

Lipidomics suggests a new role for ceramide synthase in phagocytosis

Divya Pathak^{1,3}, Neelay Mehendale^{2,3}, Shubham Singh², Roop Mallik¹,
Siddhesh S. Kamat^{2,*}.

¹Department of Biological Sciences, Tata Institute of Fundamental Research,
Homi Bhabha Road, Mumbai, 400005, India

²Department of Biology, Indian Institute of Science Education and Research,
Dr. Homi Bhabha Road, Pashan, Pune 411008, India.

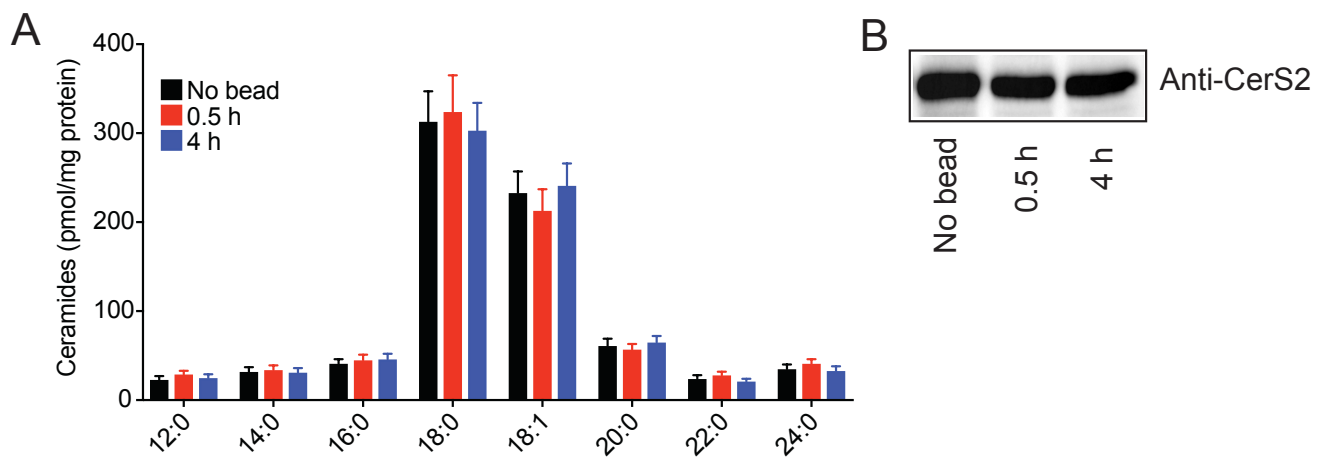
³These authors contributed equally to the work.

*To whom the correspondence should be made: siddhesh@iiserpune.ac.in

Supplementary Information

Table of contents

Supplementary Figures 1 – 8	3 – 10
Supplementary Table 1	11
• See also separate excel sheet	
Supplementary references	12



Supplementary Figure 1. Cellular concentration of ceramides and CerS2

following bead phagocytosis from RAW264.7 cells. As a control

experiment to determine whether **(A)** cellular ceramides and **(B)** cellular

CerS2 were altered in RAW264.7 cells when fed with beads, and

concentrations of ceramides and CerS2 were measured by LC-MS/MS and

western blot analysis respectively at 0.5 and 4 h post bead feeding (which

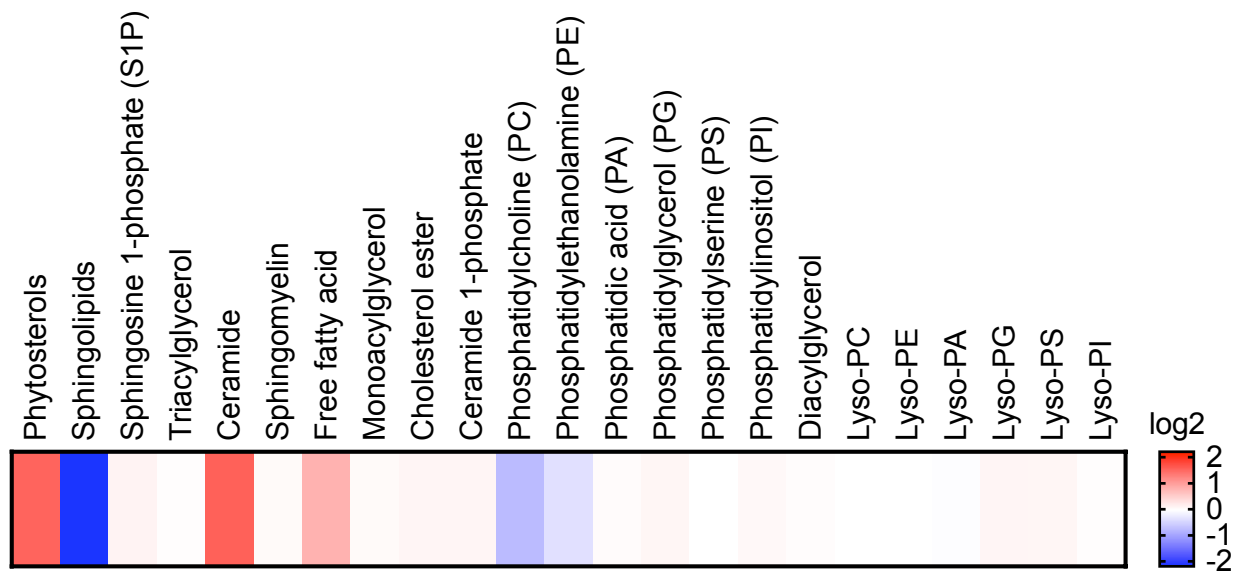
represent time points of EP and LP maturation respectively). Based on these

data, both these cellular levels are unaltered, suggesting that changes in

ceramides and CerS2 are indeed EP and LP specific. The data in panel **(A)**

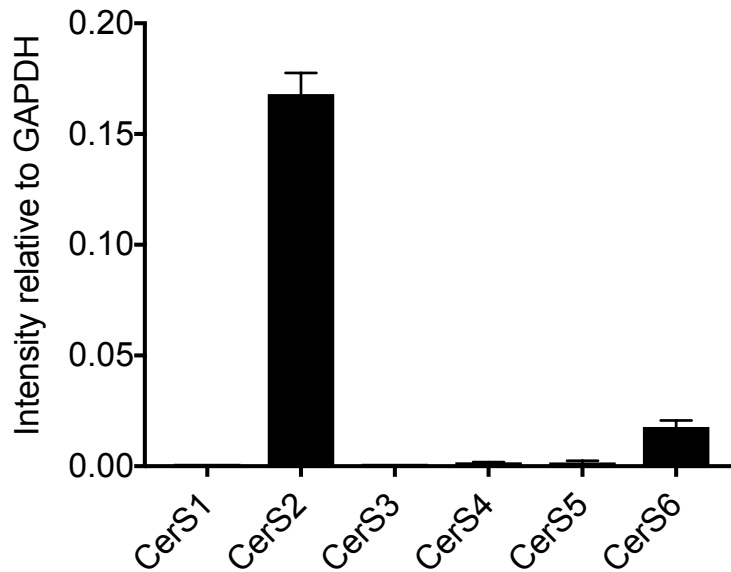
represents mean \pm s. e. m. for 4 biological replicates, while the data in panel

(B) was performed in duplicate with reproducible results.

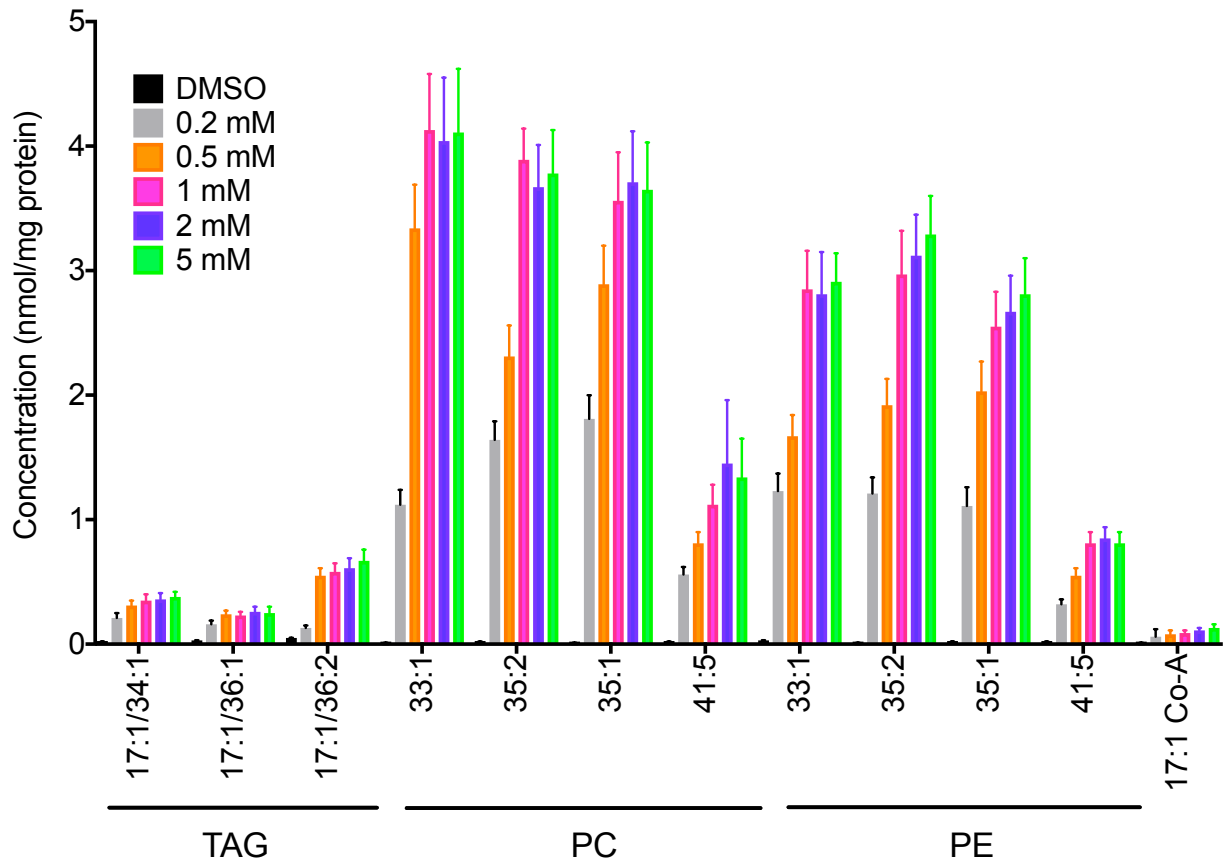


Supplementary Figure 2. Lipidomic characterization of EPs and LPs

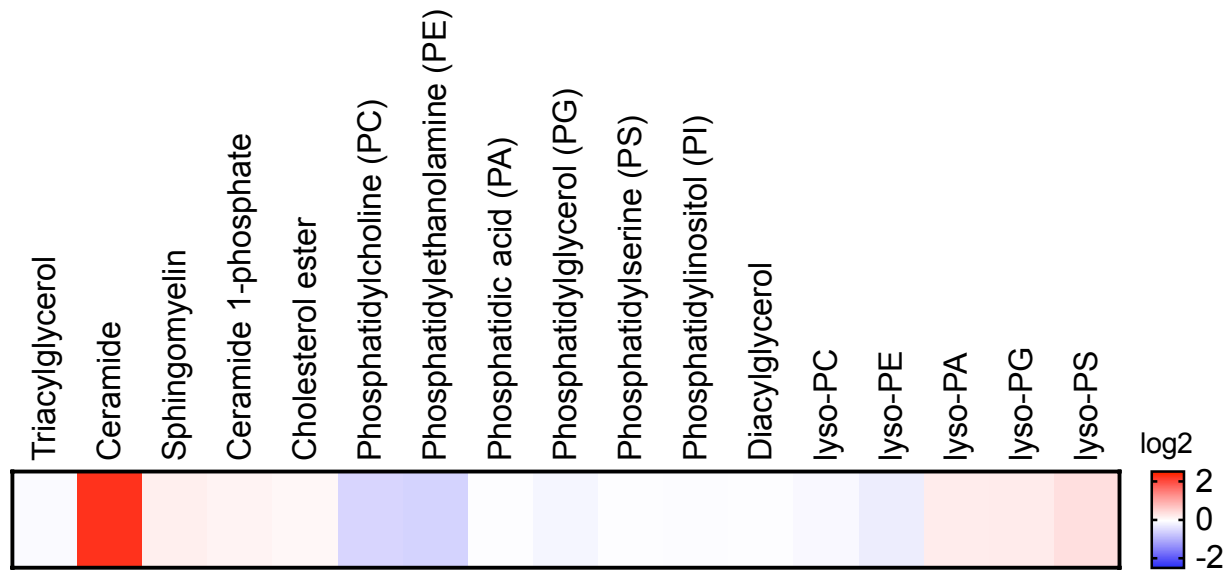
from *Dictyostelium discoideum*. Heat map plot showing the different lipid classes assessed by LC-MS/MS analysis for EPs and LPs from *Dictyostelium discoideum*. The heat map plot represents an average of fold changes (LP/EP) on a log₂ scale for different lipids from a particular lipid class. Blue and red color changes show enrichment of a particular lipid class on EP and LP respectively. Data represents 6 biological replicates per group. See **Supplementary Table 1** for complete datasets.



Supplementary Figure 3. Expression profile for different ceramide synthase isoforms in RAW264.7 mouse macrophages. The mRNA levels of ceramide synthase isoforms 1 – 6 in mammalian RAW264.7 cells relative to GAPDH from public large-scale gene expression studies (<http://biogps.org/>)¹.² The mRNA expression data suggests that CerS2 is the major ceramide synthase in RAW264.7 mouse macrophages³, with probably some contribution from CerS6. Data represents mean \pm s. d. for 2 biological replicates.

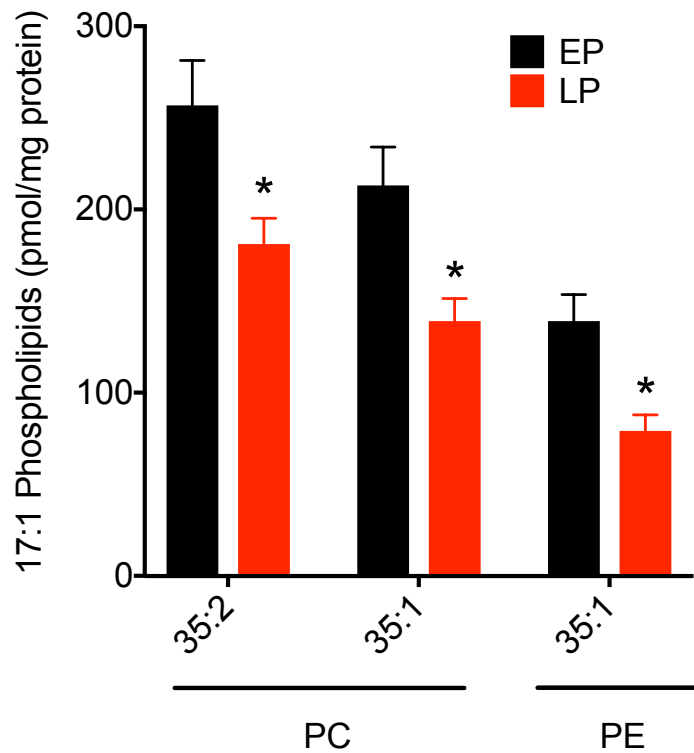


Supplementary Figure 4. Incorporation of C17:1 FFA into cellular phospholipids. Concentrations of C17:1-containing storage lipids, namely triglycerides (TAG), phosphatidylcholines (PC), phosphatidylethanolamines (PE) and co-enzyme A (Co-A) from RAW264.7 mouse macrophages, following feeding varied concentrations of C17:1 FFA (0 – 5 mM, 4 h). Data shows that the C17:1 FFA is predominantly incorporated into cellular PC and PE lipids. Data represents mean \pm s. e. m. for 4 biological replicates per group. Since there was no significant change beyond feeding 1 mM C17:1 FFA, hence this concentration was chosen for the feeding experiments.



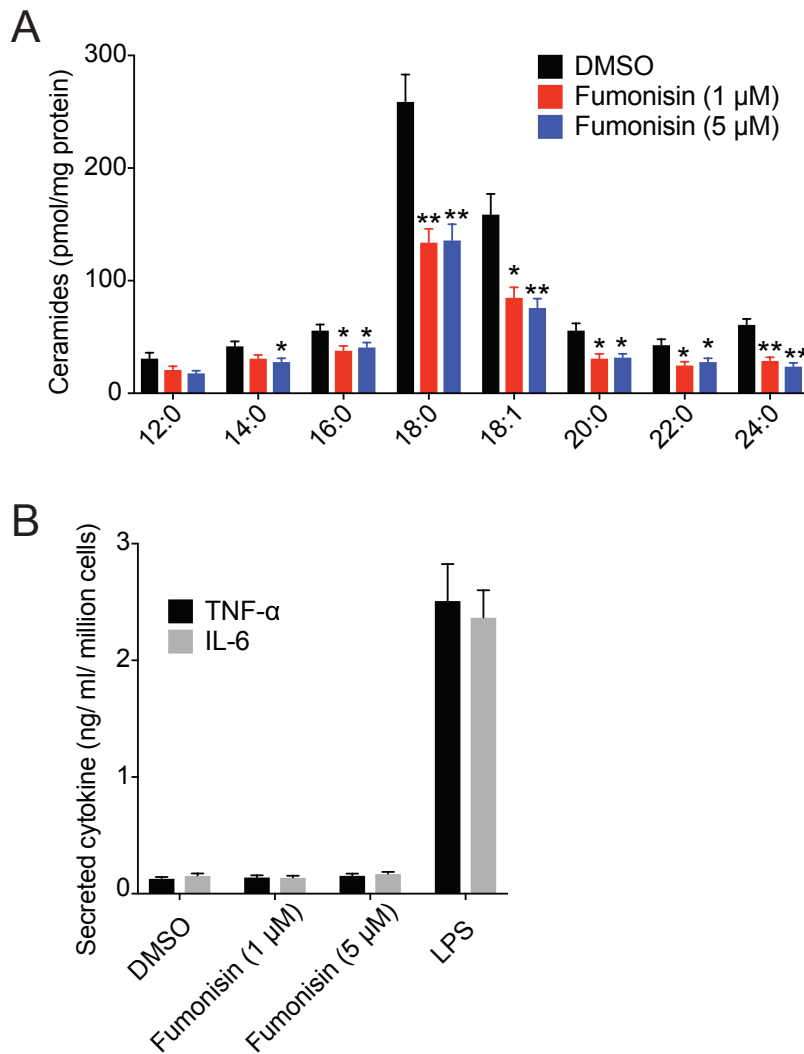
Supplementary Figure 5. Lipidomics characterization of heptadecenoic acid (C17:1)-containing lipids in EPs and LPs following C17:1 FFA

feeding to RAW264.7 mouse macrophages. Heat map plot showing the different lipid classes containing C17:1 assessed by comparative LC-MS/MS analysis for EPs and LPs from RAW264.7 cells fed with C17:1 FFA (1 mM, 4 h), prior to phagocytosis. The heat map plot represents the ratio of the mean value for LPs divided by the mean value for EPs for a particular lipid on a log₂ scale. Blue and red color changes show enrichment of a lipid class on EPs and LPs respectively. Data represents mean of 4 biological replicates per group. See **Supplementary Table 1** for complete dataset.



Supplementary Figure 6. Phospholipid concentrations of EPs and LPs following C17:1 FFA feeding to RAW264.7 mouse macrophages.

Quantitative lipidomics measurements of C17:1-containing PC and PE lipids showing changes in absolute levels between EPs and LPs groups. Data represents mean \pm s. e. m. for 4 biological replicates. * $P < 0.05$ by Student's t-test for EP versus LP preparations. See **Supplementary Table 1** for complete dataset.



Supplementary Figure 7. Pharmacological blockade of CerS2 by

fumonisin in RAW264.7 mouse macrophages. (A) Ceramide

concentrations of RAW264.7 cells following treatment with fumonisin (1 or 5 μ M) or DMSO (control) for 4 h. Data represent mean \pm s. e. m. for 4 biological replicates per group. * $P < 0.05$ and ** $P < 0.01$ by Student's t-test for treatment groups versus DMSO control.

(B) Pro-inflammatory cytokine

concentrations (TNF- α and IL-6) following treatment with fumonisin (1 or 5 μ M) or DMSO (vehicle, negative control) or lipopolysaccharide (LPS, 10 μ g mL⁻¹, positive control) for 4 h. Data represents mean \pm s. e. m. for 8 biological replicates per group.

```

CERS1_MOUSE 1 MAA-----AAAT---PRLEAPEMPSYAQMLQRSWASALAAQCGGD
CERS2_MOUSE 1 MLQ-----LYDYFWERLWLPVN-LTWADLE-----
CERS3_MOUSE 1 MFO-----TFRKWFWSERYWLPPT-IKWSGLE-----
CERS4_MOUSE 1 MSF-----SLEWLVQETVYWLPPN-VTWAELE-----
CERS5_MOUSE 1 MATAAAETLGLLWGLWSEFWLPQN-VSWADLE-----
CERS6_MOUSE 1 MAG-----ILAWFWRNERFWLPVN-VTWADLK-----
Q54S87_DICDI 1 MAL-----DENG-----VSE-IEWERLY-----

CERS1_MOUSE 40 CGWGLARRGL-AEHAHLAAPELLL-----AVLICALGWTALRWAATTHI
CERS2_MOUSE 27 -----DK-DGRVYAKASDLYI-----TLPLALLFLVIRYFFELYV
CERS3_MOUSE 27 -----DH-DGLVFWKASHLYI-----TIPYAFLLMVVRYFFEFV
CERS4_MOUSE 27 -----DR-DGLVFAHPHHVLA-----AFPVALVLVAVRIVFERFV
CERS5_MOUSE 34 -----GPGDGYGYFRAQHVLV-----VFPLAVCIFSVRMLFERFI
CERS6_MOUSE 26 -----NT-EEATFFQAEDLYL-----AFPLAFCIFMVRLIFERFI
Q54S87_DICDI 18 -----NP-DNSIFLSGRKLMSEVNAIFLVVCTNIFVIRFFQHYV

CERS1_MOUSE 82 FRPLAKRCL-----
CERS2_MOUSE 61 ATPLAALLNVKEKTRLRAPPNATLEHFYQTSKGKQKQVEVDLLSRQSGLS
CERS3_MOUSE 61 ATPLANALGIKKT-QHKIKPNAILENFKHSTSKPSHTDIYGLAKKCNLT
CERS4_MOUSE 61 ALPLSRWVGQDPIRRKIKPNVLEKYFLRMKQCPPEETQMVLLASQCLT
CERS5_MOUSE 69 AKPCALRVGKDSVNVKVEPNDTLEKVFVSVTKYPDEKRLKGLSKQLDWS
CERS6_MOUSE 60 AKPCAIALNIQANGPQTAQPNALILEKVFTAITKHPDEKRLKGLDWD
Q54S87_DICDI 59 LKPIALSFNMR-----

CERS1_MOUSE 92 -----QPRDAARLPESAKWLLFYLACWYCYAYLLGLTSYP
CERS2_MOUSE 111 GRQVERWFRRRRNQDRPSLKKFREASWRFTYLLIAFVAGMAVTD--KP
CERS3_MOUSE 110 ERQVERWLRIRQKQNKPCRLQKQFQESCWRFTYLLIMAGAVLYD--KP
CERS4_MOUSE 111 LRQTRWFRRRRNQDRPSLKKFCEACWRFFVYLCVFGGTSILYH--ES
CERS5_MOUSE 119 VRKIQCWFRRRRNQDPPTLTKFCESMWRFTYLLICYGIRFLWS--MP
CERS6_MOUSE 110 VRSIQRFRRRNQEKPSLTRFCESMWRFSFYLVFVSVGRFLKQ--TP
Q54S87_DICDI 70 -----KSYTARFLENGWYTYLIIISFFLIGSVVYSQ--ES

CERS1_MOUSE 127 FF-HDPPSVFYDWRSGMAVWDIAVAYLLQGSFYCHSIYATVYMDSWRRK
CERS2_MOUSE 159 WF-YDLRKVWEGYPIQ-SIIPSQWYMIELSFYNSLL-FSISDVKRRK
CERS3_MOUSE 158 WA-YDLWVWVNDYPRQ-PLLPQWYVYILEMSFYNSLV-FSLSTDIKRRK
CERS4_MOUSE 159 WL-WSPSLCWENYPHQ-TLNLSLWYVYLLLELGFYLSLL-ITLFPDKRRK
CERS5_MOUSE 167 WF-WDTRQWYNYPIYQ-PLSRELYYYITQLAFYWSLM-FSQFDVKKRRK
CERS6_MOUSE 158 WL-WNTRHCWYNYPIYQ-PLTADLHYVYILELSFYWSLM-VSQFTDIKRRK
Q54S87_DICDI 102 WSIFFTMNIWLGWPTQ-PPSLTFRTYLLIELSFYVHCT-IALFFETRRK

CERS1_MOUSE 176 SVVMLVGVVVTLLIASSTAFRYHNVLLVFFLGVSVVQLEFVTLNINIF
CERS2_MOUSE 206 FKEQIIEAVATIIILCFSEWFANYVRAETLIMALHVASVYLLSARMFNMA
CERS3_MOUSE 205 FLAHVIEHLAAISMSFSWCANYIRSETLVMTFHSISWLESARMFNMA
CERS4_MOUSE 206 FKEQVVHGFVAVGIGFSVNVNLRICAVVLLHSCSYLLEGGKILNMA
CERS5_MOUSE 214 FLMMFTHEMIGIMTTFSVNNMVRVICALIFCLHGFADPLLEAARMANMA
CERS6_MOUSE 205 FGIIMLHHLATIFITFSVNNMARVCTLVLCIHSASALLLEAARMANMA
Q54S87_DICDI 150 FNQMLTIEAVATFFVGCSTWYRYHRICAILWIEHIAIFLYSALALNMI

CERS1_MOUSE 226 K-----ARGGAYHRLHGLVANLGLSFCFCWFWRFLYWFPLKVLATC
CERS2_MOUSE 256 G-----WKN-----TCNNLFIVFAIVEIITRLVIMFFWLHCTM
CERS3_MOUSE 255 G-----WKQ-----TCNTLFFIFTVVFFISRFIIPFFWILYCTL
CERS4_MOUSE 256 H-----FRR-----GCDALFIMFALVFYTRLIFFFTQVIYTSV
CERS5_MOUSE 264 R-----RER-----LCTLFVIFGAAFIVSRLAIFFLWILNNTL
CERS6_MOUSE 255 K-----FQK-----MCDLFLVMFAVVEITRLGIFPLWVLTNTL
Q54S87_DICDI 200 SKEVKNKTIQI-----ICDGLFVMAVSVFVTRLIRIFFFTLKSLL

CERS1_MOUSE 269 HCSLQSPDIPYFFFNILLLLMVMNIWFLYIVAFAAKVL-TGQMREL
CERS2_MOUSE 290 IYPLELYPAFFGYFFNFMMAVLQMLHIFWAYFILRMAHKFI-TGKLI--
CERS3_MOUSE 289 ILPLHYLEPFFSYIFLNLQMLQLGLHVVYWGCFILKMLNRCI-FTQNV--
CERS4_MOUSE 290 YDSIKNSGPFYFFYFVLLVLMQLIHHVYWFCLILRMLYSFLHKGQMT--
CERS5_MOUSE 298 FESWEIIGPYPSWMLFNALLLILQVLHAIWSYLIQVASKALSRGKVS--
CERS6_MOUSE 289 FESWEIVGYPYPSWVFNLLLLLQGLNCFWSYLIIVKIACKTVSKGKVS--
Q54S87_DICDI 241 TEAYVSVVEFPLFYFTNVALTLTLILHMFVFLIARIYIKLFSKSKDF--

CERS1_MOUSE 318 EDLRE-YDTLEAQTAKPCK-AEK----PLRNGL-VKDKLF-----
CERS2_MOUSE 337 EDERSDREETESSEGEETA-AGAGAKSRLLANG-----HPIL-----NN
CERS3_MOUSE 336 QDVRSDNEEEEEEEEEAEETKGETEYLNKGL-GTNRHLIA-----NG
CERS4_MOUSE 338 EDIRSDVEEPDSSDDEPVS-EGPQLKNGMARGS-----RVAVT-----NG
CERS5_MOUSE 346 KDDRSDVESSEEEDETTTH-KNN-LSGSSSSNGANCMNGYMGSSHAESEQ
CERS6_MOUSE 337 KDDRSDISSSDEDESEPP-GKK-PHSSTTNGTSGTNGYLLTGPCSDV-
Q54S87_DICDI 289 DDIRSDSDEDEVKPTQK-LEA-EPTRTNKNTNNKNNKLTQTKVSKA-

CERS1_MOUSE -----
CERS2_MOUSE 375 NHPK-----ND
CERS3_MOUSE 380 QHG-----R
CERS4_MOUSE 377 PRSRAAAC--LTN-GHTRAT
CERS5_MOUSE 394 GTCKATGNLHFRASPHLHSCD
CERS6_MOUSE 384 -----D
Q54S87_DICDI 336 AQKK-----NE

```

Lag1P motif

Supplementary Figure 8. Sequence alignment of mammalian (mouse) ceramide synthase isoforms (CerS 1 – 6) with ceramide synthase from *Dictyostelium discoideum* (CrsA, Q54S87). The residues highlighted in green represent the invariant catalytically important residues, while the residues underlined in red constitute the highly conserved *Lag1P* motif of ceramide synthase enzymes^{4, 5}.

Supplementary Table 1: MRM transitions and complete lipidomics data.

- Tab 1 ('MRM transitions'): MRM transitions, and MS parameters for all lipids assessed in this study.
- Tab 2 ('RAW cells EP and LP data'): Complete lipidomics dataset (all identified and quantified lipids) for EPs and LPs from RAW264.7 mouse macrophages.
- Tab 3 ('Dictyostelium EP and LP data'): Complete lipidomics dataset (all identified and quantified lipids) for EPs and LPs from *Dictyostelium discoideum*.
- Tab 4 ('RAW cells 17_1 feeding expt'): Concentrations of C17:1-containing lipids from EPs and LPs from RAW264.7 mouse macrophages post-feeding 1 mM C17:1 FFA for 4 h.
- Tab 5 ('CerS2 inhibition RAW cells'): Concentrations of sphingolipids from EPs and LPs after treating RAW264.7 mouse macrophages with fumonisin (5 μ M, 4 h).

References:

- [1] Wu, C., Jin, X., Tsueng, G., Afrasiabi, C., and Su, A. I. (2016) BioGPS: building your own mash-up of gene annotations and expression profiles, *Nucleic Acids Res* 44, D313-316.
- [2] Wu, C., Orozco, C., Boyer, J., Leglise, M., Goodale, J., Batalov, S., Hodge, C. L., Haase, J., Janes, J., Huss, J. W., 3rd, and Su, A. I. (2009) BioGPS: an extensible and customizable portal for querying and organizing gene annotation resources, *Genome Biol* 10, R130.
- [3] Halasiddappa, L. M., Koefeler, H., Futerman, A. H., and Hermetter, A. (2013) Oxidized phospholipids induce ceramide accumulation in RAW 264.7 macrophages: role of ceramide synthases, *PLoS One* 8, e70002.
- [4] Spassieva, S., Seo, J. G., Jiang, J. C., Bielawski, J., Alvarez-Vasquez, F., Jazwinski, S. M., Hannun, Y. A., and Obeid, L. M. (2006) Necessary role for the Lag1p motif in (dihydro)ceramide synthase activity, *J Biol Chem* 281, 33931-33938.
- [5] Levy, M., and Futerman, A. H. (2010) Mammalian ceramide synthases, *IUBMB Life* 62, 347-356.