Modulation of Ceramide Synthase Activity via Dimerization

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Ceramide, the backbone of all sphingolipids, is synthesized by a family of ceramide synthases (CerS) that each use acyl-CoAs of defined chain length for N-acylation of the sphingoid base. CerS mRNA expression and enzymatic activity do not always correlate with the sphingolipid acyl chain composition of a particular tissue, suggesting post-translational mechanism(s) of regulation of CerS activity. We now demonstrate that CerS activity can be modulated by dimer formation. Under suitable conditions, high M₄ CerS complexes can be detected by Western blotting, and various CerS co-immunoprecipitate. CerS5 activity is inhibited in a dominant-negative fashion by co-expression with catalytically inactive CerS5, and CerS2 activity is enhanced by co-expression with a catalytically active form of CerS5 or CerS6. In a constitutive heterodimer comprising CerS5 and CerS2, the activity of CerS2 depends on the catalytic activity of CerS5. Finally, CerS dimers are formed upon rapid stimulation of ceramide synthesis by curcumin. Together, these data demonstrate that ceramide synthesis can be regulated by the formation of CerS dimers and suggest a novel way to generate the acyl chain composition of ceramide (and downstream sphingolipids), which may depend on the interaction of CerS with each other.

In mammals, the important signaling and structural lipid, ceramide (1), is synthesized by a family of ceramide synthases (CerS)², which each display a remarkable specificity toward the fatty acyl-CoA moiety used for N-acylation of the sphingoid long chain base (2–4). For instance, CerS2 uses C22-C24-acyl-CoAs (5), whereas CerS5 and -6 use C16-acyl-CoA (6, 7).

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| TABLE 1 | Primes used in this study |
|---------|--------------------------|
| **Construct/Primer** | **Primers** |
| CerS5-HA | CCGGTACCAGCTGGCAAGCGG |
| A | GGGATCCAGCTGGCAAGCGG |
| B | CCGGTACCAGCTGGCAAGCGG |
| CerS5:CerS5-HA | GGGATCCAGCTGGCAAGCGG |
| C | GGGATCCAGCTGGCAAGCGG |
| D | GGGATCCAGCTGGCAAGCGG |
| CerS5:TM:CerS5-HA | GGGATCCAGCTGGCAAGCGG |
| E | GGGATCCAGCTGGCAAGCGG |
| F | GGGATCCAGCTGGCAAGCGG |
| CerS5:TM:CerS5HH-HA | GGGATCCAGCTGGCAAGCGG |
| G | GGGATCCAGCTGGCAAGCGG |
| H | GGGATCCAGCTGGCAAGCGG |
| CerS5:TM:CerS2-HA | GGGATCCAGCTGGCAAGCGG |
| | GGGATCCAGCTGGCAAGCGG |
| CerS5:TM:CerS5HC-HA | GGGATCCAGCTGGCAAGCGG |
| | GGGATCCAGCTGGCAAGCGG |
| FLAG-CerS5ΔC32–392 | GGGATCCAGCTGGCAAGCGG |
| | GGGATCCAGCTGGCAAGCGG |
| | GGGATCCAGCTGGCAAGCGG |

*As described under “Experimental Procedures.”

Biolabs. Phusion DNA polymerase was from Finnzymes (Woburn, MA). Silica gel 60 TLC plates were from Merck. All solvents were of analytical grade and were purchased from Biolab (Jerusalem, Israel).

Cloning—Human CerS2 with an HA tag at the C terminus (CerS2-HA) was cloned as described (5). Primers for subcloning are given in Table 1. A CerS5-HA fragment was amplified by PCR using primers A and B. The PCR product was digested with Kpnl and EcoRI followed by ligation into a pcDNA3 vector. A CerS5 constitutive dimer was synthesized by Genscript (Piscataway, NJ) after modifying and optimizing the coding sequence for use with a mammalian expression system (supplemental Fig. 1). The sequence was subcloned into a pcDNA3 vector and an HA tag added at the 3' end (CerS5:CerS5-HA) using primers C and D (Table 1). Insertion of a transmembrane (TM) domain 3 between the two monomers of the dimer (CerS5:TM:CerS5-HA) was performed using primers E and F (supplemental Fig. 2). Mutagenesis of two His residues in CerS5 (CerS5:TM:CerS5-HA) was performed using primers G and H, and mutagenesis of H220A and H221A in the C-terminal monomer (CerS5:TM:CerS5HH-HA) using primers I and J (Table 1). Cloning of a CerS5:CerS2 heterodimer (CerS5:TM:CerS2-HA) was performed using primers K and L to amplify CerS2; a second PCR reaction was performed in which the CerS2 fragment replaced the 3' CerS5 fragment by restriction-free cloning (30). Mutagenesis of CerS5:TM:CerS2-HA was performed using primers E and F (supplemental Fig. 3). A truncated CerS5 chimera was cloned using primers M and N, followed by restriction cleavage using BamHI and EcoRI and ligation into a PCMV-2B vector (CerS5ΔC32–392). The sequences of all constructs were confirmed prior to use.

Cell Culture and Transfection—Human embryonic kidney (HEK) 293 and HepG2 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. Transfection with the various CerS constructs was performed using the PEI transfection reagent (Sigma). Twenty four hours after transfection, cells were collected by trypsinization and stored at −80 °C. Expression levels after transfection were confirmed by Western blotting.

CerS Assay—Cells were homogenized in 20 mm HEPES-KOH, pH 7.2, 25 mm KCl, 250 mm sucrose, and 2 mm MgCl2 containing a protease inhibitor mixture (Sigma). Protein was determined using the Bradford reagent (Bio-Rad). Homogenates were incubated with 0.25 μCi of [4,5-3H]sphinganine/15 μM sphinganine/20 μM defatted bovine serum albumin/50 μM fatty acyl-CoA for 20 min at 37 °C (5, 9, 31). Lipids were extracted and separated by TLC using chloroform:methanol:2 M sphinganine/20 M sphinganine/15 M sphinganine for 20 min. The reaction was terminated by addition of methanol. Lipids were extracted (33), subjected to alkaline hydrolysis (34) and separated by TLC using chloroform:methanol (50:3:5; v/v) as the developing solvent. [3H]-Labeled lipids were visualized using a phosphorimaging screen (Fuji, Tokyo, Japan), recovered from the TLC plates by scraping the silica directly into scintillation vials and quantified by liquid scintillation counting.

Metabolic Labeling—[3H](Dihydro)ceramide synthesis was analyzed in cultured cells as described (6) with some modifications. Briefly, cells were incubated with 0.33 μCi/ml [4,5-3H]sphinganine and 3.5 μM sphinganine for 20 min. The reaction was terminated by addition of methanol. Lipids were extracted (33), subjected to alkaline hydrolysis (34) and separated by TLC using chloroform:methanol (50:3:5; v/v) as the developing solvent. [3H]Ceramide was visualized and quantified as above.

ESI-MS/MS—Sphingolipid analysis by ESI-MS/MS was conducted using a PE-Sciex API 3000 triple quadrupole mass spec-
trometer and an ABI 4000 quadrupole-linear ion trap mass spectrometer (6, 35–38).

Cross-linking with Formaldehyde—HEK cells were transfected with FLAG-CerS (31). Twenty four hours after transfection, live cells were incubated with 1% formaldehyde for 15 min. Cross-linking was terminated by addition of 125 mM glycine for 5 min. Cells were collected and CerS dimer formation analyzed by Western blotting.

Co-immunoprecipitation of CerS—Cells were co-transfected with CerS5-HA and FLAG-CerS1–6. Twenty four hours after transfection, cells were collected, homogenized in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40) containing a protease inhibitor mixture, and centrifuged (2,700 × g, 4 °C, 10 min). Pellets (containing the non-soluble material) were discarded, and the supernatant was incubated for 5 h with 2 μl of an anti-FLAG antibody in an orbital shaker at 4 °C. Following incubation, 30 μl of agarose beads conjugated to protein A (Bio-vision, Milpitas, CA) was added overnight. Beads were collected subsequently by centrifugation and washed three times with lysis buffer. Proteins were removed from the beads by boiling in sample buffer (30 mM Tris-HCl, pH 6.8, 0.7 M glycerol, 35 mM sodium dodecyl sulfate, 0.1 M dithiothreitol, 0.14 mM bromphenol blue). Eluates were blotted with either anti-FLAG or anti-HA antibodies (1:10,000 dilution). A similar procedure was performed for endogenous CerS2 and CerS6 in HepG2 cells using anti-CerS2 and anti-CerS6 antibodies (both at a dilution of 1:1,000).

Western Blotting—Western blotting was performed as described (5, 9). When cross-linking was performed using formaldehyde prior to Western blotting, homogenates were not boiled in the sample buffer. When non-denaturing conditions were used, proteins were treated with a non-reducing sample buffer (30 mM Tris-HCl, pH 6.8, 0.7 M glycerol, 35 mM sodium dodecyl sulfate, 0.14 mM bromphenol blue) and heated to 50 °C for 5 min. Protein levels were quantified by densitometry.

CerS Reconstitution—CerS5-HA and CerS5:TM:CerS5-HA were reconstituted in liposomes by a similar method used previously for CerS5 (39). Briefly, following expression of either of the two constructs in HEK cells, protein was solubilized using 20 mM HEPES KOH, pH 7.4, 25 mM KCl, 250 mM sucrose, 2 mM MgCl₂, and 1% digitonin containing a protease inhibitor mixture. The proteins were incubated for 3 h with an anti-HA antibody followed by overnight incubation with agarose beads conjugated to protein A. The beads were washed three times with the solubilization buffer, and protein was eluted using 100 mM glycine, pH 2.5; 1 M Tris buffer, pH 11, was added immediately after elution. Eluted proteins were concentrated using centrifugal filter units (Millipore) and were reconstituted with 1,2-dioleoyl-sn-glycero-3-phosphocholine liposomes.

Immunofluorescence—The intracellular localization of various HA-tagged CerS constructs was determined by confocal laser scanning microscopy (6) using Bip as an endoplasmic reticulum marker.

RESULTS

CerS Form High Molecular Weight Complexes and Co-Immunoprecipitate—To determine whether CerS can oligomerize, HEK cells overexpressing each of the FLAG-tagged CerS were treated with formaldehyde for 15 min. Upon analysis by Western blotting, a high Mₙ band was detected, which was approximately twice the Mₙ of monomeric FLAG-CerS (Fig. 1B). Bands with a similar Mₙ could be detected when samples were treated with a non-reducing sample buffer and heated to 50 °C (Fig. 1C). No higher Mₙ bands could be detected in Western blots under denaturing conditions (Fig. 1A), consistent with earlier studies examining CerS expression under denaturing conditions.
CerS Interact in Vivo—To determine whether dimerization modulates CerS activity, HEK cells were co-transfected with various combinations of CerS. In the first experiment, cells were transfected with a catalytically active full-length CerS5-HA together with increasing amounts of a catalytically inactive FLAG-CerS5 (FLAG-CerS5\(^{AC332–392}\)) lacking the last putative transmembrane domain. The two proteins were co-immunoprecipitated (data not shown) using both anti-FLAG and anti-HA antibodies for immunoprecipitation. CerS5-HA activity decreased upon transfection with increasing amounts of FLAG-CerS5\(^{AC332–392}\) (Fig. 3), demonstrating that FLAG-CerS5\(^{AC332–392}\) acts in a dominant-negative fashion to inhibit the activity of full-length CerS5-HA.

Next, HEK cells were transfected with FLAG-CerS2 and CerS5-HA or CerS6-HA and assayed for CerS2 activity using C\(_{22}\)-CoA. Of all of the CerS, CerS2 displays the lowest activity of full-length CerS5-HA or CerS6-HA and assayed for CerS2 activity using C\(_{22}\)-CoA. The first dimer that was generated, consisting of two monomers of CerS5 directly attached to each other via the N terminus and reconstitution in 1,2-dioleoyl-sn-glycero-3-phosphocholine liposomes, CerS5:TM:CerS5-HA displayed keratin 50% (Fig. 5\(B\)).

**CerS Activity Is Modified in CerS-constitutive Dimers**—To directly determine the regulation of CerS activity by dimer formation, a series of constitutive CerS dimers were generated. The first dimer that was generated, consisting of two monomers of CerS5 directly attached to each other via the N terminus of one monomer and the C terminus of the other monomer (CerS5:TM:CerS5-HA), did not display catalytic activity (Fig. 5\(A\)). However, insertion of a transmembrane domain between the two monomers (CerS5:TM:CerS5-HA) generated a dimer with somewhat higher levels of activity than the CerS5-HA monomer (Fig. 5\(A\)). After immunoprecipitation and reconstitution in 1,2-dioleoyl-sn-glycero-3-phosphocholine liposomes, CerS5:TM:CerS5-HA displayed ~6-fold higher activity than CerS5-HA (Fig. 5\(B\)). As expected, mutation of two residues (H220A and H221A) involved in catalytic activity (CerS5\(^{H220A,221A}\):TM:CerS5\(^{H220A,221A}\)-HA) completely abrogated CerS5 activity in the constitutive dimer (Fig. 5\(A\)).
CerS5:CerS2 heterodimers were also generated and assayed for activity using C16-CoA (for CerS5) and C22-CoA (for CerS2). CerS5:TM:CerS2-HA displayed slightly more activity using C16-CoA as substrate than CerS5, but remarkably, CerS2 activity measured using C22-CoA was elevated by ~3-fold (Fig. 6). This increase in CerS2 activity was abolished using a non-catalytically active form of CerS5 in the constitutive dimer (CerS5HH:TM:CerS2-HA), demonstrating that optimal CerS2 activity depends on an interaction with a catalytically active form of CerS5.

To exclude the possibility that the modulation of CerS2 activity by CerS5 is due to altered subcellular localization, the intracellular localization of CerS5:TM:CerS5-HA and CerS5:TM:CerS2-HA were compared with that of CerS5. No differences in intracellular localization were detected (Fig. 7).

CerS Dimers Are Formed Rapidly upon Stimulation of Ceramide Synthesis—Finally, we examined the relevance of CerS dimer formation for ceramide synthesis in vivo. HEK cells overexpressing FLAG-CerS2 or FLAG-CerS5 were incubated with curcumin, which rapidly activates ceramide synthesis via the de novo pathway (19, 23). Within 5 min after curcumin treatment, high Mr bands corresponding to FLAG-CerS2 and FLAG-CerS5 dimers could be detected (data not shown), which became more pronounced after 30 min and remained elevated for up to 180 min (Fig. 8A). Next, ceramide synthesis was measured in non-transfected curcumin-treated cells, in which a significant increase of [4,5-3H]sphinganine incorporation into [3H]ceramide (Fig. 8B) was observed. Furthermore, levels of various ceramide species (particularly C20-, C18-, C26:1) were elevated after curcumin treatment (Fig. 8) consistent with the idea that curcumin treatment induces formation of both homo- and heterodimers of CerS5 and CerS2.
heterodimers. Together with the data showing dimer formation in overexpressing cells, these results imply that curcumin elevates ceramide synthesis by the rapid formation of CerS dimers.

DISCUSSION

In the current study, a variety of techniques were used to demonstrate that CerS activity is regulated by dimer formation. This suggestion is consistent with earlier studies demonstrating co-immunoprecipitation of some CerS in both mammalian cells (22) and in yeast (41) and with a kinetic study examining the mode of inhibition of CerS by FTY720 (42). In the latter, the uncompetitive inhibition of sphinganine binding to CerS by FTY720 suggested that there may be two sphinganine-binding sites that act allosterically with respect to one another, or that CerS may form dimers that interact allosterically.

The current study is consistent with a model in which the binding of a substrate to one monomer of the dimer allosteri-
trans manner.

Irrespective of the precise details of the mode of interaction of the CerS in the dimer, or of the order and location of the substrate binding sites, the finding that this interaction modulates CerS activity is of great importance for understanding ceramide and CerS biology. Previously, it had been assumed that the acyl chain composition of ceramide (and consequently of downstream sphingolipids) is likely to simply reflect the CerS expression pattern in a particular tissue (see Ref. 5 for details). However, this is now unlikely to be the major determinant of acyl chain composition, which will rather depend on the combinatorial expression of the different CerS and their ability to interact. Moreover, CerS splice variants, many of which are unlikely to have activity by themselves (2), could act in a dominant-negative manner to regulate CerS activity. Specifically, we suggest that maximal CerS2 activity depends on its interaction with other CerS; of all of the CerS, CerS2 has the lowest activity in vitro (43) but has the widest tissue distribution and is the main CerS responsible for synthesis of very long acyl chain (C_{22-24}) ceramides (5).

The yeast CerS, Lac1 and Lag1, form a high $M_c$ complex, but the role of possible homo- or heterodimeric Lag1-Lac1 complexes in regulating ceramide synthesis has not been evaluated. Moreover, it is unlikely that yeast require the same finesse of CerS regulation because yeast ceramides only contain one kind of fatty acid (namely $C_{16}$) compared with the wide variety of fatty acids found in mammalian cells. However, levels of ceramide synthesis, in both yeast and mammals, are likely to be regulated by dimer formation since the activity of a CerS5 homodimer is somewhat higher than that of the monomer.

Molecular and structural details of how the CerS interact as either homo- or heterodimers in the endoplasmic reticulum membrane are lacking. The constitutive dimers generated in this study do not help in this regard but may give clues about the topology of the CerS because an additional transmembrane domain was required for activity of the constitutive dimers; this may suggest that the CerS contain an odd number of transmembrane domains (i.e. Ref. 5, and see Ref. 7) in which the N and C termini are located on opposite sides of the endoplasmic reticulum. Indeed, in the endoplasmic reticulum, CerS may exist in equilibrium between monomers and dimers, and the formation/dissociation of the dimers might be a major way of regulating their activity. Dimers are formed relatively quickly, and this mechanism provides a rapid way to increase levels of ceramide synthesis under various physiological conditions. This could be particularly important in such cases where de novo ceramide synthesis plays a role in one or other signaling pathway and thus needs to be activated by a rapid, post-translational mechanism.

In summary, we suggest a novel means of modulating CerS activity by dimer formation and dissociation. Whether other enzymes of sphingolipid metabolism are also regulated by such means is not known (with the exception of sphingosine palmitoyl transferase, where heterodimer formation is required for activity (32)). CerS dimer formation adds a new element to our understanding of how ceramide synthesis is regulated.

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REFERENCES

1. Merrill, A. H., Jr. (2011) Sphingolipid and glycosphingolipid metabolic pathways in the era of sphingolipidomics. Chem. Rev. 111, 6387–6422
2. Pewzner-Jung, Y., Ben-Dor, S., and Futerman, A. H. (2006) When do Lasses (longevity assurance genes) become CerS (ceramide synthases)? Insights into the regulation of ceramide synthesis. J. Biol. Chem. 281, 25001–25005
3. Mullen, T. D., Hannun, Y. A., and Obeid, L. M. (2012) Ceramide synthases at the centre of sphingolipid metabolism and biology. Biochem. J. 441,
Modulation of Ceramide Synthase Activity via Dimerization

789–802

4. Levy, M., and Futerman, A. H. (2010) Mammalian ceramide synthases. *ILRMB Life* 62, 347–356

5. Laviad, E. L., Albee, L., Pankova-Kholmynsky, I., Epstein, S., Park, H., Merrill, A. H., Jr., and Futerman, A. H. (2008) Characterization of ceramide synthase 2: Tissue distribution, substrate specificity, and inhibition by sphingosine 1-phosphate. *J. Biol. Chem.* 283, 5677–5684

6. Riebeling, C., Allegood, J. C., Wang, E., Merrill, A. H., Jr., and Futerman, A. H. (2003) Two mammalian longevity assurance gene (LAG1) family members, trh1 and trh4, regulate dihydroceramide synthesis using different fatty acyl-CoA donors. *J. Biol. Chem.* 278, 43452–43459

7. Mizutani, Y., Kihara, A., and Igarashi, Y. (2005) Mammalian Lass6 and its related family members regulate synthesis of specific ceramides. *Biochem. J.* 390, 263–271

8. Kageyama-Yahara, N., and Riezman, H. (2006) Transmembrane topology of ceramide synthase in yeast. *Biochem. J.* 398, 585–593

9. Tidhar, R., Ben-Dor, S., Wang, E., Kelly, S., Merrill, A. H., Jr., and Futerman, A. H. (2012) Acyl chain specificity of ceramide synthases is determined within a region of 150 residues in the Tram-Lag-CLN8 (TLC) domain. *J. Biol. Chem.* 287, 3197–3206

10. Mesika, A., Ben-Dor, S., Laviad, E. L., and Futerman, A. H. (2007) A new functional motif in Hox domain-containing ceramide synthases: identification of a novel region flanking the Hox and TLC domains essential for activity. *J. Biol. Chem.* 282, 27366–27373

11. 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

12. Denic, V., and Weissman, J. S. (2007) A molecular caliper mechanism for determining very long chain fatty acid length. *Cell 130*, 663–677

13. Mondal, M. S., Ruiz, A., Hu, J., Bok, D., and Rando, R. R. (2001) Two histidine residues are essential for catalysis by lecithin retinol acyl transferase. *FEBS Lett.* 498, 14–18

14. Ben-David, O., Peszewn-Jung, Y., Brenner, O., Laviad, E. L., Kogot-Levin, A., Weissberg, I., Biton, I. E., Pleinik, R., Wang, E., Kelly, S., Alroy, J., Raas- Rothschield, A., Friedman, A., Brügger, B., Merrill, A. H., Jr., and Futerman, A. H. (2011) Encephalopathy caused by ablation of very long acyl chain ceramide synthase 2, 5, and 6 confer distinct roles in radiation-induced apoptosis in HeLa cells. *Cell. Signal.* 22, 1300–1307

15. Moustawi, M., Assi, K., Gómez-Muñoz, A., and Salh, B. (2006) Curcumin mediates ceramide generation via the de novo pathway in colon cancer cells. *Carcinogenesis* 27, 1636–1644

16. Basnakian, A. G., Ueda, N., Hong, X., Galitovsky, V. E., Yin, X., and Shah, D. (2010) Apoptosis: An alternative mechanism for generating death signals. *Kolesnick, R.* (1995) Ceramide synthase mediates daunorubicin-induced apoptosis. *Am. J. Physiol. Renal Physiol.* 278, F308–314

17.货车, R. C., Haimovitz-Friedman, A., Persaud, R. S., McLoughlin, M., Ehleiter, D., Zhang, N., Gatei, M., Lavin, M., Kolesnick, R., and Fuks, Z. (1999) Ataxia telangiectasia-mutated gene product inhibits DNA damage-induced apoptosis via ceramide synthase. *J. Biol. Chem.* 274, 17908–17917

18. Mesicek, J., Lee, H., Feldman, T., Jiang, X., Skobeleva, A., Berdyshev, E. V., Haimovitz-Friedman, A., Fuks, Z., and Kolesnick, R. (2010) Ceramide synthases 2, 5, and 6 confer distinct roles in radiation-induced apoptosis in HeLa cells. *Cell. Signal.* 22, 1300–1307

19. Panjarian, S., Kozhaya, L., Arayssi, S., Yehia, M., Bielawski, J., Bielawska, A., Usta, J., Hannun, Y. A., Obeid, L. M., and Dbaibo, G. S. (2008) *De novo* N-palmitoylsphingosine synthesis is the major biochemical mechanism of ceramide accumulation following p53 up-regulation. *Prostaglandins Other Lipid Mediat.* 86, 41–48

20. Yu, J., Novgorodov, S. A., Chudakov, D., Zhu, H., Bielawski, A., Bielawski, J., Obeid, L. M., Kindy, M. S., and Gudz, T. I. (2007) INK3 signaling pathway activates ceramide synthase leading to mitochondrial dysfunction. *J. Biol. Chem.* 282, 25940–25949

21. Sridivi, P., Alexander, H., Lavidi, E. L., Peszewn-Jung, Y., Hannink, M., Futerman, A. H., and Alexander, S. (2009) Ceramide synthase 1 is regulated by proteasomal mediated turnover. *Biochim. Biophys. Acta* 1793, 1218–1227

22. Mizutani, Y., Kihara, A., and Igarashi, Y. (2006) LASS3 (longevity assurance homologue 3) is a mainly testis-specific (dihydro)ceramide synthase with relatively broad substrate specificity. *Biochem. J.* 398, 531–538

23. Hirschberg, K., Rodger, J., and Futerman, A. H. (1993) The long chain sphingoid base of sphingolipids is acylated at the cytosolic surface of the endoplasmic reticulum in rat liver. *Biochem. J.* 290, 751–757

24. van den Ent, F., and Löwe, J. (2006) RF cloning: A restriction-free method for inserting target genes into plasmids. *Biochem. Biophys. Methods* 67, 67–74

25. Lahiri, S., Lee, H., Mesicek, J., Fuks, Z., Haimovitz-Friedman, A., Kolesnick, R. N., and Futerman, A. H. (2007) Kinetic characterization of mammalian ceramide synthases: Determination of Km values towards sphingosine. *FEBS Lett.* 581, 5289–5294

26. Hanada, K. (2003) Sphingomyelin synthase, a key enzyme of sphingolipid metabolism. *Biochim. Biophys. Acta* 1632, 16–30

27. Felch, J., Lees, M., and Sloane-Stanley, G. H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226, 497–509

28. Zhao, L., Spassieva, S. D., Lucius, T. J., Shultz, L. D., Shick, H. E., Macklin, W. B., Hannun, Y. A., Obeid, L. M., and Ackerman, S. L. (2011) A deficiency of ceramide biosynthesis causes cerebellar purkinje cell neurodegeneration and lipofuscin accumulation. *PLoS Genet.* 7, e1002063

29. Venkataraman, K., Riebeling, C., Bodennec, J., Riezman, H., Allegood, J. C., Sullards, M. C., Merrill, A. H., Jr., and Futerman, A. H. (2002) Upstream of growth and differentiation factor 1 (ugfl), a mammalian homolog of the yeast longevity assurance gene 1 (LAG1), regulates N-stearoyl-sphinganine (C18-(dihydro)ceramide) synthesis in a fumonisin B1-independent manner in mammalian cells. *J. Biol. Chem.* 277, 35642–35649

30. Sullards, M. C., and Merrill, A. H., Jr. (2001) Analysis of sphingosine 1-phosphate, ceramides, and other bioactive sphingolipids by high-performance liquid chromatography-tandem mass spectrometry. *Sci. STKE* 2001, pl1

31. Merrill, A. H., Jr., Sullards, M. C., Allegood, J. C., Kelly, S., and Wang, E. (2005) Sphingolipidomics: High-throughput, structure-specific, and quantitative analysis of sphingolipids by liquid chromatography tandem mass spectrometry. *Methods* 36, 207–224

32. Shaner, R. L., Allegood, J. C., Park, H., Wang, E., Kelly, S., Hayes, C. A., Sullards, M. C., and Merrill, A. H., Jr. (2009) Quantitative analysis of sphingolipids using triple quadrupole and quadrupole linear ion trap mass spectrometers. *J. Lipid Res.* 50, 1692–1707

33. Lahiri, S., and Futerman, A. H. (2005) LASS5 is a bona fide dihydroceramide synthase that selectively utilizes palmitoyl-CoA as acyl donor. *J. Biol. Chem.* 280, 33735–33738

34. Peszewn-Jung, Y., Park, H., Lavidi, E. L., Silva, L. C., Lahiri, S., Stiban, J., Erez-Roman, R., Brügger, B., Sachsenheimer, T., Wieland, F., Prieto, M., Merrill, A. H., Jr., and Futerman, A. H. (2010) A critical role for ceramide
synthase 2 in liver homeostasis: I. alterations in lipid metabolic pathways. 
*J. Biol. Chem.* **285**, 10902–10910

41. Vallée, B., and Riezman, H. (2005) Lip1p: A novel subunit of acyl-CoA ceramide synthase. *EMBO J.* **24**, 730–741

42. Lahiri, S., Park, H., Laviad, E. L., Lu, X., Bittman, R., and Futerman, A. H. (2009) Ceramide synthesis is modulated by the sphingosine analog FTY720 via a mixture of uncompetitive and noncompetitive inhibition in an acyl-CoA chain length-dependent manner. *J. Biol. Chem.* **284**, 16090–16098

43. Lahiri, S., and Futerman, A. H. (2007) The metabolism and function of sphingolipids and glycosphingolipids. *Cell Mol. Life Sci.* **64**, 2270–2284