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Supplemental Information

The Role of Ceramide Synthases in the Pathogenicity of *Cryptococcus neoformans*

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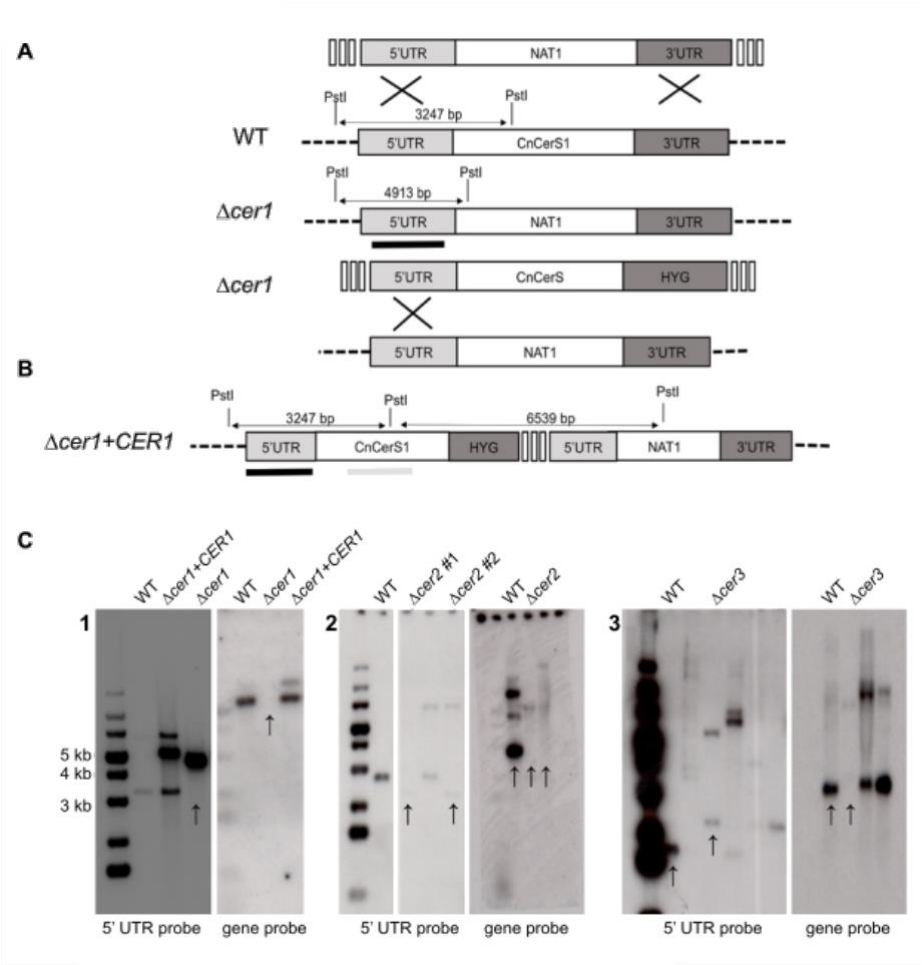
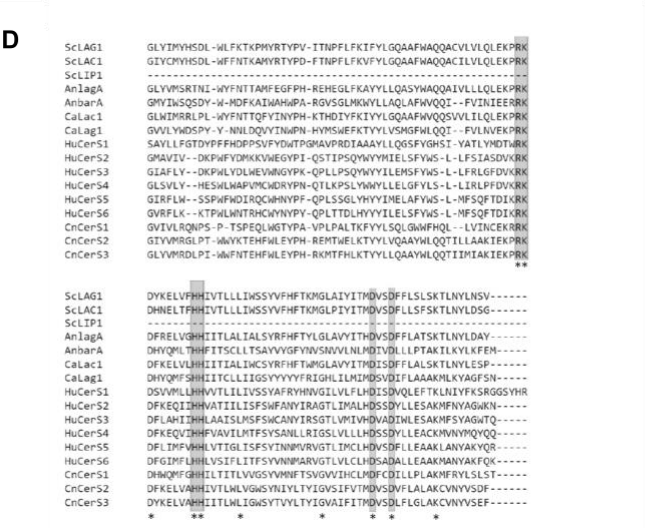
Supplemental information:

Supplementary Figures:

Figure S1 [Deletion and reconstitution of the *Cn* Cer1/Cer2/Cer3 gene, and alignment of fungal and mammalian ceramide synthases], Related to Figures 1,2,3,4,5 :





Supplementary figure 1: Deletion and reconstitution of the *Cn* Cer1/Cer2/Cer3 gene: (A) General strategy for the deletion of ceramide synthases in *C. neoformans* wild-type (WT) and creation of the mutant strain *Δcer*. (B) Strategy for the generation of the complemented strain *Δcer+CER*.

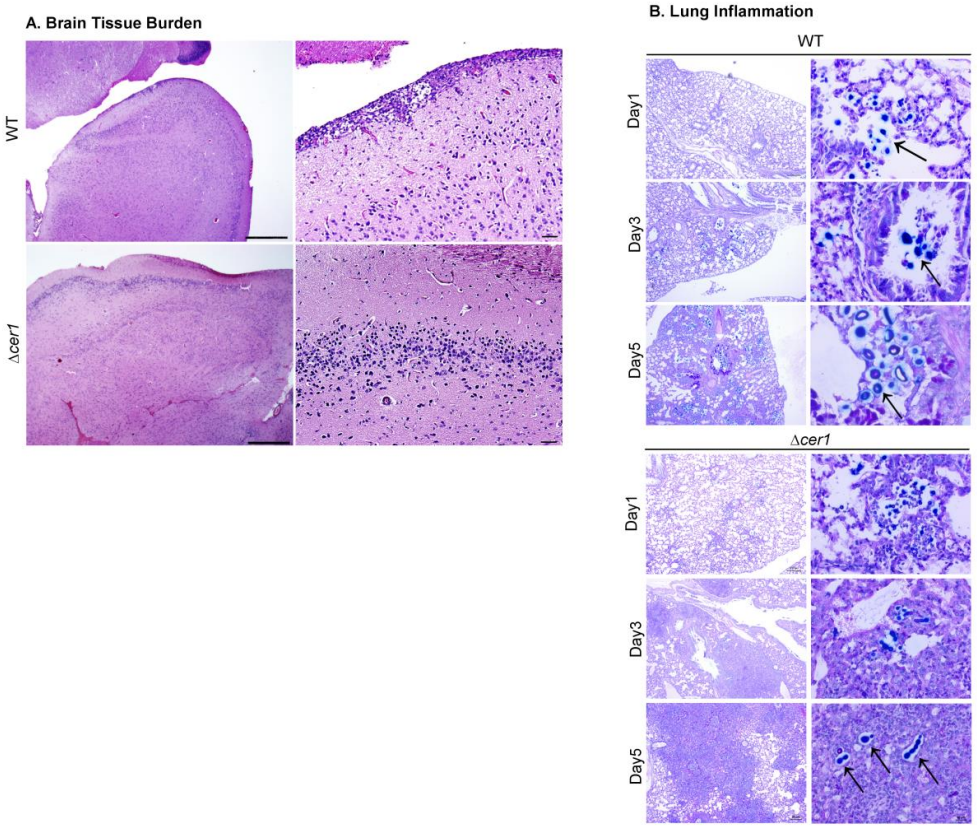
(C) Southern blot analysis for confirmation of transformants of *Δcer1, Δcer2 and Δcer3*.

Lanes: 1-4 5'UTR probe for *Δcer1*. Lane 1- 1kb marker, lane 2-WT *Cn*, lane 3- *Δcer1+CER1*, lane 4- *Δcer1*. Lanes (6-8) gene probe for *Δcer1* selection. 6-WT, 7- *Δcer1*, 8- *Δcer1+CER1*. Lane (9-16) 5'UTR and gene probes for *Δcer2*. 9-1 kb marker, 10-WT band, 11, 13- *Δcer2* transformants, 12-WT band. Lanes (17-23) 5'UTR and gene probes for *Δcer3*. 17-1 kb marker, 18-WT band, 19- *Δcer3*, 20-WT, 21- *Δcer3*, 22, 23- negative transformants.

5' UTR, 5' untranslated region; 3' UTR, 3' untranslated region; NAT1, nourseothricin 1; Cer1, ceramide synthase1 1; HYG. Hygromycin B.

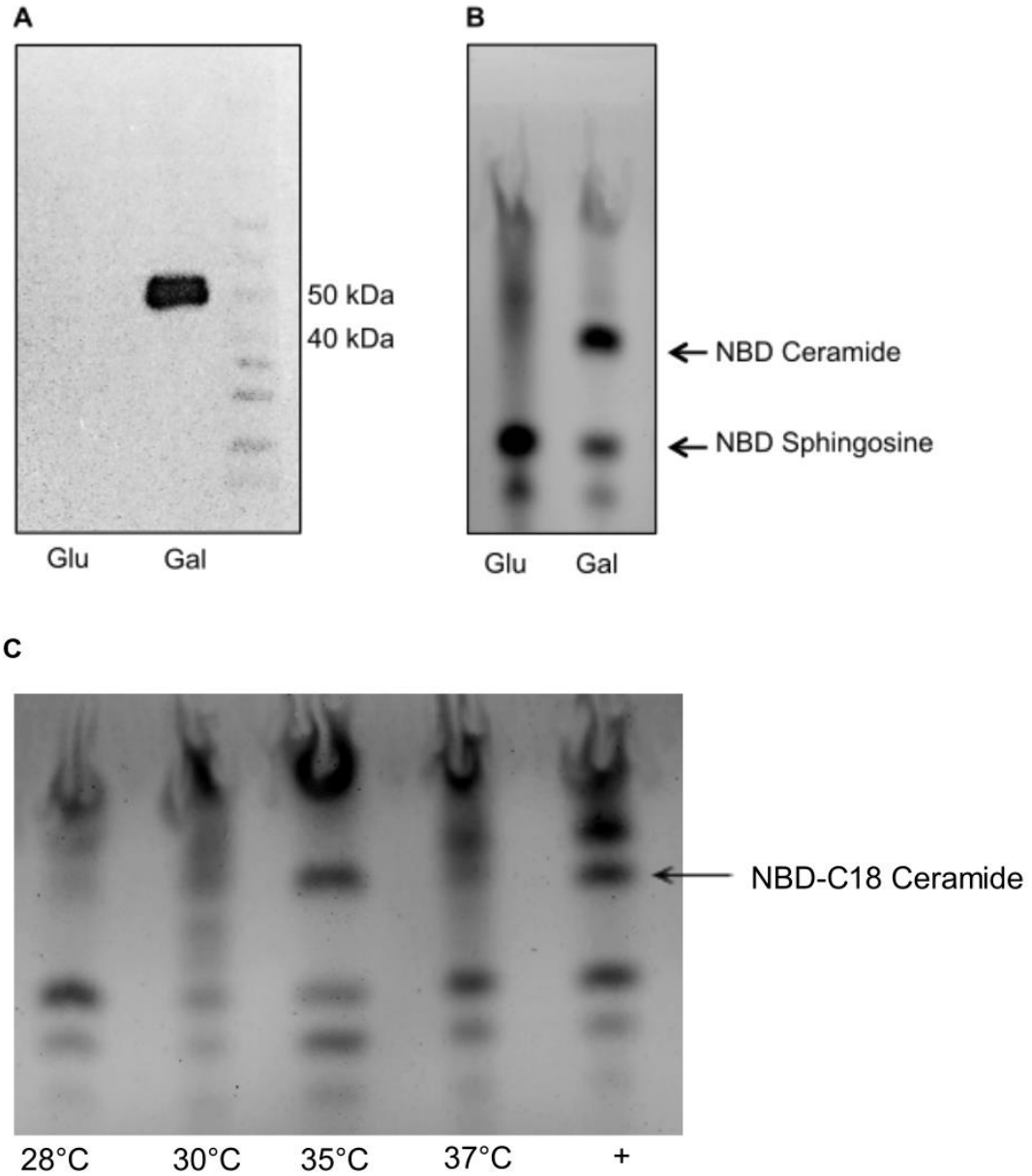
(D) Alignment of amino acid sequences of fungal and human ceramide synthases using Clustal Omega algorithm '*' indicated conserved residues. Grey boxes are conserved residues that have been reported to be important for ceramide synthase activity. *Sc, S.cereviaise; Ca, C.albicans; An, A. nidulans; Hu, Homo sapiens; Cn, C. neoformans*. Related to Figure 1

Figure S2 [Histopathology of Brain and lung sections of mice infected intranasally], Related to Figure 2:



Supplementary figure 2: (A) Histopathology of Brain sections of mice infected intranasally with WT (at death) or *Δcer1* (day 60). Sections stained with haematoxylin & Eosin. Bar=1mm (left), 100µm (right) (B) Histopathology of lungs obtained from CBA/J mice infected intranasally with wildtype (WT) or *Δcer1* at days 1,3 and 5 post infection. Infection with WT *Cn* shows a progression of inflammation along with replication of cells from day 1-5. Infection with Δ*cer1* shows a reduction in the number of cells from day 1-5 and cells start showing an elongated phenotype within 24-48 hours in the lung. Inflammation is observed during this time. Sections stained with Periodic acid Schiff's stain/Alcian Blue and Haematoxylin. Bar = 20µm. Black arrows show *Cn* cells**.**

Figure S3 [Expression of Cer1 in *S. cerevisiae*], Related to Figure 1B:



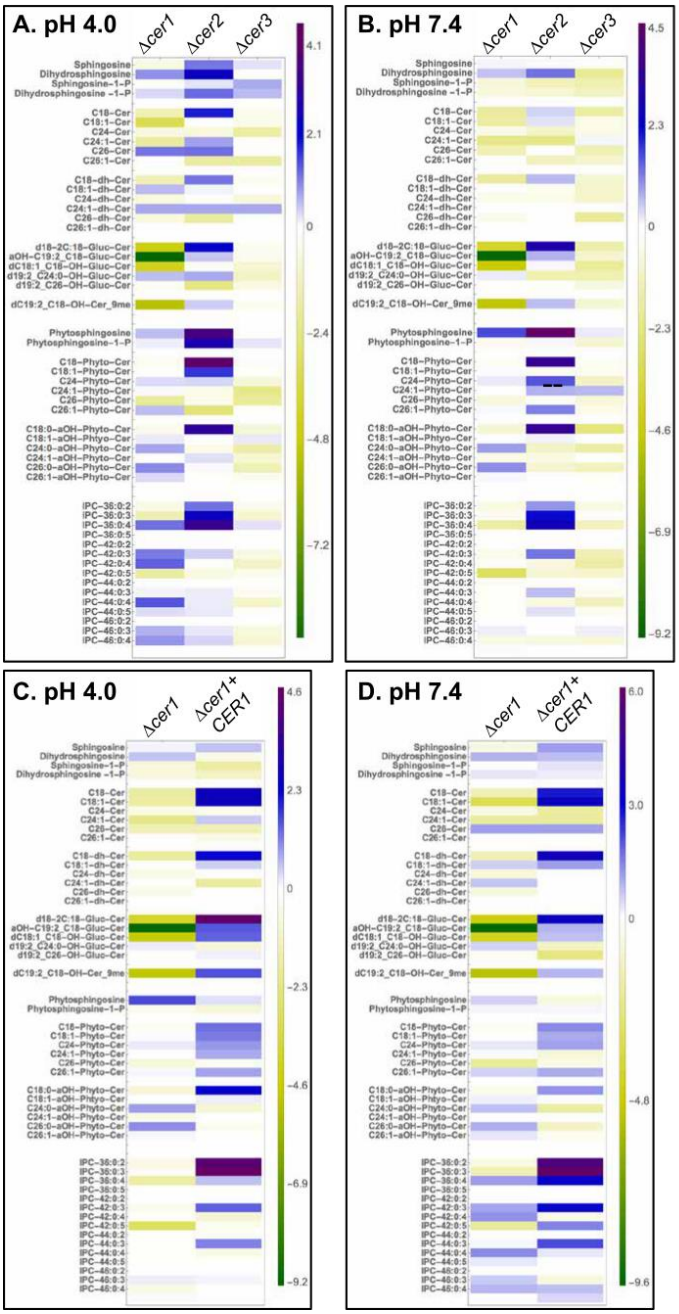
Supplementary figure 3: Expression of Cer1 in *S. cerevisiae*

(A) Western blot using microsomal protein from Cer1 expressed in 2% glucose or 2% galactose. using anti-6XHis antibody. 150µg microsomal protein was used in each lane.

(B) Ceramide synthase assay confirming activity of Cer1 in *Sc*. 150µg microsomal protein was used in each lane.

(C) Cer1 activity is temperature dependent. Ceramide synthase assay using 150µg microsomal protein of Cer1 at temperature 28℃, 30℃, 35℃, and 37℃. Formation of NBD-ceramide was detected by thin layer chromatography. Mammalian Cer1 microsomal protein was used as a positive control.

Figure S4 [Heat map of the sphingolipid profile for ceramide synthase deletion strains], Related to Figure 3:



Supplementary figure 4: Heat map of the sphingolipid profile for ceramide synthase deletion strains. The amount of lipid species are represented as relative abundance to corresponding WT lipid values. Blue bars represent lipid amount higher than WT. Green bars represent amount of lipid lower than WT. White bars are equal to WT values.

A. Lipid profile at pH 4.0/intracellular conditions.

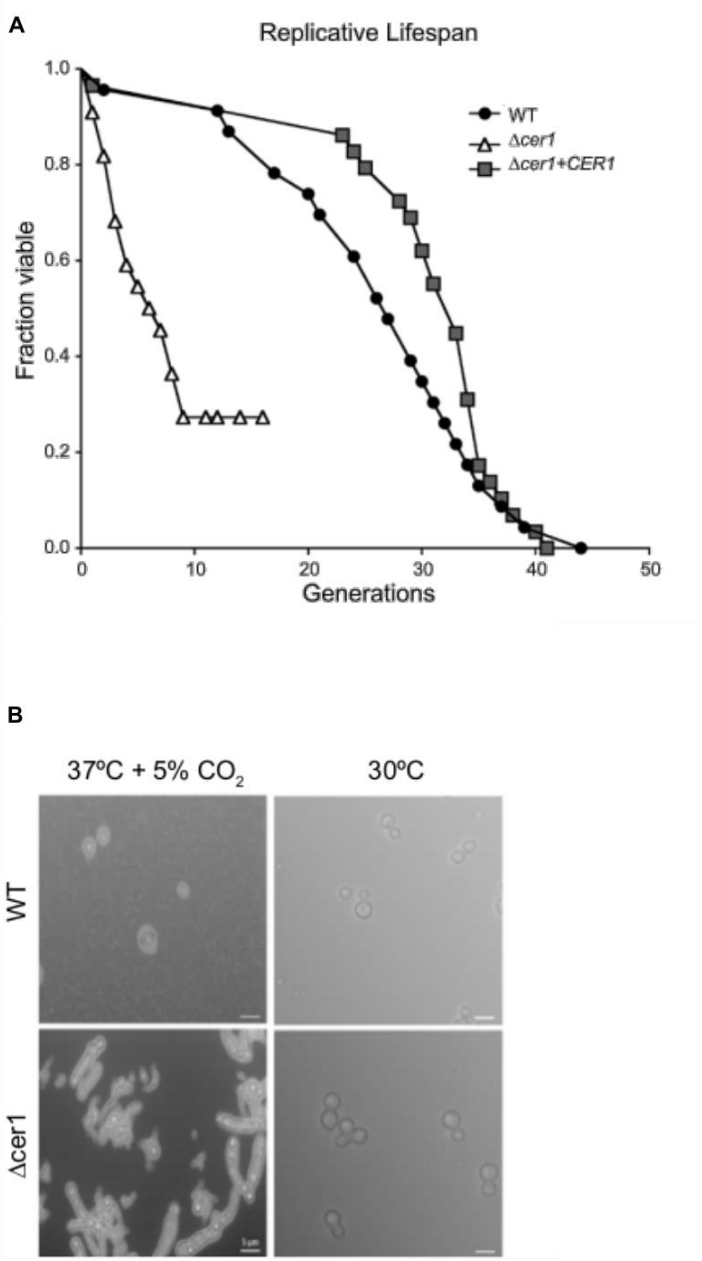
B. Lipid profile at pH 7.4/extracellular conditions. The scale is $\log_2$.

(C & D) Heat map of the sphingolipid profile for Δ*cer1*, and Δ*cer1 +CER1* strains

C. Lipid profile at pH 4.0/intracellular conditions.

D. Lipid profile at pH 7.4/ extracellular conditions. The scale is log_2.

Figure S5 [Replicative lifespan studies for WT, Δ*cer1*, and Δ*cer1 +CER1*], Related to Figure



Supplementary figure 5 (A) Replicative lifespan studies for WT, Δ*cer1*, and Δ*cer1 +CER1* shows that deletion of Cer1 leads to a drastic reduction of lifespan to an average 6.5 generations. While WT and Δ*cer1 +CER1* has a lifespan of average 27 generations.

(B) Light microscopy reveals cell wall defects in Δ*cer1*.

India ink staining shows that Δ*cer1* undergoes abnormal budding when exposed to physiological conditions. Cells of Δ*cer1* show an elongated phenotype as an outcome of incomplete cell division. These cells appear similar to WT at 30℃ and rich media.

Table S1: Primers used in this study. Related to experimental procedures:

Number	Name	Sequence 5'-3'
1	CNAG_067175UTRF	CTGGATCCGCGTCAAGTGGGTATTTCGT
2	CNAG_067175UTRR	CTACTAGTAGCTCGTGGGTGTTTGGTTA
3	CNAG_067173UTRF	CTGATATCTCTTGGATAGCCTGCGACTT
4	CNAG_067173UTRR	CTGGGCCCGACGTCAGGAAGCCTTTGTC
5	CNAG_0202865UTRF	CTGGATCCGGCCGTGAAGAGGAATAACA
6	CNAG_020865UTRR	CTACTAGTGTTGTCGAGATGTGGCTGAA
7	CNAG_020863UTRF	CTCTCGAGGGTGATCGTGGCTTGCTT
8	CNAG_020863UTRR	CTGGGCCCTAGCTGTTCTACGTCAAGTGGTC
9	CNAG_020875UTRF	CTGGATCCGGTGATCGTGGCTTGCTT
10	CNAG_020875UTRR	CTACTAGTTAGCTGTTCTACGTCAAGTGGTC
11	CNAG_020873UTRF	CTCTCGAGCAGGTGATGCACCGTGAGA
12	CNAG_020873UTRR	CTGGGCCCGAAGGACCTTTCCCAACTCC
13	pRS425_Ahomo_gal1p	ccctcgaggtcgacggtatcgataagcttgatatcgaattcctgcagccc GATCCACTAGTACGGATTAGAAGCC
14	pYES/ct_86homo	GACCTTCGCCGATGGCTGGGCTTATTTGGTGTCACTGCTGGAC CGGGCAT GTTTTTTCTCCTTGACGTTAAAGTATAGAG
15	pYES/ct_87homo	GTTACACTGGACGCTCGCCTTCGGTGAAGATGTTTATGCCTTT GGGACAT GTTTTTTCTCCTTGACGTTAAAGTATAGAG
16	86_ATG_fwd	ATGCCCGGTCCAGCAGTG
17	87_ATG_fwd	ATGTCCCAAAGGCATAAACATCTTC

18	86_TAA_pRS425Homo	agctggagctccaccgcggtggcggccgctctagaactagtggatccccc TTACTCAGCCTTCACCTTCACTTC
19	87_TGA_pRS425Homo	agctggagctccaccgcggtggcggccgctctagaactagtggatccccc TCATTCTTCCTTCGCTTCATCC
20	IDTMMREC67F	ACATCACACTGCGGCCTCATCGTGCCTCTCCTTTTC
21	IDTMMREC67R	GTCAAGCTAAGCGGCCCAAGTCGCAGGCTATCCAA
22	MMREC86F	ACATCACACTGCGGCCGCGGCCGTGAAGAGGAATAACA
23	MMREC86R	GTCAAGCTAAGCGGCCGCCGGAAACATCACTCAAGCAA
24	MMREC87F	ACATCACACTGCGGCCGCGTGATCGTGGCTTGCTTGAG
25	MMREC87R	GTCAAGCTAAGCGGCCGCCTATCCGTCTACTGAACGATTA

Table S2. Lipid species composition of SL biosynthetic pathway mutants. Related to Figure 3, 5

Lipid species	*Cn* WT	*Δgcs1*	*GAL7::IPC1*	*Δcer1*	*Δcer2*	*Δcer3*
C18 Ceramide species	680.95	6037.44	1082.24	9.82	443.3	674.97
α-OH-Δ4-Δ8, 9Me-GlcCer	3105.48	0.00	12823.82	4.96	1685.93	2821.22
Phyto-Sph	0.16	0.04	0.38	0.34	3.34	0.50
Phyto-Sph-1P	0.17	0.21	0.22	0.18	0.05	0.33
PhytoC6	0.02	0.65	0.96	0.16	0.12	0.18
PhytoC14-Cer	0.18	0.25	0.85	0.69	4.16	1.20
PhytoC16-Cer	43.19	238.22	45.56	13.66	209.03	48.89
PhytoC18:1-Cer	178.33	190.04	120.55	9.51	98.66	188.16
PhytoC18-Cer	754.08	3245.00	121.75	17.37	569.30	541.11
PhytoC20:1-Cer	5.54	1.34	4.02	4.33	124.60	4.36
PhytoC20-Cer	8.84	11.69	2.95	5.37	94.65	5.25

PhytoC22:1-Cer	0.98	2.73	0.88	0.48	11.49	0.71
PhytoC22-Cer	44.25	10.79	17.04	21.34	67.46	12.64
PhytoC24:1-Cer	2.28	1.92	4.27	5.15	2.34	1.47
PhytoC24-Cer	583.66	136.33	260.58	667.00	742.17	350.59
PhytoC26:1-Cer	59.04	12.34	33.44	45.23	54.57	38.60
PhytoC26-Cer	86.78	34.83	41.82	119.93	284.50	57.28
PhytoC28:1-Cer	9.02	4.08	4.37	7.78	24.26	10.00
PhytoC28-Cer	4.78	5.32	6.03	3.58	19.37	3.93
αOH-PhytoC14-Cer	302.47	8.04	25.19	4.44	11.47	6.39
αOH-PhytoC16-Cer	9.61	0.00	0.00	0.00	0.00	0.00
αOH-PhytoC18:1-Cer	9.16	0.00	1.93	0.08	1.41	0.94
αOH-PhytoC18-Cer	111.05	98.39	185.51	65.06	623.64	90.02
αOH-PhytoC20:1-Cer	15.00	0.00	0.16	0.00	0.72	0.12
αOH-PhytoC20-Cer	29.65	30.15	31.78	20.74	42.00	19.00
αOH-PhytoC22:1-Cer	1.45	0.00	0.00	0.05	0.00	0.00
αOH-PhytoC22-Cer	67.94	91.14	65.80	62.26	92.91	42.93
αOH-PhytoC24:1-Cer	0.00	0.71	0.00	0.00	2.99	0.53
αOH-PhytoC24-Cer	2456.35	2217.29	4060.07	2796.37	1192.94	1391.92
αOH-PhytoC26:1-Cer	0.00	0.19	0.00	1.66	1.34	0.34
αOH-PhytoC26-Cer	125.07	115.01	266.64	348.00	165.48	90.21
αOH-PhytoC28:1-Cer	0.00	0.09	0.00	0.06	0.00	0.00
αOH-PhytoC28-Cer	0.00	0.03	0.00	0.02	0.00	0.00

Table 2:

Lipid abundance in WT, Δ*gcs1, GAL7::IPC1,* Δ*cer1,* Δ*cer2,* and Δ*cer3* strains measured by LC-MS. Strains were grown in YNB, 2% glucose before extracting lipids (see methods). C18 ceramide species include: α-OH-Δ4-ceramide, α-OH-Δ4-Δ8 ceramide, and α-OH-Δ4-Δ8, 9Me ceramide. All concentrations are represented as pmol/mg dry lipid weight.

Table S3: C18 ceramide abundance of *GAL7::IPC1* at 6 hours, 12 hours, 24 hours and 48 hours post glucose inoculation. Related to Figure 5

Time	WT pH 4.0	*GAL7::IPC1 pH 4.0*	WT pH 7.0	*GAL7::IPC1 pH 7.0*
6 hours	23.93	10.90	18.64	6.49
12 hours	31.80	3.152	30.46	0.043
24 hours	11.46	37.534	18.41	6.90
48 hours	20.53	34.64	25.473	8.59

Table 3: C18 ceramide changes of WT and *GAL7::IPC1* upon glucose transfer. When Ipc1 is genetically downregulated, a transient decrease is observed in C18 ceramide levels because cells do not tolerate high levels of ceramides. As the cells start to adapt, growth is restored likely due to a parallel increase in C18 ceramides. These C18 ceramides then lead to an increase in GlcCer which is highly abundant in *GAL7::IPC1* at 48 hours post glucose inoculation (Supplementary table 2).

WT and *GAL7::IPC1* were grown overnight in galactose containing media. Strains were transferred to glucose media after 16-22 hours and samples were collected for lipid analysis at mentioned time points. Lipid extraction and analysis was performed as mentioned in the methods. Values represented as pmol/mg dry lipid weight.

Experimental procedures

Strains, Plasmids and Media

The strains used in this study are *Cryptococcus neoformans var. grubii* strain H99 as wildtype (WT) and *S. cerevisiae* BY4741. Bacterial strain used was Escherichia coli DH5-α™ Max Efficiency® (Invitrogen, Carlsbad, CA) as competent cells. Plasmid pCR topo 2.1 was used for cloning and biolistic transformation. Cloning was carried out using TOPO® TA Cloning® Kit, with pCR™2.1-TOPO® (Invitrogen, Carlsbad, CA). The three putative ceramide synthase genes in this study have the following identifiers: CNAG_06717 (Genbank accession number XM_012192296), CNAG_02086 (Genbank accession number XM_012194542), CNAG_02087 (Genbank accession number XM_012194543). For overexpression studies, pYES2/CT was used for expression of CNAG_06717 in *S.cerevisiae* BY4741 while pRS425 was used to express CNAG_02086 and CNAG_02087. *Cn* strains were routinely grown in YPD broth at 30°C and 0.04% CO_2 for 20-22 hours with shaking at 225 rpm. Dulbecco's

modified eagle medium (DMEM) buffered with 25mM HEPES (pH 4.0 or pH 7.4) was used to grow *Cn* at 37°C in the presence of 5% CO_2 (physiologically relevant conditions). *S. cerevisiae* transformed with pYES2/CT was grown in YNB without amino acids, 1g/L amino acid mixture lacking uracil (ura^-), 5g Ammonium sulfate, 0.4g $NaPO_4$ dibasic, and 2% Glucose or 1% Galactose + 1% Raffinose (to induce expression). Similarly, *S. cerevisiae* transformed with pRS425 was grown in synthetic leucine (leu^-) dropout media. Strains containing both vectors were grown in synthetic leu^-ura^- dropout media. Bacterial strains were grown at 37°C in Luria–Bertani media containing 75mg/L of ampicillin (Sigma).

Isolation and cloning of *C. neoformans* ceramide synthase genes

To independently delete each of the three ceramide synthase genes from the genome of *C. neoformans*, plasmids using nourseothricin acetyltransferase (NAT1) (Werner BioAgents, Germany) selectable marker deletion strategy were constructed. Each NAT1 deletion plasmid contained 1.5 kilobases (Kb) of 5' untranslated region (UTR) upstream of the ORF as well as 1.5 kilobases of the 3'UTR downstream of the ORF. Generally, the 5'UTR and 3'UTR of the gene of interest was constructed flanking NAT1 gene, whose expression is under the control of actin promoter. The 5'UTR and 3'UTR were generated by PCR using specific primers containing restriction sites on genomic *C. neoformans* H99 DNA. These fragments were then cloned into pCR2.1 TOPO vector generating plasmids pCR-5UTR and pCR-3UTR and sequenced for each of the three genes of interest (Cer1- 5'UTR, Cer1-3'UTR, Cer2- 5'UTR, Cer2-3'UTR. Cer3-5'UTR, Cer3-3'UTR). The 3'UTR was then sub-cloned into plasmid pCR-NAT1 vector, generating plasmid pCR-3UTR:NAT1. The 5'UTR was subcloned into pCR-3'UTR::NAT1 generating pCR 5'UTR::NAT1::3'UTR for each ceramide synthase. These constructs were named pΔcer1, pΔcer2, and pΔcer3. *C. neoformans* wildtype strain H99 was independently transformed with each of the three constructs *pΔcer1, pΔcer2*, and *pΔcer3*, by biolistic transformation according to (Singh, Qureshi et al. 2011). Transformants were grown on Yeast peptone dextrose (YPD) plates containing 100 µg/ml of nourseothricin. Resistant colonies were chosen randomly and purified through serial passage on selective media. Correct integration of DNA cassettes was examined by southern blot analysis and performed according to (Singh, Qureshi et al. 2011). Transformants for each ceramide synthase gene, showing deletion of the gene and insertion of the plasmid cassette were obtained and were chosen and designated Δcer1 strain, Δcer2 strain, and Δcer3 strain. To reintroduce the genes back in their respective knockout mutants, reconstituted constructs, pCR-Cer1-ACT-HYG, pCR-Cer2-ACT-HYG, pCR-Cer3-ACT-HYG plasmid constructs were generated as follows: A fragment (4.5 kb) containing the entire ORF of the gene and 1.5 kb of the upstream (5'UTR) was generated by PCR using wildtype H99 genomic DNA as a template and

was cloned into the pCR2.1-TOPO vector generating plasmid containing 5'UTR –GENE. This construct was then sub cloned into pSK-ACTIN-HYG plasmid containing Hygromycin resistant marker forming pSK-Cer1-ACT-HYG, pSK-Cer2-ACT-HYG, pSK-Cer3-ACT-HYG. The Δ*cer1,* Δ*cer2,* and Δ*cer3* mutant strains were each transformed with pSK-Cer1-ACT-HYG, pSK-Cer2-ACT-HYG, and pSK-Cer3-ACT-HYG plasmid respectively using biolistic delivery of DNA using the scheme as shown (Supp. Fig 1). Transformants were grown on YPD plates containing 100 µg/ml of hygromycin B. Stable transformants were selected, grown on YPD, followed by extraction of DNA and confirmation with southern blot using gene sequence probes. These reconstituted strains were named *Δcer1+CER1, Δcer2+CER2,* and *Δcer3+CER3*.

Phylogenetic analysis of putative ceramide synthase genes

Representative fungal ceramide synthase genes and the three computationally annotated ceramide synthase genes were aligned with ClustalOmega using default parameters. Exact maximum likelihood phlyogentic tree construction was performed using TreePuzzle (Schmidt, Strimmer et al. 2002, Schmidt and von Haeseler 2007) with 1000 quartet puzzling steps. Human ceramide synthase 1 was selected as the outgroup, with the exact neighbor-joining tree method used to find the parameter estimates. The Meuller-Vingron model of substitution was used to calculate mismatch penalties. Dendroscope (Huson, Richter et al. 2007, Huson and Scornavacca 2012) was used to visualize the resulting phylogenetic tree.

Biochemical characterization of *Cn* ceramide synthases

Cn ceramide synthase enzymes were further biochemically characterized using a fluorescent assay using different combinations of substrates and buffer pH. Specifically, NBD-sphingosine, NBD-phytosphingosine were used to check for formation of ceramides and phytoceramides. Fatty Acyl CoA chain lengths C18, C24 and C26 were used tested for chain length specificity. pH dependence of ceramide synthase activity was assessed by using a range of buffers from 3.0-10.0. For all further assays, three buffers: Sodium acetate trihydrate, $CH_3COONa \bullet 3H_2O$, Acetic acid NaOAc buffer (pH 4.0), $Na_2HPO_4 – NaH_2PO_4$ (pH 7.0) and Sodium Carbonate $Na_2CO_3 \bullet 10H_2O$ – Sodium Bicarbonate $NaHCO_3$ (pH 10.0) were used.

Generation of *S. cerevisiae* strains expressing *Cn* ceramide synthases

3 plasmids were constructed respectively for the genes CNAG_06717, CNAG_02086 and CNAG_02087. Gene fragment for CNAG_06717 containing V5 and 6X histidine tags and overlapping ends with pYES2/CT vector was

generated using IDT gblocks gene fragments. The construct was inserted into vector pYES2/CT by plasmid gap repair using the gene fragment with flanking homology to the linearized plasmid vector. Positive colonies were purified through serial passage on ura$^-$ media. The resulting colonies were sequenced to confirm correct integration. Similarly, plasmid pRS425 was used to insert genes CNAG_02086, and CNAG_02087. These plasmids do not contain a Gal1 promoter. Therefore, the Gal1p was amplified from plasmid pYES2/CT. Primers were designed to amplify Gal1p with overlap of part of plasmid pRS425, gene CNAG-02086 ATG forward primer, CNAG_02086 with TAA and overlap of pRS425, as well as CNAG-02087 ATG forward primer, CNAG_02087 with TAA and overlap of pRS425. These fragments were then co-transformed along with the linearized vector into *Sc* BY4741. Transformants were selected on synthetic leu$^-$ dropout media. Two additional strains were constructed by co-transforming pYES2/CT+Cer1 along with pRS425+Cer2 or pRS425+Cer3. These transformants were passaged on synthetic leu$^-$ura$^-$ media to obtain pure isolates.

Protein microsomal preparation

Microsomal isolation method was adapted from (Ternes, Wobbe et al. 2011) with modifications. Briefly, cells of *S. cerevisiae* strain BY4741 expressing gene of interest (Cer1, Cer2, Cer3, Cer1+Cer2, or Cer1+Cer3) was grown in 10 ml YNB (containing the appropriate amino acid dropout mix) + 2 %glucose, overnight at 30°C. The next day, these cells were washed twice with PBS and transferred to 300 ml YNB+1% galactose+1% raffinose media and allowed to grow overnight. These cells were then centrifuged and the pellet was resuspended in 1-2 ml lysis buffer (20mM HEPES/KOH pH 7.4, 25mM KCl, 2mM $MgCl_2$, 250mM sorbitol) and 50µl protease inhibitor cocktail (Thermo scientific, Waltham, MA). ~1ml volume of glass beads was added to 1ml lysate in a tube and this slurry was vortexed vigorously for ~2 hours. Cell debris were removed by centrifugation at 1000×g, 4°C, for 3 mins. Supernatant was loaded on to a 60% sucrose cushion (w/w), and spun in an ultracentrifuge at 4°C, 24,000 RPM, for 1 hour. The microsomes were isolated from the interphase with a Pasteur pipette and stored at -80°C.

Fluorescent cryptococcal ceramide synthase assay

An assay for ceramide synthase activity was adapted from (Kim, Qiao et al. 2012, Tidhar, Sims et al. 2015) with minor changes. Microsomes of overexpressed ceramide synthase were used as enzymes for these reactions. Briefly, NBD- Sphingosine or NBD-Phytosphingosine (Avanti Polar lipids, Alabaster, AL) was combined with Fatty Acyl CoA of varying chain lengths (C18, C24, C26) (Avanti Polar lipids, Alabaster, AL) as a substrate mixture. A 100µl reaction was carried out using reaction buffer (20 mM Hepes, pH 7.4, 25 mM KCl, 2 mM $MgCl_2$, 0.5 mM DTT,

0.1% (w/v) fatty acid-free BSA) along with 10μM NBD sphingosine and 50μM fatty acyl CoA. 150μg of microsomal protein was added per reaction, as measured in a Bradford assay (effective protein amount empirically determined). The reactions were then incubated at 35°C for 90 minutes. The reactions were then stopped with 2:1 chloroform:methanol, followed by gentle vortexing. The lipids were then extracted and dried in a speed vacuum (SPD 2010) followed by resuspension in 100% methanol. The reaction was analyzed by thin layer chromatography, using chloroform/methanol/water (8:1:0.1, v/v/v) as the solvent mixture.

Virulence studies and histology analysis in a murine mouse model of cryptococcosis

3-4 weeks old female CBA/JCrHsd (Harlan Laboratories, Indianapolis, IN, USA) mice were used for all experiments. Mice were anesthetized with 60 μl xylazine/ketamine mixture containing 95 mg ketamine and 5 mg xylazine per kilogram of body weight prior to infection. *Cn* strains WT H99, *Δcer1, Δcer2, Δcer3 and Δcer1+CER1* were grown overnight in YPD broth at 30°C. The next day, cells were pelleted, washed twice and resuspended in PBS at a concentration of 3.5×10^7 cells/ ml. For survival studies, ten CBA/JCrHsd mice per strain were infected with 7×10^5 cells for each strain in a volume of 20 μl through nasal inhalation. For tissue burden analysis, 9 mice per strain were used. Lung, brain, kidney, liver and spleen were excised and homogenized in 10ml PBS using stomacher 80 (Seward, UK) for 2 min at high speed. Serial dilutions were plated in duplicate on YPD agar plates and incubated for 48-72 hours at 30°C for assessment of CFU per organ. For histopathology analysis, 3 mice per experimental group were used. Mice organs were fixed in 3.7% formaldehyde in paraffin and stained with haematoxylin and eosin and mucicarmine. Staining was performed in part by McClain Labs (Smithtown, NY), as well as by Research Histology Core at Stony Brook University.

Extraction and mass spectrometry analysis of yeast sphingolipids

For extraction of lipids, cells of wildtype, mutant and reconstituted strains were grown overnight in YPD at 30°C. The next day these cells were washed and transferred to DMEM (pH 4.0 or 7.4) and grown in shaking condition at 37°C + 5% CO_2 for about 16 hours. These cells were washed and counted for lipid extraction. Briefly, 10^8 cells for each replicate were pelleted in a glass tube in which mandala extraction buffer was added and extraction was performed as described in (Mandala, Thornton et al. 1995). Further extraction was performed according to the methods of Bligh and Dyer (Bligh and Dyer 1959). After measuring the dry weights, the samples were subject to base hydrolysis (Clarke and Dawson 1981). The extracts were dried in a centrifuge under a vacuum (SPD 2010, ThermoFisher Scientific, Waltham, MA). All internal standards were added prior to lipid extraction. The following

internal standards from Avanti Polar Lipids (Alabaster, AL) were used: Sphingosine (d17:1), D-erythro-sphingosine (C17 base), N-08:0 Phytosphingosine (N-octanoyl-4-hydroxysphinganine)(Saccharomyces Cerevisiae), sphinganine (d17:0) D-erythro-sphinganine (C17 base), Sphingosine-1-Phosphate (d17:1) D-erythro-sphingosine-1-phosphate (C17 base) and C17 Ceramide (d18:1/17:0) N-heptadecanoyl-D-erythro-sphingosine.

For the mass spectrometry analysis, the dried extracts were separated on a Thermo Accela HPLC system (San Jose, CA) after dissolving in 150 µL of ammonium formate (1mM) with 0.2% formic acid in methanol. We used a Peeke Scientific Spectra C8 (Redwood City, CA) HPLC column (150 x 3 mm) into which 10 µl samples were injected. The buffers used for the runs were as follows: Buffer A (2mM ammonium formate and 0.2% formic acid(FA)) and buffer B, ammonium formate (1mM) with 0.2% FA in methanol. A gradient using buffer A and B was used, starting with 70% B with an increase to 90% over 5 minutes, followed by a ramp to 99% B over 9 minutes. The column was equilibrated with initial conditions for 8 minutes at a flow rate of 500 µL/min. The HPLC was coupled to the HESI source of a Thermo TSQ Quantum Ultra triple quadrupole mass spectrometer (San Jose, CA). The sphingolipid profile was performed using positive ion mode. With the high voltage set to 3.5 kV, vaporizer temperature at 400ºC, sheath gas pressure at 60, auxiliary gas pressure at 15 and a capillary temperature of 300ºC. The collision cell was operated at 1.5 mTorr of argon. For the duration of the run, transitions for each lipid species were monitored at 100 ms or 50 ms dwell time. 20 lipid standards for our profile from Avanti (Alabaster, AL) were used to develop calibration curves and these curves were then used for lipids species to be monitored. Processing of the samples was done using Thermo Xcalibur 2.2 Quan Browser software and exported to excel for reporting results.

***In vitro* growth studies**

From overnight YPD broth cultures of *Cn* WT, *Δcer1* and *Δcer1+CER1* were washed twice in phosphate buffered saline (PBS), resuspended and diluted into 10ml DMEM (buffered with HEPES, pH 4.0 or pH 7.4) to a final density of 10^4 cells/ml and incubated in shaker incubator at 37°C with 5% CO_2. Aliquots were taken at time points indicated and serial dilutions were plated on YPD agar for assessment of CFU. For cell wall stability, cells were spotted in serial dilutions on YPD plates with 0.05% SDS. For osmotic stress, cells were spotted on YPD containing 2mM H_2O_2.

Transmission Electron microscopy

Cn strains were grown overnight in YPD at 30°C with shaking. The next day, these cells were washed, counted and transferred to DMEM (pH 4.0 or 7.4) and grown in shaking condition at 37°C +5% CO_2 to mimic physiological

conditions. After 24 hours of growth, these cells were washed with phosphate buffered saline, and fixed in 3% EM grade glutaraldehyde solution for 2 hours. For supplementation experiments, cells grown in physiological condition as mentioned earlier were supplemented with 50µM ceramides mix (Matreya LLC, PA). For sample preparation, after glutaraldehyde fixation, cells were rinsed in 0.1M phosphate buffer pH7.4 and dispersed and embedded in ultra-low gelling temperature agarose. Tubes containing these cells were then cooled and agarose samples were chopped into cubes of smaller size. Post fixation of these samples was done by rinsing with aqueous potassium permanganate, and then further rinsed and treated with sodium meta periodate. This was followed by another rinse, and ultimately dehydrated through a graded ethanol series. After dehydration samples were embedded in Spurr's resin and polymerized in a 60°C oven. For sectioning, ultrathin sections of 80nm were cut with a Leica EM UC7 ultramicrotome and placed on 300 mesh copper grids. Sections were then counterstained with uranyl acetate and lead citrate and viewed with a FEI TeCnai12 BioTwinG^2 transmission electron microscope. Digital images were acquired with an AMT XR-60 CCD Digital Camera system.

Replicative lifespan studies

Replicative lifespan for WT and mutant *Cn* strains was measured by microdissection according to (Park, McVey et al. 2002, Bouklas, Jain et al. 2017) with minor adjustments. Briefly, Cells of *Cn* were plated and incubated at 37°C. The bud of these cells were followed by identifying the first bud and following its increase in size during the cell cycle. The daughter cells were separated from mother cell at the end of each division (1-2 hours) with the help of a 50 µm fiber optic needle (Cora Styles) on a tetrad dissection Axioscope A1 microscope (Zeiss) at 100x magnification. Replicative lifespan of each cell was determined as sum of the total buds until the mother cells fail to divide any further.

Glucose dependent medium acidification to measure plasma membrane H^+-ATPase

Glucose-dependent medium acidification was monitored by a modification of a procedure described previously (Perlin, Brown et al. 1988, Soteropoulos, Vaz et al. 2000). Cultures of *Cn* strain WT, Δ*cer1*, Δ*cer1+CER1,* Δ*gcs1* were grown to mid-log

Phase in YPD. The next day, these cells were transferred to DMEM at pH 4.0 and allowed to grow under shaking conditions for 24 hours. These cells were then harvested and washed using 100mM KCl, pH 5.0. These pellets were then resuspended in 10ml KCl, pH 5.0 and incubated under shaking condition at 30 ℃. These samples were then stored at 4 ℃ overnight prior to use. For the assay, cells were concentrated to a final A590 of approximately 2.0. 20

µl cells along with 155 µl of bromophenolblue (50 µg/ml) in 100mM KCl, pH 5.0. 20 µl 20% (w/v) glucose was added to initiate the reaction. Medium acidification was monitored at 590 nm over a period of 5 hours (data point every 3 mins) in a microplate reader (SpectraMax M5).