A CRITICAL ROLE FOR CERAMIDE SYNTHASE 2 IN LIVER HOMEOSTASIS: I. ALTERATIONS IN LIPID METABOLIC PATHWAYS

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Running title: Lipid metabolism in a ceramide synthase 2 null mouse

Ceramide, an important lipid signaling molecule which plays critical roles in regulating cell behavior. Ceramide synthesis is surprisingly complex and is orchestrated by six mammalian ceramide synthases (CerS) which each produce ceramides with restricted acyl chain lengths. We have generated a CerS2 null mouse and characterized the changes in the long chain base and sphingolipid composition of livers from these mice. Ceramide and downstream sphingolipids were devoid of very long (C22-C24) acyl chains, consistent with the substrate specificity of CerS2 towards acyl CoAs. Unexpectedly, C16-ceramide levels were elevated and as a result, total ceramide levels were unaltered; however, C16-ceramide synthesis in vitro was not increased. Levels of sphinganine were also significantly elevated, by up to 50-fold, reminiscent of the effect of the CerS inhibitor, fumonisins B1. With the exceptions of glucosylceramide synthase and neutral sphingomyelinase 2, none of the other enzymes tested in either the sphingolipid biosynthetic or degradative pathways were significantly changed. Total glycerophospholipid and cholesterol levels were unaltered, although there was a marked elevation in C18:1 and C18:2 fatty acids in phosphatidylethanolamine, concomitant with a reduction in C18:0 and C20:4 fatty acids. Finally, differences were observed in the biophysical properties of lipid extracts isolated from liver microsomes, with membranes from CerS2 null mice displaying higher membrane fluidity and showing morphological changes. Together, these results demonstrate novel modes of cross-talk and regulation between the various branches of lipid metabolic pathways upon inhibition of very long acyl chain ceramide synthesis.
INTRODUCTION

Biological membranes contain thousands of different lipid species that can be broadly classified according to their backbone structure (1). Of these, sphingolipids (SLs) have become particularly prominent due to the discovery of their unexpected structural complexity and their intricate modes of cellular trafficking and metabolism (2-4). Ceramides are perhaps the most well-studied class of SLs, because of their essential roles in differentiation and in apoptosis (5-7).

Ceramides can differ in both their long chain sphingoid base (8) and fatty acid composition (9). Over the past few years, a complex mode of regulation of ceramide synthesis has been described, with each of the six mammalian ceramide synthases (CerS) (formerly known as Lass (longevity assurance)) genes generating ceramides with specific acyl chain lengths (10). Thus, CerS1 uses mostly C18-CoA (11), CerS4 uses C18- and C20-CoAs (12), CerS5 and CerS6 use mostly C16-CoA (12,13), and CerS3 uses very long chain acyl CoAs (C26 and higher) (14). CerS2 can utilize a wider range of fatty acyl CoAs, but uses mainly C22 to C24. In addition, CerS2 displays complex modes of regulation and has genomic features characteristic of a 'housekeeping' gene, although no other CerS genes display these characteristics (15).

We have now generated a CerS2 null mouse, and have systematically dissected the changes in SL levels in the livers of these mice during the first four months of their development. These analyses extend the recent study by Imgrund et al. (16), inasmuch as we provide data on the changes in SL levels during mouse development. Our study also measures changes in levels of sphingoid long chain bases, and is consistent with the idea that the dramatic pathological changes that occur in the liver of these mice (Pewzner-Jung et al., accompanying manuscript) are likely due to a combination of changes in the acyl chain composition of SLs as well as changes in long chain base (predominantly sphinganine) levels. Finally, we have analyzed the activity of a number of enzymes of SL metabolism, including neutral sphingomyelinase 2 (N-SMase 2), whose levels are significantly elevated, and have also analyzed changes in the biophysical properties of microsomal lipids. Significant and unexpected changes are observed that together suggest complex modes of interaction between components of the SL metabolic pathway.

EXPERIMENTAL PROCEDURES

Materials - D-erythro-[4,5-3H]Sphinganine (80 Ci/mmol) was synthesized as described (17). Fatty acyl-CoAs, ceramides, C6-NBD-sphingomyelin (SM), C6-NBD-glucosylceramide (GlcCer), C12-NBD-ceramide, C6-NBD-ceramide, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rho), and the internal standards for liquid chromatography electrospray ionization-tandem mass spectrometry (ESI-MS/MS) were purchased from Avanti Polar Lipids (Alabaster, AL). Sphinganine was from Matreya (Pleasant Gap, PA). Silica gel 60 thin-layer chromatography plates were from Merck. A protease inhibitor cocktail was from Sigma-Aldrich. 1,6-Diphenyl-1,3,5-hexatriene (DPH) and trans-parinaric acid (t-PnA) were from Molecular Probes (Leiden, The Netherlands). Dulbecco’s modified Eagle’s medium was from Gibco or Sigma-Aldrich. All solvents were of analytical grade and were purchased from Biolab (Jerusalem, Israel).

Mice - All mice were maintained under pathogen-free conditions and handled
according to protocols approved by the Weizmann Institute Animal Care Committee as per international guidelines.

**PCR methods and primers for genotyping mice** - Genotyping was performed by PCR using primers that detect the wild type CerS2 (CerS2⁺) and the gene-trapped CerS2 (CerS2⁻) alleles in one reaction, using a cocktail of three primers: Forward (264) 5’-GTAGTGCTCTCCATCATG-3’; Forward (564) 5’-CAGACACAGATAAGTTGC-3’; Reverse (432) 5’-CATACAAGGTCTGGAGC-3’.

PCR cycles were 95°C for 3 min (95°C for 30 sec, 57°C for 30 sec, 72°C for 1.5 min) for 35 cycles and 72°C for 3 min. Taq DNA Polymerase Master Mix (Amplicon) was used. A 1537 base pair band was detected for the CerS2⁺ allele and a 1258 base pair band for the CerS2⁻ allele (see Supplementary Experimental Procedures).

For full length RT-PCR analysis of CerS2, the following primers were used:
Forward (314)
5’-GATGCTCCAGACCTTGTATGAC-3’,
Reverse (590)
5’-TTGTTATTGAGGATGGGGTG-3’. PCR cycles were as above. Further details are given in Supplementary Materials.

**Lipid biochemistry** - CerS activity was assayed using [³H]sphinganine and acyl CoAs of different chain lengths (15,18) using 150 µg of protein. Neutral sphingomyelinase (SMase) activity was assayed using C6-NBD-SM (19), and other enzymes were measured according to the following protocols: SM synthase using C6-NBD-ceramide (19); acid, alkaline and neutral ceramidases using C12-NBD-ceramide (20); GlcCer synthase using C6-NBD-ceramide (21); acid SMase using C6-NBD-SM (19); glucosylceramidase using C6-NBD-ceramide (GlcCerase) (22).

**CerS mRNA expression** - cDNA was synthesized and determined by qPCR using TaqMan™ analysis and a 7300 Sequence Detection System (15). The following primers were used. CerS2 sense:
5’-GGCGCTAGAAGTGAGGAAAC-3’; antisense,
5’-TCGAATGACGAGAAGAGCA-3’. For CerS4, 5, 6, and for TATA binding protein (TBP), which was used as an endogenous control, see Ref. (15).

**Lipid analysis** - SL analyses by ESI-MS/MS were conducted using a PE-Sciex API 3000 triple quadrupole mass spectrometer and an ABI 4000 quadrupole-linear ion trap mass spectrometer (12,15,23,24), as were acyl CoA levels (25). Glycerophospholipids and cholesterol were measured by nano-ESI-MS/MS (26,27). All data is expressed per dry weight. Total cholesterol was measured by ferric chloride (28).

**Neutral-SMase expression** - N-SMase 1 and 2 mRNA expression was measured by RT-PCR using the following primers.
N-SMase 2 forward
5’-GAGCAGCTACACGCTACTTC-3’
N-SMase2 reverse
5’-GAGACCGTTGAGGTCCACAGC-3’ to yield a 546 base pair DNA fragment.
N-SMase 1 forward
5’-CAATCTCAACTGCTG-3’
N-SMase1 reverse
5’-GAGTCACGTAGCATTGAG-3’ to yield a 371 base pair DNA fragment.

**Membrane biophysics and morphology** – The biophysical properties of membranes
were examined after preparing lipid vesicles (29) (~0.3 mM phospholipid in phosphate buffered saline) from microsomal lipid extracts (30). During vesicle preparation, all the samples were re-equilibrated by freeze-thaw cycles. For studies with t-PnA and DPH, the samples were slowly brought to room temperature and the probe was added from an ethanol stock solution. The samples were again re-equilibrated by freeze-thaw cycles and subsequently kept overnight at 4°C. Before measurements, the samples were slowly brought to room temperature and maintained at this temperature at least for 1 h. Fluorescence anisotropy was measured (10 measurements for each analysis) in a FluoroLog®-3 fluorimeter (Horiba Jobin-Yvon, NJ, USA) using t-PnA, Rho or DPH (at final probe concentrations of 0.7 μM, 0.5 μM and 0.8 μM, respectively). All measurements were performed in 0.5 cm × 0.5 cm quartz cuvettes under magnetic stirring. The excitation (λex)/emission (λem) wavelengths were 320/405 nm for t-PnA, 358/430 nm for DPH and 570/593 nm for Rho.

Membrane morphology was studied by fluorescence microscopy using a Leica TCS SP5 (Leica Microsystems CMS GmbH, Mannheim, Germany) inverted microscope (DMI6000) with a 63x water (1.2 numerical aperture) apochromatic objective. Giant unilamellar vesicles were prepared by electroformation (31) using microsomal lipid extracts. Rho was used at a probe to lipid ratio of 1:500.

Protein phosphatase 2A - Protein phosphatase 2A (PP2A) activity was measured as described (32,33).

Statistics - All data are shown as means ± s.e.m. (unless indicated). p values were calculated using a one-tailed two-independent samples Student's t test. A p value <0.05 was considered statistically significant.

RESULTS

A CerS2 null mouse was generated from embryonic stem cells harboring a gene trap (GT) retroviral vector insertion in the first intron of the CerS2 gene (34) (Fig. 1A). Intercrossing CerS2GT/+ mice (F1 of 129S4/SvJae x C57BL/6) resulted in generation of F2 CerS2GT/GT (CerS2 null) mice (Fig. 1B), which were born with normal Mendelian inheritance ratios but weighed ~20-30% less than wild type (WT) mice (Fig. 1C). CerS2 null mice do not normally survive beyond ~16 months, although occasionally mice survived for as long as 20 months (Pewzner-Jung et al., accompanying manuscript).

CerS2 null mice display changes in the acyl chain composition of SLs - Analysis of ceramide synthesis in homogenates from mouse liver (which contains high levels of CerS2 mRNA (15)) demonstrated that synthesis of ceramides containing very long acyl chains (C22-C24) was reduced by ~95% in CerS2 null mice (Fig. 2), as predicted from the acyl chain specificity of CerS2 (15). The small residual amounts of C22-24 ceramide synthesis may be due to the sensitivity of the assay or due to the reverse activity of ceramidase; alternatively, other CerS (i.e. CerS3 and 4) may be able to synthesize small amounts of C22-24-ceramides, although the data largely support the functional non-redundancy of CerS2 with respect to acyl chain lengths.

ESI-MS/MS analysis of the changes in SL levels versus development of the CerS2 null mouse, corroborated that CerS2 null mouse liver contained only trace amounts of very long acyl chain ceramides compared to WT (Fig. 3, Table 1), and significantly reduced
levels of long acyl chain SM and HexCer (Supplementary Tables 1 and 2), at all stages of mouse development. Thus, levels of C22-, C24:0- and C24:1-SLs were reduced by 10-100-fold in the CerS2 null mouse, but no significant reduction was seen in C20-SLs. Levels of C26:1- and C26:0-SLs were relatively low in the WT, and only a small reduction was observed in their levels in the CerS2 null mouse.

Unexpectedly, levels of C16-ceramide and C16-SLs were increased (Fig. 3, Table 1 and Supplementary Tables 1 and 2), and this increase compensated for the loss of C22-24-ceramides such that total ceramide levels (i.e. the sum of individual ceramide species containing all acyl chains) were essentially unaltered (Table 2). Thus, CerS2 null mice are not depleted in total ceramide levels, but rather show a change in their acyl chain composition.

C16-ceramide was the only ceramide species whose level increased (Table 1) and the increase was detectable at birth; the extent of the increase in C16-ceramide did not alter significantly during subsequent development (up to 120 days of age) (Table 1). Likewise, C16-SM was elevated from birth to 120 days of age (Supplementary Table 2), as was C16-HexCer, though HexCer levels in liver were significantly lower than those of SM, and therefore the changes in HexCer levels were more variable (Supplementary Table 2).

CerS2 null mice display changes in levels of long chain bases - In addition to the unexpected elevation of C16-SLs, a massive elevation of sphinganine was observed (Fig. 3 and Table 3), reaching levels as high as 95 pmol/mg of tissue, an ~50-fold elevation compared to WT. Interestingly, sphinganine levels were high at birth in both WT and CerS2 null mice (Table 3) and subsequently decreased to levels of ~1-2 pmol/mg of tissue in WT mice; in contrast, in CerS2 null mice, sphinganine levels started to increase after ~21 days of age and reached levels similar to those at birth after about one month. Sphinganine was the only long chain base whose levels increased significantly, as levels of the important signaling molecules, sphingosine 1-phosphate and sphinganine 1-phosphate, were unchanged, and only a small change in sphingosine levels was observed (Table 3). However, no significant changes in levels of the second substrate of the CerS reaction, acyl CoA, were detected (Fig. 4).

Changes in levels of other enzymes of SL metabolism cannot explain the changes in the SL profile – In order to determine the mechanism responsible for the changes in SL acyl chain length and sphinganine levels, we analyzed the mRNA expression levels, and the activity of a number of enzymes in the SL metabolic pathway. mRNA levels of CerS5, which synthesizes C16-ceramide (12,13), were elevated ~2.5-fold, whereas mRNA levels of CerS6 (13), which can also synthesize C16-ceramide, were unaltered (Fig. 5). However, the increased mRNA levels do not result in changes in CerS5 (or CerS6, see also (35)) enzyme activity, since C16-ceramide synthesis in vitro was unaltered (Fig. 2, insert). CerS1 and CerS3 mRNA was undetectable (not shown).

Systematic analysis of enzymes that either utilize ceramide as a substrate, or produce it, including SM synthase, acid, alkaline and neutral ceramidases and acid SMase, revealed no change in activity in either 14-day old or 30-day old mice (Fig. 6). The only biosynthetic enzyme whose activity was altered was GlcCer synthase, which showed an ~2-fold elevation in activity (Fig. 6). In addition, levels of N-SMase 2 mRNA,
but not N-SMase 1 (Fig. 7), were elevated in CerS2 null mice, resulting in increased N-SMase activity in the liver starting at 14 days of age (Fig. 7).

Changes in glycerophospholipid and cholesterol levels - No changes were observed in glycerophospholipid levels between WT and CerS2 null mice. Thus, total phosphatidylcholine (PC) comprised ~50% of the total glycerophospholipids in both WT and CerS2 null mouse liver, and phosphatidylethanolamine comprised ~30% (Fig. 8A). However, there were significant differences in the distribution of acyl chains in phosphatidylethanolamine (PE), with a significant reduction in 18:0 fatty acids and in the polyunsaturated fatty acid, C20:4, and a significant elevation in unsaturated fatty acids (C18:1 and C18:2) (Fig. 8B). Cholesterol levels were unaltered between the WT and CerS2 mice (Fig. 9).

Functional consequences of altered SL levels - To examine the functional consequences of altered SL levels, two properties were examined that have been reported to depend on the acyl chain composition of SLs. First, small but significant differences were observed in the biophysical properties of lipid extracts isolated from liver microsomes, with membranes from CerS2 null mice displaying higher membrane fluidity (Fig. 10A) as shown by the lower anisotropy of t-PnA and DPH in CerS2 null membranes. This was confirmed by the increase in Rho anisotropy in CerS2 null membranes. In fluid membranes, Rho anisotropy increases because it distributes homogeneously in the membrane and energy homotransfer is minimized. In membranes containing ordered regions, Rho is confined to the more fluid areas leading to an increase in energy homotransfer and consequent decrease in anisotropy (29). Nevertheless, the extent of the change was not as large as might have been predicted based on the large change in the sphingolipid profile (Tables 1 and Supplementary Tables 1 and 2), suggesting that compensatory changes occur in CerS2 null mice so as to maintain membrane fluidity. Despite the small differences observed in membrane fluidity, CerS2 null membranes displayed strong morphological alterations including membrane fusion, budding, tubule formation and vesicle adhesion, compared to WT membranes (Fig. 10B).

Secondly, we examined the activity of PP2A, whose activity is known to be modified by the acyl chain composition of ceramides (36). PP2A activity was unchanged (Fig. 11), which is in agreement with studies suggesting a role of C18-ceramide, but not C16-ceramide (36) (whose levels are elevated in the CerS2 null mouse liver, Fig. 3), in regulating PP2A activity.

**DISCUSSION**

In the current study we have demonstrated that multiple and unexpected mechanisms of regulation of SL levels exist in vivo. Thus, although the mouse that we generated is defective only in synthesis of ceramides containing very long acyl chain fatty acids (i.e. C22-24), levels of ceramide and downstream SLs containing long acyl chains (i.e. C16) were elevated, as were levels of sphinganine.

The extent of the differences in the SL profile between the WT and CerS2 null mouse can be appreciated by use of a pathway relational map (9) which shows fold-differences in the amounts of SLs between each mouse (Fig. 12) using a 'heat map' format. In this map, which depicts each metabolite as a node connected to its precursor and downstream metabolites, the...
hub of the middle cluster shows the formation of sphinganine from which radiates each of the different chain-length N-acyl-sphinganines (dihydroceramides, DHCer) produced by CerS, which are next incorporated into dihydrosphingomyelins (DHSM; left branching nodes), dihydroceramide monohexoses (DHCMH; right branching nodes), or desaturated to the respective ceramides (Cer) (dashed lines to the right cluster) that are incorporated into SM, HexCers (CMH), or turned-over to sphingosine (So) and sphingosine 1-phosphate (S1P) (Fig. 12). This depiction is particularly useful for drawing attention to the metabolites that are elevated (yellow to red) versus reduced (blue), and the relationships are relatively easy to appreciate. For example, by starting at the branch labeled '16' and proceeding clockwise through the various chain-length subspecies, it is evident which subspecies are elevated and which are lower in the CerS2 null mouse. It is also apparent that sphinganine is much higher in the CerS2 null mouse, and that dihydro-SLs are elevated more than SLs.

Despite the dramatic changes in the SL profile, we have not yet delineated the precise mechanisms leading to these changes. Thus, although C16-ceramide levels are elevated, there was no difference in C16-ceramide synthesis in vitro, and none of the changes, or lack of changes in the activities of other enzymes that metabolize ceramide, could explain these changes, including N-SMase 2, which shows a preference for degradation of very long acyl chain SM (37). One possibility is that ceramides with different acyl chain lengths (i.e. long chain versus very long chain) turnover with different rates, and that these rates are affected upon depletion of very long acyl chain ceramides and SLs; unfortunately, nothing is known about the rate of turnover of SLs with different acyl chains. Another possibility is that different hepatocytes express different complements of CerS genes (see Pewzner-Jung et al., accompanying manuscript), and that deletion of CerS2 in one type of hepatocyte has unexpected consequences on the other type. For instance, CerS2 is found at high levels in periportal hepatocytes but at lower levels in centrilobular hepatocytes (see Fig. 5 in Pewzner-Jung et al., accompanying manuscript). Assuming that CerS2 is the major CerS in periportal hepatocytes, and that other CerS are found at low levels in these cells, then sphinganine would accumulate in periportal hepatocytes of the CerS2 null mouse but would be consumed in centrilobular hepatocytes, assuming that centrilobular hepatocytes express a wider array of CerS genes. Interestingly, in a recent study using siRNA to CerS2 in cultured SMS-KCNR neuroblastoma and MCF-7 breast cancer cells, no changes in sphinganine levels were observed although C16-ceramide levels did increase in a similar manner to our study (35).

Irrespective of the precise molecular mechanism responsible for sphinganine elevation, the increase in the sphinganine levels are reminiscent of those observed after fumonisin treatment (38), a CerS inhibitor (39), that results in elevation of sphinganine. Sphinganine levels have been determined after treatment with fumonisin (10 mg/kg of fumonisin, once per day for 5 days), with levels of ~50 nmol/g of wet weight of tissue (40). Assuming that ~3 g of wet tissue is equivalent to ~1 g of dry tissue (41), then sphinganine levels in the CerS2 null mouse liver are ~30 nmol/g of wet weight, a little lower than values obtained after short-term fumonisin treatment. In contrast, sphinganine 1-phosphate is significantly elevated in liver after fumonisin treatment (40), but barely changes.
in the CerS2 null mouse.

While this study, and the accompanying study were in preparation for publication, another study was published in which the same mouse was generated (16). While there are some similarities between the two studies, our current study contains significant additional information to that of Imgrund et al. (16). (i) In the Imgrund et al. study, only one time point was measured for SL analyses, namely 10 weeks. In our study, we systematically analyzed SL levels for up to 4 months, including early time points in mouse development. This is important since it permits prediction of the time at which liver pathology might be observed and will help delineate the primary events leading to pathology; interestingly, there is little pathology until about one month after birth (Pewzner-Jung et al., accompanying manuscript) even though SL levels (including sphinganine) are altered immediately after birth. (ii) Imgrund et al. did not measure long chain bases, which could lead to the incorrect assumption that any pathology observed would be only due to changes in the acyl chain composition of SLs. In contrast, our data suggest that pathology is likely due to a combination of changes in the acyl chain composition and also in sphinganine levels; this is important since considerable information is available about pathological changes that occur in mouse liver upon fumonisn consumption, which can thus be compared and contrasted to that obtained in the CerS2 null mouse. (iii) Imgrund et al. see surprisingly high levels of C18-ceramide synthesis in WT liver relative to the low amount of C18-ceramide, -SM and -HexCer that is found in liver (15,16); in addition, they see a large reduction in C18-ceramide synthesis in the CerS2 null mouse. This is rather unexpected, since CerS2 does not synthesize significant levels of C18-ceramide (15), suggesting that expression or activity of CerS1 (or CerS4) (which both synthesize C18-ceramide (42)) have been inexplicably altered under their experimental conditions. (iv) Total ceramide, SM and HexCer levels are reduced by 30-50% in the liver of mice generated by Imgrund et al., whereas there are essentially no statistically significant changes in the total levels of these lipids in our study. This difference may be related to the one specific time point studied by Imgrund et al. However, it is of important functional consequence, since we are able to exclude that any pathology we observe is due to changes in total SL levels.

Although inactivation of the CerS2 gene has a major effect on the acyl chain composition of SLs and on levels of SL metabolites, membrane fluidity is changed to a smaller extent than might be predicted, suggesting that the membrane lipid composition of CerS2 null mice might be fine-tuned to maintain overall fluidity. In WT mice this property appears to be mainly determined by the balance between very long chain saturated SLs (which give a higher membrane order) and unsaturated SLs (which give higher membrane fluidity) (Table 1 and Supplementary Tables 1 and 2). In the CerS2 null mouse, membranes are depleted in unsaturated SLs and thus membrane order should increase. However, there is a concomitant enrichment in saturated long chain (i.e. C16) SLs, which induce an overall order lower than very long chain saturated SLs (43) leading to an overall decrease in order (fluidity increase). Despite the similar degree of order in CerS2 null and WT membranes, strong differences in membrane shape and curvature were observed. Membrane morphology plays an important role in cellular processes such as development and maintenance of organelles, endocytosis, vesiculation, and lipid and protein sorting (44). Indeed, it was recently
shown that structural motifs such as $\alpha$-helices and alkyl chains are able to sense differences in membrane curvature and are susceptible to membrane curvature-induced redistribution and sorting (45). In addition, the endocytic sorting of lipids and proteins commonly involves vesicular and tubular membranes, where the differences in lipid packing/membrane fluidity determine the route of the sorting molecule (46,47). Tubular structures were also shown to play a major role in cell-to-cell communication and intercellular transfer of organelles (48). Therefore, changing the lipid profile in CerS2 null mice induces major alterations in membrane morphology that may affect both intra- and inter-cellular signaling. Furthermore, changes in the membrane lipid composition and concomitant changes in membrane morphology in CerS2 null mice may disrupt several trafficking pathways with various pathophysiological consequences.

In summary, the unexpected changes observed in the SL and long chain base profile of the CerS2 null mouse liver, and in the biophysical properties, implies much more complex pathways of regulation of SL metabolism than previously envisaged. Moreover, these changes cause a dramatic hepatopathy, as reported in the accompanying study (Pewzner-Jung et al.).

ACKNOWLEDGMENTS

We thank Reut Pienik and Hani Dekel for excellent technical assistance, and Dr. Yisrael Parmet for help with statistical analyses. This work was supported in part by the Israel Science Foundation (1404/07), the National Institutes of Health (GM076217) and by the Minerva Foundation.

REFERENCES

1. Fahy, E., Subramaniam, S., Brown, H. A., Glass, C. K., Merrill, A. H., Jr., Murphy, R. C., Raetz, C. R., Russell, D. W., Seyama, Y., Shaw, W., Shimizu, T., Spener, F., van Meer, G., VanNieuwenhze, M. S., White, S. H., Witztum, J. L., and Dennis, E. A. (2005) *J Lipid Res* **46**, 839-861
2. Hanada, K., Kumagai, K., Yasuda, S., Miura, Y., Kawano, M., Fukasawa, M., and Nishijima, M. (2003) *Nature* **426**, 803-809
3. Alvarez-Vasquez, F., Sims, K. J., Cowart, L. A., Okamoto, Y., Voit, E. O., and Hannun, Y. A. (2005) *Nature* **433**, 425-430
4. D'Angelo, G., Polishchuk, E., Tullio, G. D., Santoro, M., Campli, A. D., Godi, A., West, G., Bielawski, J., Chuang, C. C., van der Spoel, A. C., Platt, F. M., Hannun, Y. A., Polishchuk, R., Mattjus, P., and De Matteis, M. A. (2007) *Nature* **449**, 62-67
5. Futerman, A. H., and Hannun, Y. A. (2004) *EMBO Reps.* **5**, 777-782
6. Hannun, Y. A., and Obeid, L. M. (2008) *Nat Rev Mol Cell Biol* **9**, 139-150
7. Spiegel, S., and Milstien, S. (2003) *Nat Rev Mol Cell Biol* **4**, 397-407
8. Pruett, S. T., Bushnev, A., Hagedorn, K., Adiga, M., Haynes, C. A., Sullards, M. C., Liotta, D. C., and Merrill, A. H., Jr. (2008) *J Lipid Res* **49**, 1621-1639
9. Merrill, A. H., Jr., Wang, M. D., Park, M., and Sullards, M. C. (2007) *Trends Biochem. Sci.* **32**, 457-468
10. Pewzner-Jung, Y., Ben-Dor, S., and Futerman, A. H. (2006) *J. Biol. Chem.* **281**, 25001-25005
11. Venkataraman, K., Riebeling, C., Bodennec, J., Riezman, H., Allegood, J. C., Sullards, M. C., Merrill, A. H., Jr., and Futerman, A. H. (2002) *J Biol Chem* **277**, 35642-35649
12. Riebeling, C., Allegood, J. C., Wang, E., Merrill, A. H., Jr., and Futerman, A. H. (2003) *J Biol. Chem.* **278**, 43452-43459
13. Mizutani, Y., Kihara, A., and Igarashi, Y. (2005) *Biochem. J.* **390**, 263-271
14. Mizutani, Y., Kihara, A., and Igarashi, Y. (2006) *Biochem J* **398**, 531-538
15. Laviad, E. L., Albee, L., Pankova-Kholmyansky, I., Epstein, S., Park, H., Merrill, A. H., Jr., and Futerman, A. H. (2008) *J. Biol. Chem.* **283**, 5677-5684
16. Imgrund, S., Hartmann, D., Farwanah, H., Eckhardt, M., Sandhoff, R., Degen, J., Gieselmann, V., Sandhoff, K., and Willecke, K. (2009) *J. Biol. Chem.* **284**, 33549-33560
17. Hirschberg, K., Rodger, J., and Futerman, A. H. (1993) *Biochem. J.* **303**, 751-757
18. Lahiri, S., Lee, H., Mesicek, J., Fuks, Z., Haimovitz-Friedman, A., Kolesnick, R. N., and Futerman, A. H. (2007) *FEBS letters* **581**, 5289-5294
19. Futerman, A. H., Stieger, B., Hubbard, A. L., and Pagano, R. E. (1990) *J. Biol. Chem.* **265**, 8650-8657
20. Tani, M., Okino, N., Mitsutake, S., and Ito, M. (1999) *Biochem J* **258**, 746-749
21. Futerman, A. H., and Pagano, R. E. (1991) *Biochem. J.* **280**, 295-302
22. Meivar-Levy, I., Horowitz, M., and Futerman, A. H. (1994) *Biochem. J.* **303**, 377-382
23. Sullards, M. C., and Merrill, A. H., Jr. (2001) *Sci STKE* (http://stke.sciencemag.org/cgi/content/full/OC_sigtrans;2001/67/pl1) 2001, PL1
24. Merrill, A. H., Jr., Sullards, M. C., Allegood, J. C., Kelly, S., and Wang, E. (2005) *Methods* **36**, 207-224
25. Haynes, C. A., Allegood, J. C., Sims, K., Wang, E. W., Sullards, M. C., and Merrill, A. H., Jr. (2008) *J Lipid Res* **49**, 1113-1125
26. Brugger, B., Glass, B., Haberkant, P., Leibrecht, I., Wieland, F. T., and Krausslich, H. G. (2006) *Proc Natl Acad Sci U S A* **103**, 2641-2646
27. Brugger, B., Sandhoff, R., Wegehingel, S., Gorgas, K., Malsam, J., Helms, J. B., Lehmann, W. D., Nickel, W., and Wieland, F. T. (2000) *J Cell Biol* **151**, 507-518
28. Courchaine, A. J., Miller, W. H., and Stein, D. B., Jr. (1959) *Clin Chem* **5**, 609-614
29. Silva, L. C., Futerman, A. H., and Prieto, M. (2009) *Biophys J* **96**, 3210-3222
30. Stiban, J., Caputo, L., and Colombini, M. (2008) *J Lipid Res* **49**, 625-634
31. Pinto, S. N., Silva, L. C., de Almeida, R. F., and Prieto, M. (2008) *Biophys J* **95**, 2867-2879
32. Donella Deana, A., Mac Gowan, C. H., Cohen, P., Marchiori, F., Meyer, H. E., and Pinna, L. A. (1990) *Biochim Biophys Acta* **1051**, 199-202
33. Baykov, A. A., Evtushenko, O. A., and Avaeva, S. M. (1988) *Anal Biochem* **171**, 266-270
34. Friedrich, G., and Soriano, P. (1993) *Methods Enzymol* **225**, 681-701
35. Spassieva, S. D., Mullen, T. D., Townsend, D. M., and Obeid, L. M. (2009) *Biochem J* **415**, 203-213
36. Mukhopadhyay, A., Saddoughi, S. A., Song, P., Sultan, I., Ponnusamy, S., Senkal, C. E., Snook, C. F., Arnold, H. K., Sears, R. C., Hannun, Y. A., and Obeid, L. M. (2009) *Faseb J* **23**, 751-763
37. Marchesini, N., Osta, W., Bielawski, J., Luberto, C., Obeid, L. M., and Hannun, Y. A. (2004) *J Biol Chem* **279**, 25101-25111
38. Riley, R. T., and Voss, K. A. (2006) *Toxicol Sci* **92**, 335-345
39. Wang, E., Norred, W. P., Bacon, C. W., Riley, R. T., and Merrill, A. H. (1991) J. Biol. Chem. 266, 14486-14490
40. Kim, D. H., Yoo, H. S., Lee, Y. M., Kie, J. H., Jang, S., and Oh, S. (2006) J Toxicol Environ Health A 69, 2071-2082
41. Yang, J., and Miyazaki, N. (2003) Environmental Pollution 121, 345-347
42. Venkataraman, K., Riebeling, C., Bodennec, J., Riezman, H., Allegood, J. C., Sullards, M. C., Merrill, A. H., Jr., and Futerman, A. H. (2002) J. Biol. Chem. 277, 35642-35649
43. Bjorkqvist, Y. J., Brewer, J., Bagatolli, L. A., Slotte, J. P., and Westerlund, B. (2009) Biochim Biophys Acta 1788, 1310-1320
44. Doherty, G. J., and McMahon, H. T. (2009) Annual review of biochemistry 78, 857-902
45. Hatzakis, N. S., Bhatia, V. K., Larsen, J., Madsen, K. L., Bolinger, P. Y., Kunding, A. H., Castillo, J., Gether, U., Hedegard, P., and Stamou, D. (2009) Nat Chem Biol
46. Mukherjee, S., Soe, T. T., and Maxfield, F. R. (1999) J. Cell Biol. 144, 1271-1284
47. Tian, A., and Baumgart, T. (2009) Biophys J 96, 2676-2688
48. Rustom, A., Saffrich, R., Markovic, I., Walther, P., and Gerdes, H. H. (2004) Science 303, 1007-1010

FOOTNOTES

1 Abbreviations used:

CerS, ceramide synthase;
DPH, 1,6-diphenyl-1,3,5-hexatriene;
ESI-MS/MS, electrospray ionization-tandem mass spectrometry;
GlcCer, glucosylceramide;
GlcCerase, glucosylceramidase;
HexCer, hexosylceramide;
HPRT, hypoxanthine guanine phosphoribosyl transferase 1;
Rho, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl);
PC, phosphatidylcholine;
PE, phosphatidylethanolamine;
PP2A, protein phosphatase 2A;
SMase, sphingomyelinase;
SL, sphingolipid;
SM, sphingomyelin;
t-PnA, trans-parinaric acid;
WT, wild type.
### Table 1. Ceramide composition of CerS2 WT and null mouse liver.

Measurements were made by ESI-MS/MS. For day 0, n=4, and for all other time points, n=2. Statistical analysis was performed as indicated.

| Age (days) | C14   | C16   | C18:1 | C18   | C20   | C22   | C24:1 | C24   | C26:1 | C26   |
|------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 0          | 1.4 ± 0.1 | 80.4 ± 13.9 | 2.9 ± 1.3 | 14.0 ± 3.0 | 6.1 ± 0.9 | 36.6 ± 6.5<sup>a</sup> | 74.1 ± 13.4 | 63.5 ± 15.8 | 2.3 ± 0.3 | 1.7 ± 0.2 |
| 7          | 2.1 ± 0.4 | 73.5 ± 2.4<sup>a</sup> | 2.2 ± 1.6 | 6.2 ± 0.9 | 2.6 ± 3.7 | 12.7 ± 3.6<sup>d</sup> | 70.6 ± 18.0 | 48.4 ± 8.6 | 1.5 ± 0.2 | 1.2 ± 0.0 |
| 14         | 1.2 ± 0.1 | 23.1 ± 0.4<sup>b</sup> | 2.0 ± 0.3 | 6.2 ± 0.2 | 2.4 ± 0.2 | 10.8 ± 0.3<sup>c</sup> | 26.3 ± 0.8<sup>h</sup> | 30.8 ± 0.3 | 0.7 ± 0.2 | 0.8 ± 0.0 |
| 21         | 1.1 ± 0.3 | 29.9 ± 2.8 | 1.2 ± 0.1 | 8.7 ± 1.5 | 3.6 ± 0.1 | 20.3 ± 1.9<sup>f</sup> | 50.1 ± 1.8<sup>i</sup> | 34.9 ± 4.5 | 1.3 ± 0.1 | 1.0 ± 0.1 |
| 30         | 0.6 ± 0.1 | 17.5 ± 8.4 | 1.8 ± 0.0 | 4.8 ± 1.8 | 4.2 ± 0.1 | 37.2 ± 2.7<sup>g</sup> | 66.2 ± 45.4 | 36.4 ± 11.6 | 1.1 ± 0.2 | 1.2 ± 0.2 |
| 60         | 0.66    | 19.2 ± 12.1 | 0.99   | 3.1 ± 1.2 | 4.4 ± 2.0 | 48.7 ± 32.0 | 44.2 ± 22.8 | 52.6 ± 30.9 | 1.4 ± 0.2 | 1.2 ± 0.2 |
| 120        | 0.8 ± 0.1 | 17.7 ± 2.5 | 2.8 ± 0.3 | 3.4 ± 0.4 | 6.2 ± 0.3 | 60.4 ± 10.0 | 52.9 ± 8.8 | 42.2 ± 0.4 | 4.8 ± 1.5 | 2.0 ± 0.4 |

| Age (days) | C14   | C16   | C18:1 | C18   | C20   | C22   | C24:1 | C24   | C26:1 | C26   |
|------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 0          | 2.3 ± 0.5 | 173 ± 43 | 3.1 ± 0.8 | 9.8 ± 2.1 | 5.5 ± 1.0 | 4.2 ± 1.0 | 1.2 ± 0.3 | 3.0 ± 0.7 | 0.5 ± 0.2 | 0.8 ± 0.3 |
| 7          | 2.2 ± 0.2 | 101 ± 6 | 3.2 ± 0.3 | 2.8 ± 0.1 | 1.7 ± 0.1<sup>j</sup> | 1.7 ± 0.2 | 0.4 ± 2.5 | 1.2 ± 0.2 | 0.3 ± 0.2 | 0.2 ± 0.1 |
| 14         | 3.0 ± 0.6 | 101 ± 15 | 3.2 ± 0.5 | 5.8 ± 0.6 | 3.6 ± 1.0<sup>k</sup> | 3.0 ± 0.4 | 0.5 ± 0.0 | 1.3 ± 0.2 | 0.1 ± 0.0 | 0.2 ± 0.0 |
| 21         | 1.5 ± 0.6 | 104 ± 34 | 1.1 ± 0.7 | 4.2 ± 2.3 | 2.5 ± 1.4<sup>l</sup> | 2.5 ± 1.2 | 0.5 ± 0.1 | 1.2 ± 0.4 | 0.2 ± 0.0 | 0.2 ± 0.0 |
| 30         | 1.7 ± 0.3 | 108 ± 1 | 7.1 ± 0.2 | 10.9 ± 2.0 | 8.6 ± 1.0<sup>m</sup> | 7.6 ± 0.8 | 1.3 ± 0.1 | 2.5 ± 0.0 | 0.4 ± 0.2 | 1.3 ± 0.4 |
| 60         | 222 ± 170 | 15.1 ± 11.7 | 10.3 ± 8.0 | 9.7 ± 0.8 | 1.4 ± 0.8 | 3.0 ± 2.1 | 0.6 ± 0.2 | 1.1 ± 0.5 |
| 120        | 1.2 ± 0.4 | 186 ± 24 | 10.0 ± 0.8 | 11.0 ± 2.9 | 8.2 ± 1.1 | 10.3 ± 2.1 | 11.3 ± 7.6 | 6.9 ± 4.2 | 1.0 ± 0.3 | 1.4 ± 0.1 |

<sup>a</sup> versus <sup>b</sup>, <sup>h</sup> versus <sup>i</sup>, p<0.01; <sup>c</sup> versus <sup>d</sup>, <sup>e</sup> versus <sup>f</sup>, <sup>e</sup> versus <sup>g</sup>, <sup>j</sup> versus <sup>k</sup>, <sup>i</sup> versus <sup>m</sup>, p<0.05
Table 2. Total SL levels in WT and CerS2 null mouse liver. The data are the sum of the individual SL species shown in Tables 1 and Supplementary Tables 1 and 2. With the exception of ceramide on day 7, SM on day 120, and HexCer on days 7 and 14 (p<0.05), total ceramide, SM and HexCer levels between WT and CerS2 null mice were statistically indistinguishable.

| Age (days) | Ceramide | SM | HexCer |
|------------|----------|----|--------|
|            | pmol/mg of tissue |            |         |
| WT         | CerS2 null | WT | CerS2 null | WT | CerS2 null |
| 0          | 282 ± 49   | 203 ± 47   | 1153 ± 238 | 1402 ± 510 | 54.0 ± 9.6 | 52.8 ± 9.4 |
| 7          | 220 ± 30   | 114 ± 7    | 979 ± 215  | 707 ± 29   | 75.1 ± 3.5 | 48.0 ± 6.0 |
| 14         | 104 ± 0    | 121 ± 18   | 707 ± 109  | 827 ± 47   | 86.0 ± 11.1 | 50.1 ± 1.3 |
| 21         | 152 ± 12   | 117 ± 41   | 920 ± 16   | 1253 ± 304 | 99.3 ± 20.2 | 57.2 ± 15.7 |
| 30         | 171 ± 70   | 149 ± 4    | 859 ± 302  | 887 ± 42   | 40.1 ± 23.3 | 29.6 ± 1.6 |
| 60         | 175 ± 99   | 264 ± 199  | 780 ± 280  | 1093 ± 423 | 37.5 ± 19.5 | 48.9 ± 25.9 |
| 120        | 193 ± 23   | 247 ± 15   | 559 ± 62   | 1180 ± 69  | 19.1 ± 6.8  | 49.1 ± 15.7 |
Table 3. Long chain base composition of WT and CerS2 null mouse liver. Measurements were made by ESI-MS/MS. For day 0, n=4 and for all other time points, n=2. Differences between sphinganine levels in the WT versus the CerS2 null mice were statistically significant (p<0.05). The only other statistically significant differences in long chain base levels in WT versus CerS2 null mice were between sphingosine levels on days 30 and 120 (p<0.05), and in sphinganine 1-phosphate levels on days 120 (p<0.05).

| Age (days) | Sphingosine | Sphinganine | Sphingosine 1-phosphate | Sphinganine 1-phosphate |
|------------|-------------|-------------|-------------------------|-------------------------|
|            | WT          | CerS2 null  | WT                      | CerS2 null              |
|            | pmol/mg of tissue | pmol/mg of tissue | pmol/mg of tissue | pmol/mg of tissue |
| 0          | 31.8 ± 8.0  | 43.1 ± 15.0 | 7.0 ± 1.5               | 62.0 ± 22.1             | 0.2 ± 0.0 | 0.3 ± 0.1 | 0.9 ± 0.03 | 1.2 ± 0.6 |
| 7          | 13.1 ± 1.9  | 10.5 ± 1.0  | 4.7 ± 1.4               | 19.3 ± 0.7              | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.2 ± 0.1 |
| 14         | 9.7 ± 0.7   | 9.6 ± 0.6   | 2.9 ± 0.6               | 14.0 ± 3.3              | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 |
| 21         | 11.0 ± 1.7  | 16.3 ± 0.9  | 3.1 ± 0.1               | 26.8 ± 1.1              | 0.1 ± 0.0 | 0.1 ± 0.0 | 2.1 ± 0.0 | 0.1 ± 0.0 |
| 30         | 12.0 ± 0.9  | 20.7 ± 0.3  | 1.8 ± 0.5               | 54.3 ± 0.6              | 0.1 ± 0.0 | 0.1 ± 0.0 | 2.0 ± 0.0 | 0.1 ± 0.0 |
| 60         | 17.2 ± 5.7  | 38.7 ± 21.3 | 1.8 ± 0.3               | 94.4 ± 61.2             | 0.1         | 0.1         | 0.1         | 0.2         |
| 120        | 15.3 ± 2.2  | 32.8 ± 4.6  | 1.3 ± 0.4               | 73.3 ± 22.1             | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.4 ± 0.1 |

<sup>a</sup> versus <sup>b</sup>, <sup>b</sup> versus <sup>c</sup>, <sup>d</sup> versus <sup>e</sup>, p<0.05  
<sup>e</sup> versus <sup>f</sup>, p<0.01
FIGURE LEGENDS

Fig. 1. Generation and characterization of CerS2 null mice. (A) CerS2 null mice were created by crossing CerS2<sup>GT/+</sup> mice generated from a gene trap embryonic stem (ES) cell line (stock number 013236-UCD, Mutant Mouse Regional Resource Center). The upper panel shows the Rosafary gene trap retroviral vector used to generate CerS2 gene trap ES cells (34). A splice acceptor (SA) is located upstream to a β-geo gene (neo resistance + LacZ genes), which is followed by a polyadenylation sequence (pA). A selectable marker for hygromycin (hyg) resistance is expressed under the PGK promoter, followed by a splice donor (SD) sequence, which is within two <i>ftr</i> sites that are recognized by Flp recombinase. Elements of this vector are marked as grey boxes. The vector was inserted between exons 1 and 2 of the CerS2 gene (dashed lines). Exons are marked as black boxes and translational start and stop sites are shown as arrows. A spliced transcript of the WT locus is also shown. The lower panel shows the integration of the retroviral vector into the CerS2 gene, leading to generation of two transcripts. Transcript 1 is generated by splicing exon 1 of CerS2 (5'-UTR) into the Rosafary splice acceptor, resulting in expression of LacZ under the CerS2 promoter. The second transcript is driven by the PGK promoter of the hyg gene which is spliced into the second exon of CerS2. (B) CerS2 null mice do not express full-length transcript 2, demonstrated using primers that amplify the full length CerS2 transcript. (C) Weight of CerS2 null mice. * p<0.01, n = 5.

Fig. 2. Ceramide synthesis in CerS2 null mouse liver. The synthesis of C22- or C24-ceramides is highly reduced in the CerS2 null mouse; the insert shows C16-ceramide synthesis, which is unchanged. * p<0.05, n = 3.

Fig. 3. Ceramide and long chain base levels in CerS2 null mouse liver. Ceramide and sphinganine levels in CerS2 null mice versus age of the mice, measured by ESI-MS/MS. C24:1-ceramide is shown as an example of a very long acyl chain ceramide; data for the other very long acyl chain ceramides are in Table 1. For day 0, n = 4; for other time points, n = 2. * p<0.05; ** p<0.01.

Fig. 4. Levels of acyl CoAs. Levels of fatty acyl CoAs were analyzed by ESI-MS/MS in 30 day-old WT and CerS2 null mouse livers, and are shown as a ratio. n = 2. There were no statistically significant differences between WT and the CerS2 null mouse.

Fig. 5. Levels of CerS mRNA expression. mRNA was isolated from 1 month-old WT and CerS2 null mice. n = 5, * p<0.005.

Fig. 6. Activity of enzymes of ceramide metabolism. Activity was measured in liver homogenates (15-200 μg of protein) using fluorescent (NBD) lipid analogs. n = 3-4, * p<0.01.

Fig. 7. N-SMase activity. The left-hand panel shows RT-PCR of N-SMase 1 and 2 in WT versus CerS2 null mice at one month of age; HPRT was used as a loading control. The right-hand panel shows N-SMase activity. n = 3, * p<0.05.

Fig. 8. Glycerophospholipid levels. Glycerophospholipid levels were analyzed by nano-ESI-MS/MS in 30 day-old WT and CerS2 null mouse livers. (A) Glycerophospholipid levels are
shown as a percent of total glycerophospholipids. (n = 4 ± s.d.). (B) The distribution of fatty acid chains in PE is shown. n = 2. aPC, phosphatidylcholine (acyl chains); ePC, phosphatidylethanolamine (ethyl chains); PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol. (n = 3 ± s.d.). * p< 0.05.

Fig. 9. Cholesterol levels. (A) Total cholesterol levels were measured by the ferric chloride method for 14 day- and 30 day-old mouse liver (n = 3 ± s.d.). (B) Free (unesterified) cholesterol was measured by nano-ESI-MS/MS for 30 day-old liver (n = 4).

Fig. 10. Membrane biophysical properties and morphology of microsomal lipids. (A) Fluorescence anisotropy of three different probes in microsomal lipid extracts obtained from membranes of CerS2 null and WT mice at 30 days of age. n = 3, * p<0.001. (B) Confocal fluorescence microscopy showing the morphological features of microsomal lipid extracts from WT (upper panel) and CerS2 null mice (lower panel). Scale bar = 10 µm.

Fig. 11. Protein phosphatase 2A (PP2A) activity. PP2A activity was measured in homogenates from WT and CerS2 null mouse liver. n = 3.

Fig. 12. A pathway relational map showing differences in the relative amounts of sphingolipids in 60-day old CerS2 null versus WT mouse liver. The upper scheme (diagonal from lower left to upper right) summarizes the biosynthetic pathway of SLs beginning with condensation of serine and palmitoyl-CoA, to 3-ketosphinganine (3-KetoSa) then sphinganine (Sa), which is either N-acylated (to N-AcylSa, dihydroceramide, DHCer) or phosphorylated (Sa1P). N-AcylSa can be desaturated to N-acylsphingosine (N-AcylSo, Cer), and both DHCer and Cer can be converted into sphingomyelins (SM) or glycosylated to ceramide monohexoses (CMH). Also shown is the hydrolysis of Cer to So (upper right), which can undergo phosphorylation to S1P. Other intermediates and products of this scheme can also undergo turnover; some additional reactions (such as ceramide phosphate formation) are not shown since their amounts are small. The lower part of the figure depicts this pathway with all of the measured individual molecular subspecies as nodes that have been colored in the style of a 'heat map'. The colors display the fold-difference in the amounts of each compound in CerS2 null versus WT mouse liver (i.e., CerS2/WT) using the color scale shown at the lower right. Thus, the light blue circle at the bottom left is for palmitoyl-CoA, followed by 3-ketoSa (which is shown smaller and faded to reflect that the amounts in both samples were too low for detection, but there was no evidence for accumulation of this intermediate in CerS2 null mice), then by the node for Sa, which is deep red because Sa was substantially higher in the CerS2 null mice. Radiating from this hub are each N-acyl chain length metabolite of Sa (for examples, N-palmitoyl-Sa is labeled '16' and N-nervonoyl-Sa is labeled '24:1') (note that the former is elevated and the latter reduced), followed by the respective DHCer (left nodes) and DHCMH (right nodes). Dashed lines relate each N-acyl-chain length DHCer to the respective Cer, which can be converted to SM and CMH (outer nodes) or hydrolyzed to Sa (at the hub of this diagram). The phosphorylation products of Sa and So are also shown, labeled Sa1P and S1P. The data used for this figure are from Tables 1, 2 and 3. The layout of this scheme is from Ref. (9).
Fig. 1

**A**

![Diagram A](image1)

**B**

WT null +/-

CerS2 (1144bp)

1000bp

**C**

WT □ null

Weight (g)

|          | P0 | P7 | 5 months |
|----------|----|----|----------|
| WT       | 1.6| 0.8| 40       |
| null     | 1.2| 0.4| 30       |

Fig. 2

**CerS activity (pmol/mg/min)**

| Acyl chain length | C16 | C18 | C20 | C22 | C24 | C26 |
|-------------------|-----|-----|-----|-----|-----|-----|
| WT                | 10  | 10  |     |     |     |     |
| null              | 10  | 10  |     |     |     |     |

* indicates statistical significance.
Fig. 3

- **C24:1-ceramide**
  - WT
  - null

- **C16-ceramide**

- **Total ceramides**

- **Sphinganine**

```
Age (days)
0  7  14  21  30  60  120
```

Fig. 4

```
Fold change CerS2 null/WT
```

```
14:0  16:0  18:1
18:0  18:0-OH  20:0
22:0  24:1  24:0  26:1  26:0
```
Fig. 5

![Bar chart showing fold increase of CerS2 null/WT for CerS4, CerS5, and CerS6.]

Fig. 6

![Graphs showing enzyme activities for GlcCer synthase, SM synthase, Acid ceramidase, Alkaline ceramidase, and Neutral ceramidase across different ages (14 and 30 days)].

**GlcCer synthase**

**SM synthase**

**Acid ceramidase**

**Alkaline ceramidase**

**Neutral ceramidase**
Fig. 11

![Bar chart showing phosphate levels in WT and Null strains.](image)

- **Y-axis:** Phosphate (pmol/µg protein)
- **X-axis:** Days (14 and 30)
- **Legend:**
  - WT
  - Null

Fig. 12

![Biological pathway diagram.](image)

- **Nodes:** Various fatty acids and ceramides
- **Edges:** Metabolic pathways
- **Color scale:** Fold difference CerS2 null/WT
A critical role for ceramide synthase 2 in liver homeostasis: I. Alterations in lipid metabolic pathways
Yael Pewzner-Jung, Heyjung Park, Elad L. Laviad, Liana C. Silva, Sujoy Lahiri, Johnny Stiban, Racheli Erez-Roman, Britta Brugger, Timo Sachsenheimer, Felix Wieland, Manuel Prieto, Alfred H. Merrill, Jr. and Anthony H. Futerman

J. Biol. Chem. published online January 28, 2010

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