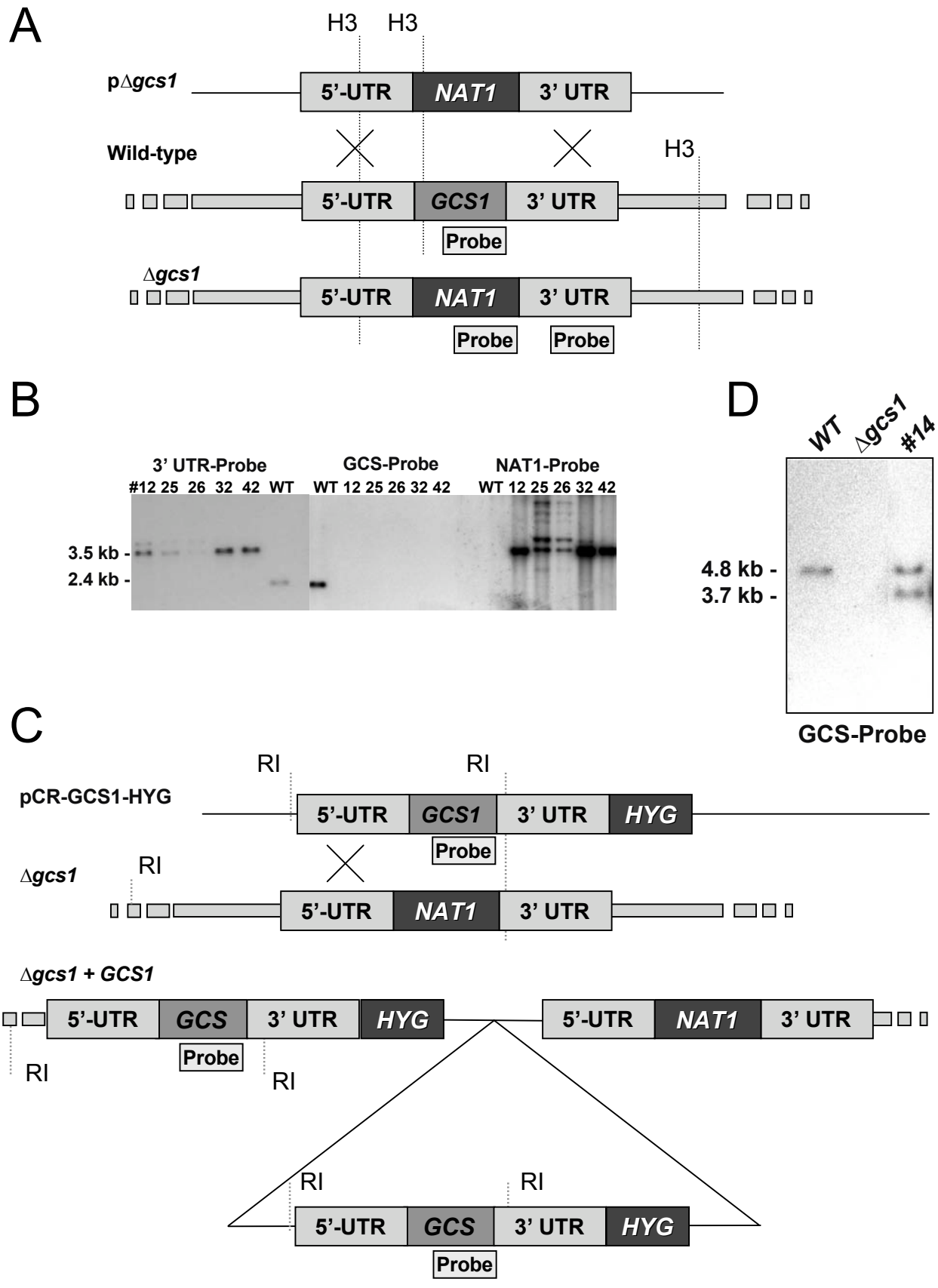
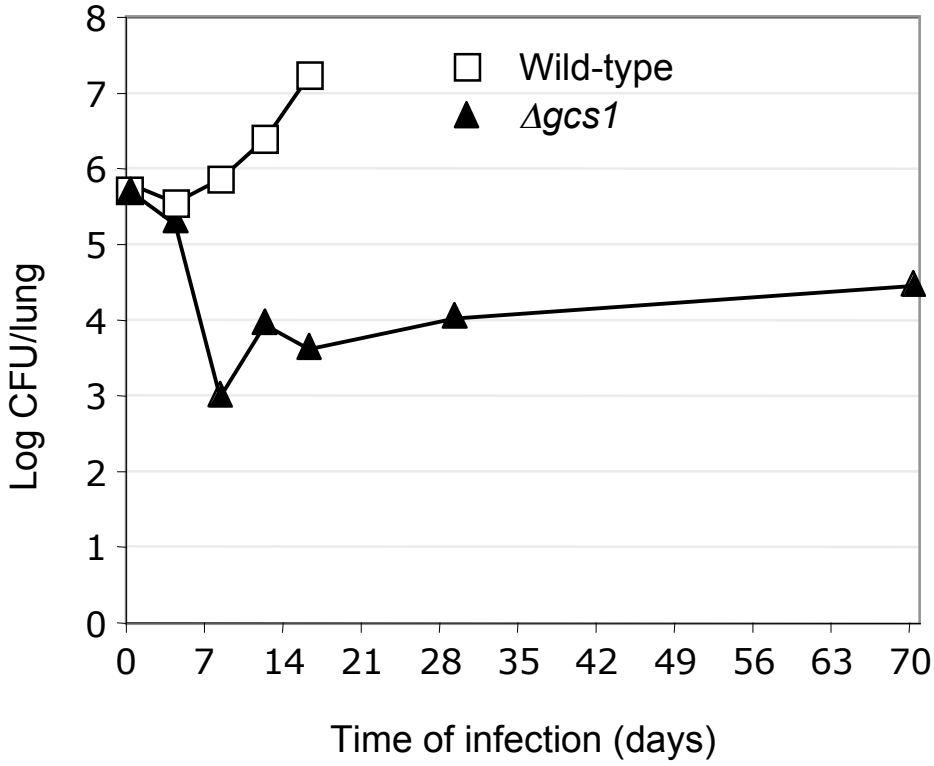


Supplemental Figure 1



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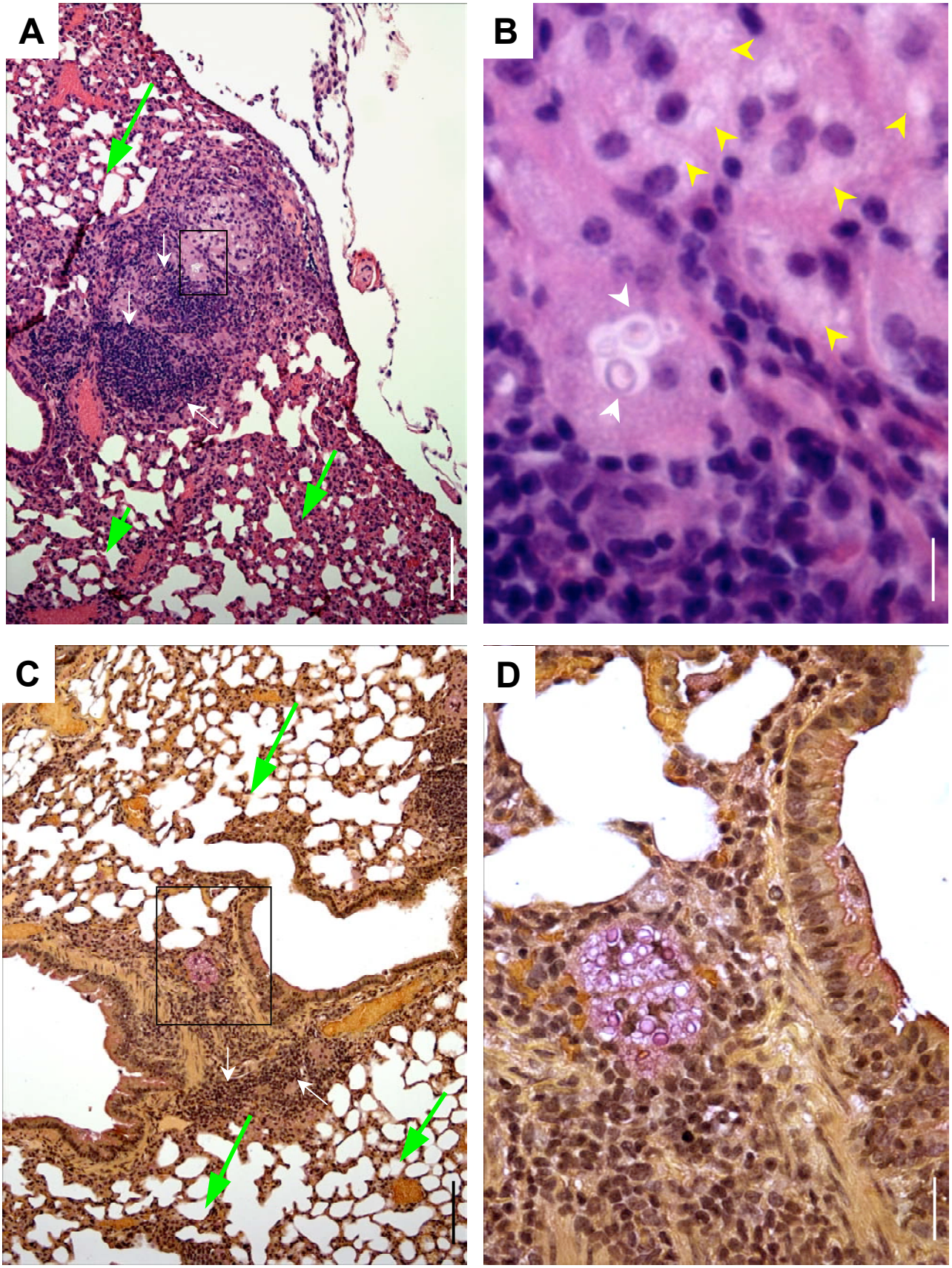

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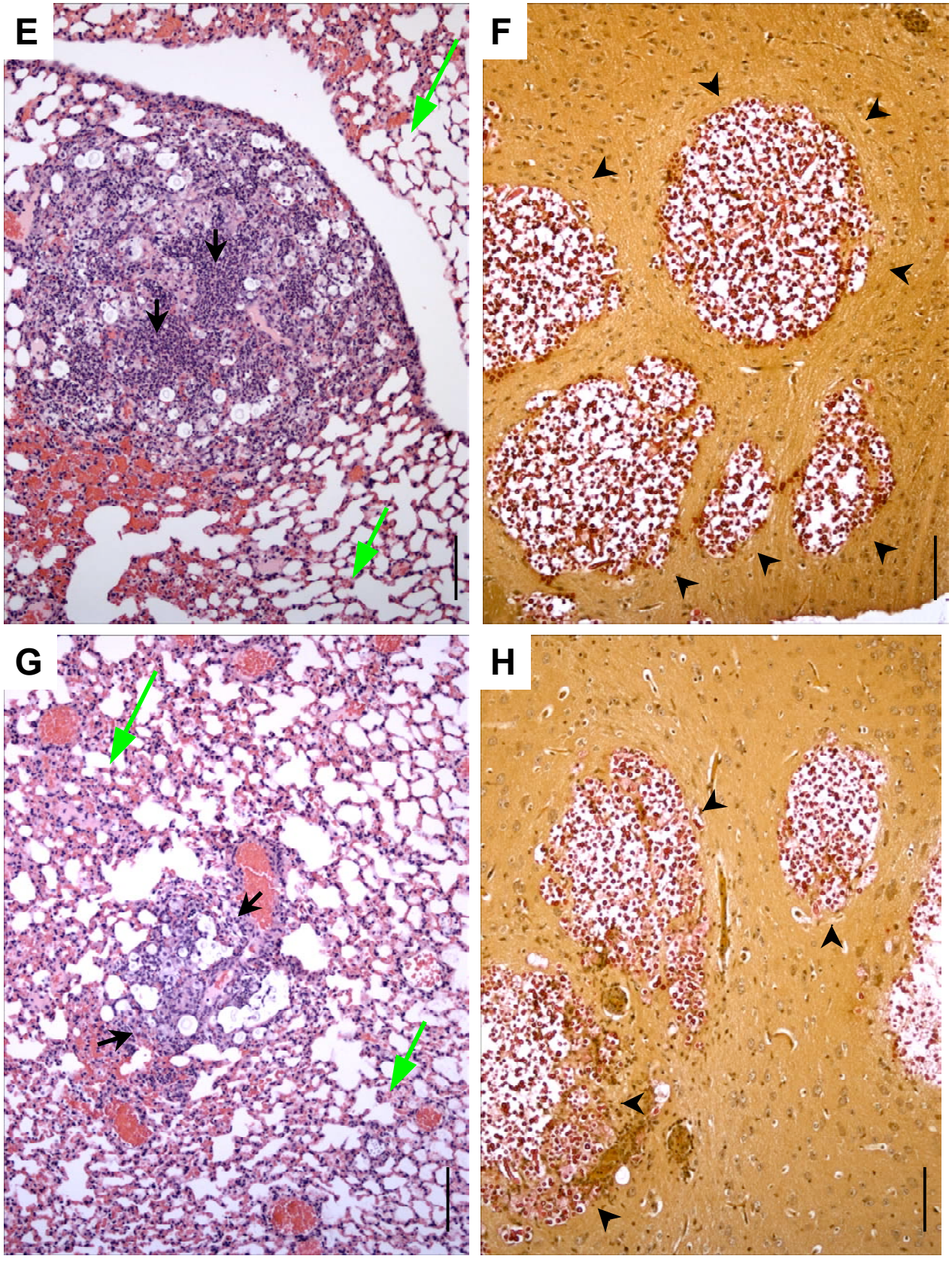

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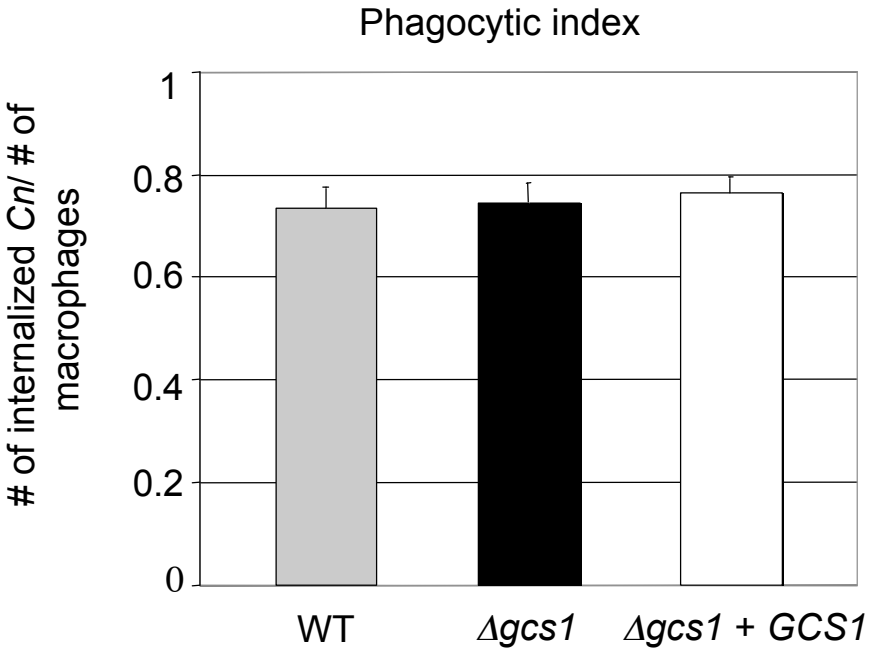

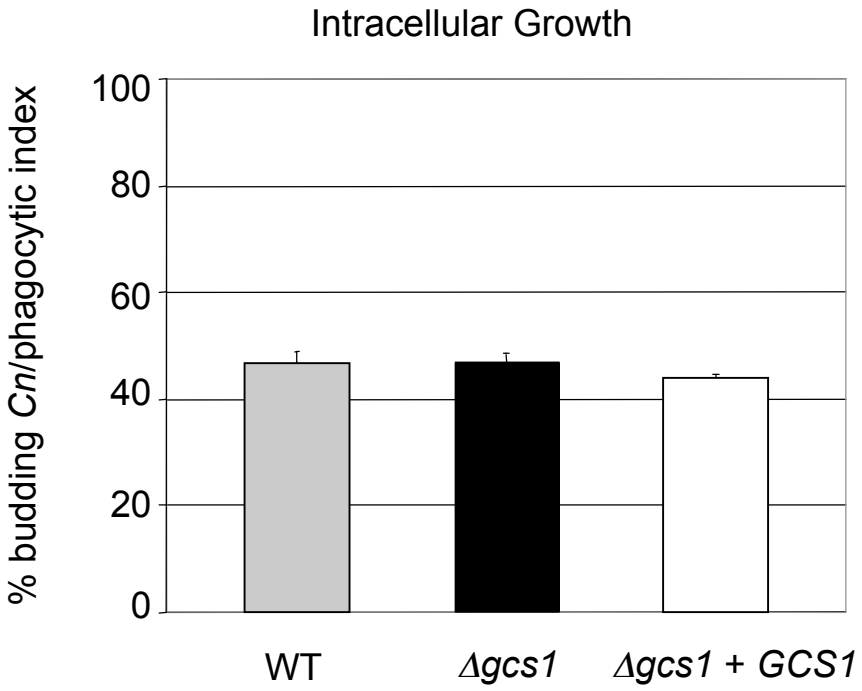

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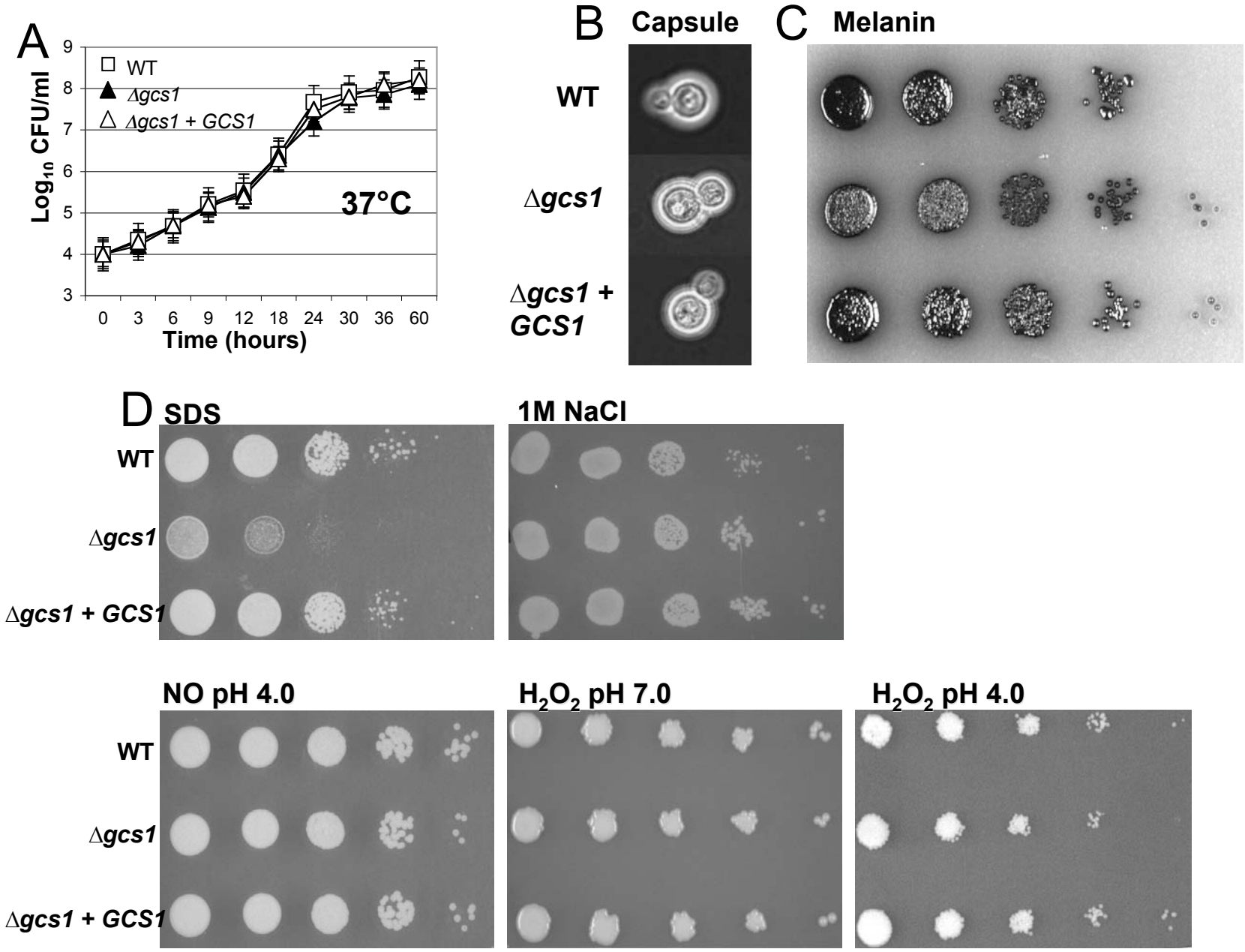

Supplemental Figure 5

	^{1}H [ppm]	^{13}C [ppm]	*J* [Hz]
Hex-H1’	4.17	103.11	J_{12} = 7.7
Hex-H2’	3.01	72.96	J_{23} = 8.9
Hex-H3’	3.20	66.17	J_{34} = 8.2
Hex-H4’	3.09	69.55	J_{45} = 9.1
Hex-H5’	3.14	76.45	
Hex-H6a’	3.49	60.70	
Hex-H6b’	3.72		

Supplemental Table 1