Fatty acid transport protein 2 interacts with ceramide synthase 2 to promote ceramide synthesis

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Dihydroceramide is a lipid molecule generated via the action of (dihydro)ceramide synthases (CerSs), which use two substrates, namely sphinganine and fatty acyl-CoAs. Sphinganine is generated via the sequential activity of two integral membrane proteins located in the endoplasmic reticulum. Less is known about the source of the fatty acyl-CoAs, although a number of cytosolic proteins in the pathways of acyl-CoA generation modulate ceramide synthesis via direct or indirect interaction with the CerSs. In this study, we demonstrate, by proteomic analysis of immunoprecipitated proteins, that fatty acid transporter protein 2 (FATP2) (also known as very long-chain acyl-CoA synthetase) directly interacts with CerS2 in mouse liver. Studies in cultured cells demonstrated that other members of the FATP family can also interact with CerS2, with the interaction dependent on both proteins being catalytically active. In addition, transfection of cells with FATP1, FATP2, or FATP4 increased ceramide levels although only FATP2 and 4 increased dihydroceramide levels, consistent with their known intracellular locations. Finally, we show that lipofemerta, an FATP2 inhibitor which is believed to directly impact tumor cell growth via modulation of FATP2, decreased de novo dihydroceramide synthesis, suggesting that some of the proposed therapeutic effects of lipofemerta may be mediated via (dihydro)ceramide rather than directly via acyl-CoA generation. In summary, our study reinforces the idea that manipulating the pathway of fatty acyl-CoA generation will impact a wide variety of downstream lipids, not least the sphingolipids, which utilize two acyl-CoA moieties in the initial steps of their synthesis.

The sphingolipid (SL) biosynthetic pathway begins with the condensation of an amino acid, normally serine, with a fatty acyl-CoA, normally palmitoyl CoA, to generate 3-ketosphinganine, followed by the action of 3-ketosphinganine reductase to generate sphinganine (1). The latter is then N-acylated by (dihydro)ceramide synthases (CerSs), which can also N-acylate the sphingosine that is generated in the recycling pathway (2). Thus, dihydroceramide generation requires the input of two acyl-CoAs. Whereas serine palmitoyltransferase has a relativity-restricted ability to use acyl-CoAs of different chain lengths (3), the CerS can use a much broader variety of acyl-CoAs (4). For instance, of the six mammalian CerS (5), CerS2 uses very long-chain (VLC) acyl-CoAs (i.e., C22–26) (6), whereas CerS5 and 6 use long-chain (LC) acyl-CoAs (i.e., C16) (7, 8).

Unexpected and complex modes of regulation of this pathway have been uncovered over the past few years. Thus, CerS can be regulated via dimerization (9) or phosphorylation (10), or via interactions with other proteins, including a number related to either the elongation of acyl-CoAs, such as ELOVL1 (11), the supply of acyl-CoAs, such as acyl-CoA-binding protein (12), and generation of LC-acyl-CoAs via ACSL5 and DGAT2, which are subsequently incorporated into 1-O-acyl-ceramides (13).

We now identify an additional protein family which directly interacts with the CerSs and specifically with CerS2, namely VLC–acyl-CoA synthetase, encoded by Slec27a2, also known as fatty acid transport protein 2 (FATP2). FATP2 is one of the six members of the FATP family (14), bifunctional proteins that mediate the ATP-dependent import of LC-fatty acids into the cell (15, 16) as well as catalyzing the conversion of LC- and VLC-fatty acids to acyl-CoA thioesters (17). FATPs can be distinguished from LC-acyl-CoA synthetases by their VLC–fatty acyl-CoA synthetase activity (17–19). Furthermore, members of the FATP family differ in their tissue distribution, levels of expression in different cell types, and in subcellular localization (20).

We now demonstrate that the FATPs directly interact with the CerS and mainly focus on the interaction between FATP2 and CerS2, which depends on the catalytic activity of both. Moreover, inhibition of FATP2 using lipofemerta decreases ceramide synthesis, suggesting that some of the effects ascribed to FATP2 inhibition in various cancers (21, 22) may actually be mediated via the ceramide pathway.

Results

FATPs interact with CerS2 and modulate cellular SL levels

To identify proteins that may interact with CerS2, we analyzed proteins that were immunoprecipitated from mouse liver homogenates using an anti-CerS2 antibody. Three
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hundred seventy-nine proteins were identified by LC-MS/MS (Table S1). Comparison with proteins immunoprecipitated from homogenates obtained from CerS2 null mouse liver (23) revealed 15 proteins that were differentially immunoprecipitated >1.5-fold (Table 1). Among these were CerS2, as expected, and a number of proteins which do not seemingly appear to be related to the SL or associated metabolic pathways, such as glutamine synthetase and retinal dehydrogenase. These latter proteins may either interact nonspecifically with CerS2 upon immunoprecipitation after homogenization in 1% digitonin or may be associated with the CerS via as yet unknown mechanisms. However, a number of the immunoprecipitated proteins appeared to be bone fide candidates to interact with the SL metabolic pathway, including FATP2.

Table 1
Putative CerS2-interacting proteins identified by LC-MS/MS

| Protein ID | Protein Description | Gene | Ratio WT:CerS2 null | p value |
|------------|---------------------|------|---------------------|---------|
| Q92424     | Ceramic acid synthase 2 | CerS2 | 227 | <0.001 |
| P15105     | Glutamine synthetase | Glul | 103 | <0.01 |
| Q91914     | Formimidoyltransferase-cyclodeaminase | Ftdc | 8.52 | <0.05 |
| O35488     | Very long-chain acyl-CoA synthetase | Scs2a2 | 6.58 | <0.05 |
| P24549     | Retinal dehydrogenase 1 | Aldh1a1 | 5.22 | <0.01 |
| P16015     | Carbonyl reductase 3 | Ca3 | 4.13 | <0.01 |
| P33267     | Cytochrome P450 2F2 | Cyp2f2 | 3.68 | <0.05 |
| P17363     | Selenium-binding protein 1/Selenium-binding protein 2 | Selbp1/Selbp2 | 3.07 | <0.01 |
| O35490     | Betaine–homocysteine 5-methyltransferase 1 | Bhmt | 2.88 | <0.05 |
| O08709     | Peroxiredoxin-6 | Prdx6 | 2.49 | <0.05 |
| P00329     | Alcohol dehydrogenase 1 | Adh1 | 2.25 | <0.05 |
| Q99114     | Maleylacetoacetate isomerase | Gsat1 | 2.24 | <0.05 |
| P04869     | Hydroxymethylglutaryl-CoA synthase, mitochondrial | Hmgcs2 | 2.15 | <0.005 |
| P17751     | Triphosphosphate isomerase | Tap1 | 2.15 | <0.01 |
| Q8BBW1     | 3-ketoacyl-CoA thiolase, mitochondrial | Aca2 | 1.72 | <0.05 |

Proteins were immunoprecipitated from WT mouse liver homogenates using an anti-CerS2 antibody and compared to those immunoprecipitated from CerS2 null mouse liver homogenates. Three mice were used per group. Very long-chain acyl-CoA synthetase is also known as fatty acid transporter protein 2. A complete list of all immunoprecipitated proteins is given in Table S1 with the 15 differentially immunoprecipitated proteins indicated. *It was not possible to distinguish between Selbp1/2 and Selbp2 by LC-MS/MS.

Catalytically active CerS and FATP are required for their interaction

Based on the assumption that FATPs interact with CerS in order to supply the acyl-CoAs needed for ceramide synthesis, we examined the extent of coimmunoprecipitation of the two proteins upon inhibition of their activity. First, Hek293T or HepG2 cells (which contain high levels of FATP2 (24)) were transfected with FATP2-Flag and treated with the CerS inhibitor, fumonisin B1 (FB1) (25). The extent of coimmunoprecipitation of CerS2 with FATP2 decreased in both cell types by ~75% upon incubation with FB1 (Fig. 3A). Next, we compared the extent of coimmunoprecipitation using a splice variant of FATP2, FATP2b, which lacks exon 3 and is unable to synthesize VLC–acyl-CoAs even though it maintains its ability to import free fatty acids (26). This splice variant also showed significantly less binding (~70%) to CerS2 (Fig. 3B). Together, these two results are consistent with the notion that the interaction of CerS2 and FATP2 depends on the maintenance of catalytic activity, either via the supply of VLC–acyl-CoAs and their utilization by CerS for the N-acylation of sphinganine, although we cannot exclude the possibility that FATP2 binds to CerS2 via the sequence encoded by exon 3.

The C-terminus of CerS2 may regulate the interaction with FATP2

To attempt to determine which structural domains of CerS2 are involved in the binding to FATP2, we took advantage of a series of CerS2 mutations and deletion constructs (10, 27, 28). Deletion of the Hox-like domain (27) had no effect on the extent of immunoprecipitation of...
CerS2 with FATP2 in CerS2−/− HEK cells and neither did replacement of the two putative active site residues (HH212–213AA) (28) (Fig. 4). The reason that incubation with FB1 decreases the extent of immunoprecipitation between CerS2 and FATP2, whereas deletion of the putative active site residues does not, is currently unknown. However, it should be noted that FB1 inhibits CerS by competition with sphinganine (due to their structural similarities), perhaps inducing a structural, kinetic, or allosteric change which does not occur upon deletion of the putative active site histidine residues (29).

Unexpectedly, deletion of the C-terminus, deletion of a novel heptapeptide found uniquely in the C-terminus of CerS (30), or replacement of four putative phosphorylation sites (10) in the C-terminus increased the extent of coimmunoprecipitation (Fig. 4). The latter is of particular interest since phosphorylation of these four residues has been implicated in the regulation of de novo ceramide synthesis (10, 31, 32), and our
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Figure 2. Cellular dihydroceramide and ceramide levels are elevated upon transfection with FATPs. Hek293T cells were transfected with FATP1, 2, or 4 for 48 h prior to analysis of (A) dihydroceramide (d18:0) and (B) ceramide (d18:1) levels by LC-MS/MS. The data are means ± S.E.M., n = 3. *p < 0.05; **p < 0.01; ***p < 0.001. FATP, fatty acid transport protein; HEK, human embryonic kidney.

data imply that phosphorylation may actually regulate the mode of interaction of CerS with FATP2, which could directly impinge upon CerS activity. Alternatively, the C-terminus of CerS appears to be unstructured in most structural prediction programs, and it may be that mutating or deleting this region nonspecifically enhances the accessibility of the CerS to interact with FATP. However, none of the five mutations or deletion constructs reduced the extent of coimmunoprecipitation with CerS2, such that further work is required to unambiguously determine the precise structural features by which CerS and FATPs interact.

An FATP2 inhibitor down-regulates de novo dihydroceramide synthesis

Lipofermata is an inhibitor of FATP2 (33) and is believed to directly impact tumor cell growth via inhibition of FATP2 and to also prevent lipotoxicity induced by fatty acids (21, 22, 34, 35). We examined whether the inhibition of FATP2 by lipofermata affects the rate of dihydroceramide synthesis in HepG2 cells by metabolic labeling using NBD-Sphinganine, which is converted to NBD-dihydroceramide. Levels of both VLC- and LC-NBD-dihydroceramide synthesis decreased in a concentration-dependent manner (Fig. 5A) upon incubation with lipofermata. The levels of VLC-NBD-dihydroceramide synthesis also decreased in a time-dependent manner (Fig. 5B), but reverted to control levels after 4 h incubation, suggesting that the efficacy of lipofermata decreases after longer times of incubation. This data implies that any potential therapeutic effects of lipofermata may not only be related to its ability to inhibit FATP2, but may also be affected by the levels of other lipids whose levels can be modulated by FATP2, among them (dihydro)ceramide, a critical lipid involved in regulating normal cell and tumor cell growth (36).

Discussion

In the current study, we extend the number of proteins that have been shown to interact with the CerSs by demonstrating that the six isoforms of FATP interact with CerS2 to some extent. While this interaction appears to be generic, suggesting that common structural features determine the mode of interaction, the specific interaction between CerS2 and FATP2 appears most relevant since both proteins are located in the ER (6, 37), and both are related to either the generation or utilization of VLC-acyl-CoAs. This is consistent with lipidomics analysis in which FATP2 (and FATP4) directly impacted the de novo SL synthesis pathway (note that FATP4 is also located in the ER), whereas FATP1 did not impact de novo synthesis but did affect SLs associated with the recycling pathway, consistent with the suggested subcellular localization of FATP1 in the plasma membrane (38, 39), perhaps suggesting that FATP family members need to be adjacent to the CerS to allow their interaction in cells. Thus, although CerS2 may be able to interact with all FATPs upon coimmunoprecipitation, the interaction with FATP2 may be of most relevance in cell physiology.

FATP joins the growing pantheon of proteins that intersect with the metabolic pathway of ceramide synthesis (Fig. 6). While the generation of sphinganine occurs in the membrane of the ER via the sequential action of two ER-embedded proteins, the generation of acyl-CoA occurs in a wider variety of
subcellular locations. Thus, free fatty acids can be taken-up by cells at the plasma membrane via a variety of transporters (40), including some FATPs, prior to the generation of fatty acyl-CoAs. The VLC–acyl-CoAs generated by FATP2 are then used for VLC-ceramide generation, via a pathway that also includes acyl-CoA–binding protein (12). Another pathway of generation of acyl-CoAs is via the action of LC-acyl-CoA synthetases, which also bind to CerS (13), and can provide acyl-CoA precursors for the elongation steps mediated via ELOVLs (11). Thus, two distinct branches become apparent upon analysis of the supply of substrates for the CerSs (Fig. 6), implying multiple finely tuned mechanisms (41) involved in the intricate regulation of the rate of (dihydro)ceramide synthesis. In addition, CerS activity can also be regulated by dimer formation, which depends on a short C-terminal motif (30), and by interaction with another member of the same protein family, FAM57B (42). While many other metabolic pathways are also tightly regulated, the critical roles that ceramide plays as a precursor to a whole gamete of SLs (43), and as a cellular signaling molecule, apparently necessitates these remarkably complex mechanisms of regulation.

One of the signaling pathways with which ceramide is associated is cell death, with ceramide generally considered to be proapoptotic (44, 45), although the roles of dihydroceramide in cell death pathways are less well established (46). Interestingly, incubation of cells with the FATP inhibitor, lipofermata, reduced levels of dihydroceramide synthesis. Lipofermata has been proposed as an anticancer agent (21, 22) via its interaction with FATP, although no studies are available prior to the current one examining whether lipofermata also impacts the ceramide pathway. Our data now show that this is the case and strengthen the notion that manipulating the

Figure 3. Catalytically active CerS2 and FATP2 are required in order for them to interact. A, Hek293T and HepG2 cells were transfected with FATP2-Flag for 48 h. The cells were incubated with or without FB1 (20 μM) for 24 h prior to harvesting, lysis, and immunoprecipitation using an anti-Flag antibody. CerS2 was identified by Western blotting using an anti-CerS2 antibody (upper panel; IP-Flag). Levels of expression of CerS2 and FATP2-Flag are shown in the lower panel (Input), and GADPH is shown as a loading control. The experiment was repeated three times. Mr markers (kDa) are indicated. B, Hek293T cells were transfected with full-length FATP2 or with a splice variant (FATP2b) and immunoprecipitation performed as in A. The experiment was repeated three times with similar results. Mr markers (kDa) are indicated. CerS, ceramide synthase; FATP, fatty acid transport protein; FB1, fumonisin B1; HEK, human embryonic kidney.
Figure 4. Effect of CerS2 mutations on binding to FATP2. A, schematic representation of the domains of CerS2-HA with the site of the mutations (10, 27, 28) color coded as in B. The numbers indicate the position of amino acid residues in the sequence. B, CerS2−/− Hek293T cells were transfected with pcDNA or CerS2-HA mutants along with FATP2-Flag. After 48 h, the cells were lysed and immunoprecipitated with an anti-HA antibody. FATP2-Flag was identified using an anti-Flag antibody (upper panel; IP-HA). Levels of protein expression are shown in the middle panels (input). GAPDH is shown as a loading control. Mr markers (kDa) are indicated. The experiment was repeated three times with similar results. The lower panel shows quantification of three independent coimmunoprecipitation experiments. The data are shown as a truncated violin plot with individual values. The Student t test was used to compare each group to CerS2-HA. *p < 0.05; **p < 0.01. CerS, ceramide synthase; FATP, fatty acid transport protein; HEK, human embryonic kidney.
The pathway of fatty acyl-CoA generation is likely to impact a wide variety of downstream lipids, not least the SLs, which utilize two acyl-CoA moieties in the initial steps of their synthesis.

**Experimental procedures**

**Materials**

NBD-Sphinganine and C18:0-NBD-ceramide were from Avanti Polar Lipids. Defatted-bovine serum albumin, FB1, a protease inhibitor cocktail, rabbit anti-CerS2, mouse anti-Flag, and mouse anti-GAPDH antibodies were from Sigma. Liposofermata was from Cayman Chemicals. A mouse anti-CerS2 antibody was from Santa Cruz and a rabbit anti-FATP2 antibody was from Proteintech. A rabbit anti-Calnexin antibody was purchased from Abcam. Horseradish peroxidase was from the Jackson Laboratory. An enhanced chemiluminescence detection system was from Thermo Scientific. 4x Laemmli sample buffer was from Bio-rad. Silica gel 60 TLC plates were from Merck. All the solvents were of analytical grade and were purchased from Bio-Lab. Anti-DYKDDDDK G1 Affinity Resin, pcDNA 3.1(+) vectors containing FATP1-6, and FATP2b with a C-terminus Flag-tag were from GenScript.

**Animals**

Livers from WT and CerS2 null mice (23) were perfused with cold PBS. All mouse studies were approved by the Weizmann Institute of Science Animal Care Committee according to National Institutes of Health’s Guidelines for Animal Care.

**Proteomics**

Proteomics was performed essentially as described (47); see also Supplemental Methods. Briefly, protein A agarose beads with CerS2 were incubated with 5% sodium dodecyl sulfate in 50 mM Tris-HCl and loaded onto S-Trap micro-columns (Protifit). Eluted samples after digestion with trypsin (1:50, trypsin/protein, wt/wt) were loaded onto a split-less nano ultra performance liquid chromatography column (10 kpsi nanoAcquity; Waters). The nano ultra performance liquid chromatography was coupled online through a nano-electrospray ionization emitter (10 μm tip; New Objective) to a quadrupole orbitrap mass spectrometer (Q Exactive Plus, Thermo Scientific) using a Flexion nanospray apparatus (Proxeon). Raw data was processed with MaxQuant v1.6.0.16. The data was analyzed with the Andromeda search engine against the Uniprot mouse proteome database (48). Label-free quantification intensities were calculated and used for further calculation by Perseus v1.6.0.7. Decoy hits were filtered out as well as proteins that were identified on the basis of only one modified peptide. The data was further filtered to include only proteins with at least two valid values in at least one of the groups. A Student’s t test, after logarithmic transformation, was used to identify significant

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**Figure 5. Lipofermata inhibits de novo dihydroceramide synthesis.** HepG2 cells were cultured for 4 days and were then incubated with (A) increasing concentrations of lipofermata or with (B) 10 μM lipofermata for increasing times. The cells were incubated with NBD-Sph for the last 25 min of the incubation period with lipofermata. LC (squares) and VLC-NBD-ceramides (circles) were separated by TLC, examples of which are given in each panel. The data are means ± S.D., n = 3. The Student t test was used to compare each group to untreated controls *p < 0.05; **p < 0.01. LC, long-chain; NBD-Sph, NBD-Sphinganine; VLC, very long-chain.

**Figure 6. A scheme of CerS-interacting proteins.** The upper branch shows the proteins involved in the pathway by which acyl-CoAs are supplied to the CerS, with enzymes encircled in blue and metabolic intermediates in black. The lower branch shows the enzymes (encircled in yellow) involved in ceramide synthesis. CerS are shown as a dimer (9), with a related protein, FAM57B, indicated due to its ability to regulate CerS activity and levels (42). For further details, see text. ACBP, acyl-CoA-binding protein; ACSL, long-chain acyl-CoA synthetase; CerS, ceramide synthase; DAG, diacylglycerol; DEGS, dihydroceramide desaturase; ELOVL, elongation of very long-chain fatty acids protein; FATP, fatty acid transport protein; KDSR, 3-ketosphinganine reductase; LC, long-chain; SPT, serine palmitoyltransferase; TAG, triacylglycerol; VLC, very long-chain.
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differences across biological replicates. Fold-change was calculated based on the ratio of geometric means of the different groups.

Liquid chromatography electrospray ionization tandem mass spectrometry

Sphingolipid analysis by liquid chromatography electrospray ionization tandem mass spectrometry using an ABI 4000 quadrupole-linear ion trap mass spectrometer was performed as described (30, 49).

CerS constructs

CerS constructs (Fig. 4) were subcloned using restriction-free cloning (50) from CerS2 in a pcDNA 3.1(+) vector containing a C-terminus HA tag. Primers are given in Table 2. All sequences were confirmed prior to use.

Cell culture and transfection

Human embryonic kidney 293T cells and CerS2+/− Hek293T cells (51) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 110 μg/ml sodium pyruvate. Transfections were performed with the polyethylenimine reagent using 8 μg of plasmid per 10 cm culture dish. The cells were removed from culture dishes after 48 h and washed twice with PBS on ice before immunoprecipitation. HepG2 hepatoma cells were cultured in DMEM supplemented as above but including Glutamax. The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO2.

Coimmunoprecipitation

Mouse liver was homogenized in lysis buffer (150 mM NaCl, 20 mM Hepes pH 7.5) containing 1% digitonin (v/v) and a protease inhibitor cocktail. After centrifugation (15,000gav, 10 min, 4 °C), protein was determined in the supernatant using the bicinchoninic acid reagent (Cyanagen). One microgram of a rabbit anti-CerS2 antibody was used to pull-down CerS2 from liver lysates (2 mg protein). After overnight incubation, protein A agarose beads (Santa Cruz) were added and gently rotated (4 °C on ice). The beads were heated (80 °C, 10 min) with sample buffer to elute Flag-bound proteins. The eluates were stored at −20 °C.

Western blotting

Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Flag-tagged constructs were identified using a mouse anti-Flag antibody (1:5000 dilution) and goat anti-mouse horseradish peroxidase (1:10,000 dilution) as the secondary antibody. CerS2 proteins were detected using a rabbit anti-CerS2 antibody or a mouse anti-CerS2 antibody followed by incubation with a goat anti-rabbit or antimouse horseradish peroxidase antibody (1:10,000 dilution). Equal loading was confirmed using a mouse anti-GAPDH antibody (1:5000 dilution). Detection was performed using the enhanced chemiluminescence detection system. Protein bands were imaged and quantified using the ChemiDoc MP imaging system (Bio-rad).

Immunocytochemistry

Human embryonic kidney 293T cells were fixed with methanolacetone (1:1, v/v) for 7 min on ice followed by incubation with 5% donkey serum/1% bovine serum albumin (v/v) in PBS for 1 h. Cells were then incubated with a rabbit anticalnexin antibody (1:300 dilution) or with a mouse anti-CerS2 antibody (1:100 dilution) along with a mouse anti-Flag antibody (1:1000 dilution) or a rabbit anti-FATP2 antibody (1:50 dilution) at 4 °C overnight. After three washes with PBS, the cells were incubated with Alexa-488 conjugated donkey anti-mouse and Alexa-647 dye-conjugated donkey anti-rabbit secondary antibodies (1:1000 dilution) in blocking buffer for 1 h. Hoechst 33342 (Invitrogen) was used to label nuclei, and the cells were examined by confocal laser scanning microscopy using an Olympus Fluoview FV500 imaging system.

Table 2

| Construct                  | Primers                  |
|----------------------------|--------------------------|
| CerS2ΔH212ΔH213A-HA        | F CAAGGAACAGATCATCGCCGCTGTGGCCACCATCATTC |
| CerS2Δ338-118-HA           | F GAATGATGGTGGCCACAGCGGCGATGATCTGTTCCTTG |
| CerS2Δ335–380-HA           | F GTTGCGGCGGCGGACAGGGAGGAGCAGCAGTTG |
| CerS2Δ338–344-HA           | F GGCACAAGTTCTAATACTGGAAAGTACCCATAGTGGTCAGATTAACAGTTG |
| CerS2(4A)-HA              | F GAATTCTCATTAACACTGGAAAGCTGTAAGAAGAAGACGAGGCTACAGAGGAGGAGGAGG |
| CerS2(ΔA)-HA              | R GGCCCACAAGTTCTAATACTGGAAAGTTACCCATAGTGGTCAGATTAACAGTTG |
| CerS2(ΔI)-HA              | R CCCTCCCTCCTGCTGCTCGCTCTCCGCTGCTG |

Abbreviations: F, forward; R, reverse.
Metabolic labeling

Cells were incubated in serum-free medium for 24 h prior to incubation with 20 μM lipofermata (dissolved in 0.1% DMSO) in serum-free medium. After various times of incubation with lipofermata, 3 μM NBD-Sphinganine was added for 25 min. After washing the cells with PBS, cells were homogenized in ice-cold distilled water and an aliquot taken for protein determination. The remaining homogenate was lysed in chloroform/methanol (1:2, v/v) to extract lipids (52). Lipids were dried under N2, resuspended in chloroform/methanol (9:1, v/v), and separated by TLC using chloroform/methanol/2 M NH4OH (40:10:1, v/v/v) as the developing solvent. NBD-labeled LC- and VLC-NBD-ceramides were visualized using an Amersham Typhoon 5 biomolecular fluorescence imager and quantified by ImageQuantTL software (GE Healthcare). C18:0-NBD-ceramide was used as a marker.

Statistics

Statistical significance was assessed using an unpaired two-tailed Student’s t test. *p < 0.05; **p < 0.01; ***p < 0.001.

Data availability

The mass spectrometry proteomics data was deposited in the ProteomeXchange Consortium via the PRIDE (53) partner repository with the dataset identifier PXD031088.

Supporting information—This article contains supporting information (47).

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Conflict of interest—The authors declare that they have no conflicts of interest.

Abbreviations—The abbreviations used are: CerS, ceramide synthase; ER, endoplasmic reticulum; FATP, fatty acid transport protein; FB1, fumonisin B1; Hek, human embryonic kidney; LC, long-chain; SL, sphingolipid; VLC, very long-chain.

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