INTRODUCTION

Sphingolipids are multifunctional lipids that have a variety of physiological roles, such as immunity, vascular formation, neural function, skin barrier formation, recognition of bacterial toxins, spermatogenesis, and regulation of insulin resistance.\textsuperscript{1-5} They fulfill these functions as components of biological membranes or multilamellar lipids (such as myelin sheaths or lipid

**Abbreviations:** 4,8-SPD, 4,8-sphingadiene; 6-OH, 6-hydroxy; DHS, dihydrosphingosine; DHS1P, DHS 1-phosphate; DRM, detergent-resistant membrane; ER, endoplasmic reticulum; FA, fatty acid; FADS, FA desaturase; HEK, human embryonic kidney; HexCer, monohexosylceramide; KO, knockout; LC, liquid chromatography; MRM, multiple reaction monitoring; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PHS, phytosphingosine; PHS1P, PHS 1-phosphate; PUFA, polyunsaturated FA; SPD, 4,14-sphingadiene; SPD1P, SPD 1-phosphate; SPH, sphingosine; SPH1P, sphingosine 1-phosphate; TLC, thin layer chromatography.

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lamellae in the epidermis) or as bioactive lipids. They contain a polar head group and hydrophobic backbone ceramide, which is composed of a sphingoid base (long-chain base) and fatty acid (FA). The polar head groups are phosphorycholine (in sphingomyelin) or a sugar chain (in glycosphingolipids) in mammals. Each constituent of sphingolipids—sphingoid base, FA, and polar head group—exists in various forms, and different combinations of these three components generate sphingolipid diversity, which leads to the multifunctionality of sphingolipids.

Sphingoid bases commonly have hydroxyl groups at C1 and C3 and an amino group at C2, although they differ with respect to the presence or absence of double bond(s) and/or an additional hydroxyl group. Mammals have five sphingoid bases: dihydrosphingosine (DHS), sphingosine (SPH), phytosphingosine (PHS), 6-hydroxy (6-OH) SPH, and 4,14-sphingadiene (or 4,14-sphingadienine; SPD) (Figure 1).

Of these, SPD is unique in that it has a cis double bond between C14 and C15. Although SPD was discovered about half a century ago, it remains enigmatic such that its tissue distribution, biosynthetic enzyme, and degradation pathway have not been elucidated. The hydroxyl and amino groups in sphingolipids serve as donors and acceptors in hydrogen bond formation, enhancing lipid-lipid interaction. This interaction stimulates the formation of lipid microdomains, which are platforms for signal transduction. Lipid microdomains contain tightly packed cholesterol and sphingolipids. The straight structure of saturated or trans double-bonded carbon chains strengthens lipid packing, whereas the bent structure of the cis double bond increases the distance between lipids and weakens the lipid-lipid interaction. Therefore, an SPD that contains a cis double bond may differ from other sphingoid bases in terms of its functions and lipid microdomain formation.

Sphingoid bases are produced by de novo synthesis or by the degradation of ceramides (salvage pathway). They are condensed with acyl-CoA to produce ceramides or phosphorylated by SPH kinases (SPHK1 and SPHK2) to generate sphingoid base 1-phosphates. Some sphingoid base 1-phosphates are transported extracellularly and act as lipid mediators that bind to G-protein-coupled receptors [SPH 1-phosphate (SPH1P) receptors (S1P₁–S1P₅)] and induce cellular responses such as cell proliferation, cell migration, organization of the actin cytoskeleton, and adherens junction assembly. Intracellularly, sphingoid base 1-phosphates are dephosphorylated to sphingoid bases by SPH1P phosphatases (SGPP1 and SGPP2) or cleaved to long-chain aldehydes and phosphoethanolamine by the SPH1P lyase SGPL1. The resulting long-chain aldehydes are converted to acyl-CoAs through several reactions and are mainly metabolized to glycerophospholipids.

Although SPD is known to have a cis double bond between C14 and C15, the enzyme responsible for this desaturation has not yet been identified. The FA desaturase (FADS) family of proteins has been found to catalyze the introduction of a double bond into lipids. The FADS family comprises eight members (FADS1-8) in humans. Despite being called FA desaturases, the substrates of these enzymes are not FAs but acyl-CoAs (FADS1, FADS2, FADS4/SCD5, and FADS5/SCD1) or DHS-containing ceramides (DHS ceramides) (FADS7/DEGS1); the latter produce SPH ceramides. FADS members introduce a cis double bond at a specific position into acyl-CoA: FADS1 introduces this bond between C5 and C6; FADS2 between C6 and C7; and FADS4 and FADS5 between C9 and C10. Furthermore, not all FADS members act as desaturases: FADS8/DEGS2 is a hydroxylase that introduces a hydroxyl group at the C4 of DHS ceramides to produce PHS ceramides.

The presence of SPD in the plasma, lymph, brain, and lungs of humans and/or mice has been reported. However, the process of characterizing SPD has been slower than that of other sphingoid bases. We reveal the tissue distribution of SPD, the activity of sphingoid base- or sphingoid-base 1-phosphate-metabolizing enzymes toward SPD/SPD 1-phosphate (SPD1P), the enzyme involved in SPD biosynthesis, the synthetic pathway of SPD, and the lipid microdomain localization of SPD-containing sphingolipids.

**FIGURE 1** Structures of mammalian sphingoid bases. All sphingoid bases have hydroxyl groups at C1 and C3 and an amino group at C2. DHS has no double bonds and is present in all tissues, albeit in small quantities. SPH, which has a trans double bond between C4 and C5, is the most abundant sphingoid base in mammals and exists in all tissues. PHS and 6-OH SPH each have a hydroxy group, which are found at C4 on the DHS structure and at C6 on the SPH structure, respectively. PHS is present in specific tissues such as skin, small intestine, and kidney. 6-OH SPH specifically exists in skin. SPD has a trans double bond between C4 and C5 and a cis double bond between C14 and C15. Although the existence of SPD in plasma, lymph, brain, and lung had previously been reported, its exact tissue distribution remained unclear [Color figure can be viewed at wileyonlinelibrary.com]
2 | MATERIALS AND METHODS

2.1 | Mice

C57BL/6J mice were housed in a controlled environment (temperature, 23 ± 1°C; 12 hours light/12 hours dark cycle) and fed with normal diet (PicoLab Rodent Diet 20; LabDiet, St. Louis, MO, USA) and given water ad libitum. All animal experiments were approved by the institutional animal care and use committee of Hokkaido University.

2.2 | Cell culture and transfection

Human embryonic kidney (HEK) 293T, HeLa, and human hepatoma HepG2 cells were cultured in Dulbecco’s Modified Eagle’s medium (D6429 for HEK 293T cells and D6046 for HeLa and HepG2 cells; Merck, Darmstadt, Germany). HAP1 cells, which are near-haploid human cells derived from a chronic myelogenous leukemia sample,\textsuperscript{25} were cultured in Iscove’s Modified Dulbecco’s medium (Thermo Fisher Scientific, Waltham, MA, USA). Each medium was supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), 100 units/mL of penicillin, and 100 μg/mL of streptomycin (Merck). Cells were cultured at 37°C under 5% CO\textsubscript{2}. HEK 293T cells were grown on collagen-coated dishes (IWAKI, Shizuoka, Japan). Transfections were performed using Lipofectamine Transfection Reagent combined with Plus Reagent (Thermo Fisher Scientific) in most cases, according to the manufacturer’s instructions, but PEI Max (Polysciences, Warrington, UK) was used for the transfection of CERS genes into HEK 293T cells, as follows. In one set of tubes, PEI Max (1 mg/mL; 24 μL) was added to 1.5 mL of OPTI-MEM I (Thermo Fisher Scientific) medium and incubated for 5 minutes at room temperature. In another set of tubes, plasmid (12 μg) was added to 1.5 mL of OPTI-MEM I. These two solutions were then mixed and incubated for 20 minutes at room temperature. HEK 293T cells grown on 10 cm dishes were washed with 5 mL of OPTI-MEM I, suspended in 8 mL of OPTI-MEM I, and incubated with the PEI-Max/DNA complex for 4 hours at 37°C. The medium was then changed to 10 mL of Dulbecco’s Modified Eagle’s medium supplemented with fetal bovine serum and antibiotics, and cells were incubated for another 20 hours.

2.3 | Plasmids

The mammalian expression vectors pCE-puro-3 × FLAG-1\textsuperscript{26} and pEFh-3 × FLAG-1, both for the expression of N-terminally 3 × FLAG-tagged proteins, were used. The pEFh-3 × FLAG-1 vector was derived from pEFh vector.\textsuperscript{27} For the overexpression of human CERS1-6, SGPP1, SGPP2, SGPL1, and FADS1-8, the genes were introduced into pCE-puro-3 × FLAG-1 vector and for overexpression of human SPHK1 and SPHK2, the genes were cloned into the pEFh-3 × FLAG-1 vector.

2.4 | Construction of FADS3 gene knockout (KO) HAP1 cells

FADS3 gene KO HAP1 cell lines were generated by the CRISPR/Cas9 system using the pYU417 plasmid,\textsuperscript{28} which consists of a Cas9 mutant nuclease (D10A nickase), a guide RNA cloning cassette, an EGFP expression cassette, and a puromycin N-acetyltransferase gene. Two pairs of oligonucleotides [forward and reverse FADS3 guide RNA sequences 1 (5’-TCCCCAGCAAGGTGGCAGGTTTTT-3’ and 5’-CTGCCCCACTCTTTGCTGGACGTTG-3’) and FADS3 guide RNA sequences 2 (5’-ACGCCAGCCCGCGACAAGGTTTT-3’ and 5’-CTTCGCCGGGCGCTGTTGCGTGTTG-3’)], which target exon 1 of FADS3, were each annealed and introduced into the Bael site of the pYU417 plasmid, generating the pJK34 and pJK25 plasmids. HAPI cells were transfected with the pJK34 and pJK35 plasmids. Twenty-four hours after transfection, cells were cultured in the presence of 2 μg/mL of puromycin for another 24 hours, diluted into 10 cm plates, and cultured for additional 7 days in the absence of puromycin. Individual cell colonies were isolated using cloning rings. Genotyping was performed by amplifying FADS3 by PCR using genomic DNA prepared from each clone and the primers FADS3-F (5’-AACAGGTGCGTGTTGTGGACGGTG-3’) and FADS3-R (5’-ATGCTAAGGCCCCACAGGTGGACGCG-3’), followed by DNA sequencing.

2.5 | Lipid analyses

Brain, lung, heart, skeletal muscle, spleen, kidney, liver, colon, small intestine, and testis were collected from one-month-old male mice, and epidermis was prepared from mice at postnatal Day 0. Each tissue (10 mg) was homogenized in isopropanol (SARSTEDT, Nümbrecht, Germany) using Micro Smash MS-100 (TOMY Seiko, Tokyo, Japan). After centrifugation (20 400g, room temperature, 3 min), the supernatant was recovered. The pellet was resuspended in 450 μL of chloroform/methanol/12 M formic acid (100:200:1, v/v), treated with 30 pmol of internal standard (LIPIDMAPS Mass Spec Internal Standard, Ceramide/Sphingoid Internal Standard Mix II; Avanti Polar Lipids, Alabaster, AL, USA), and mixed (4500 rpm, 4°C, 1 min) in a tube containing 300 mg zirconia beads (SARSTEDT, Nümbrecht, Germany) using Micro Smash
room temperature, 3 min), this supernatant was combined with the previous one and then mixed with 300 µL of chloroform and 540 µL of water. Phases were separated by centrifugation (20 400g, room temperature, 3 min) and the organic phase (lower phase) was recovered and dried. The lipids were dissolved in 1.5 mL of chloroform/methanol (1:2, v/v), diluted 50-fold (for brain, spleen, kidney, or epidermis) or 10-fold (for other tissues), and subjected to liquid chromatography (LC)-coupled tandem mass spectrometry (MS/MS) analysis (injection volume, 5 µL) as described below.

Quantification of SPH ceramides and SPD ceramides from HEK 293T, HeLa, and HepG2 cells was performed as follows. Cells cultured in a 12-well plate (80%-100% confluence) were recovered by scraper and transferred to a plastic tube. Cells were suspended in 100 µL of water and mixed with 375 µL of chloroform/methanol (1:2, v/v) and 2.5 pmol of internal standard (LIPIDMAPS Mass Spec Internal Standard, Ceramide/Sphingoid Internal Standard Mix II). To hydrolyze the ester bonds of glycerolipids by alkaline treatment, 25 µL of 3 M KOH was added to the samples followed by incubation for 1 hour at 37°C. After neutralization by the addition of 30 µL of 3 M formic acid, samples were mixed with 125 µL of chloroform and 125 µL of water and centrifuged (20 400 g, room temperature, 3 min). The organic phase was recovered and dried, and the lipids were dissolved in 125 µL of chloroform/methanol (1:2, v/v) and analyzed by LC-MS/MS (injection volume, 5 µL) as described below.

Lipid labeling assays using d7-SPH (Avanti Polar Lipids), d7-DHS (Avanti Polar Lipids), or C6:0 SPH ceramide (Merck) were performed as follows. Labeled cells were collected, suspended in 100 µL of water, and mixed successively with 375 µL of chloroform/methanol/12 M formic acid (100:200:1, v/v), 125 µL of chloroform, and 125 µL of water. After centrifugation, the organic phase was recovered. In the case of kinetic analyses of d7-SPH and d7-DHS, the organic phase was dried, suspended in chloroform/methanol (1:2, v/v), and subjected to LC-MS/MS analysis as described below. In the other lipid labeling experiments, lipids were subjected to alkaline treatment by adding 71 µL of 0.5 M NaOH to the organic phase for 1 hour at 37°C. After neutralization with 35.5 µL of 1 M formic acid, lipids were extracted by mixing with 135 µL of methanol and 210 µL of water. Phases were separated by centrifugation and the organic phase was recovered, dried, suspended in chloroform/methanol (1:2, v/v), and resolved by LC-MS/MS as described below.

Ceramides were quantified using an LC-coupled triple quadrupole mass spectrometer Xevo TQ-S (Waters, Milford, MA, USA) in positive ion mode by product ion scanning or by multiple reaction monitoring (MRM). LC conditions were as described previously. In product ion scanning, m/z values of [M + H - H2O]+ (for C16:0 SPH ceramide, 520.5; for C16:0 4,8-SPD ceramide and C16:0 SPD ceramide, 518.5) were set at the mass filter Q1 and collision energy was set at 20 eV. Fragment (product) ions were detected in the scanning range m/z 100-600. In MRM, ceramides were detected by setting the Q1 m/z value at [M + H - H2O]+ for the precursor ions and the Q3 m/z value as that specific to SPH (m/z 264.3) or SPD-specific fragment ions (m/z 262.3), using the appropriate collision energies (Tables 1-4), as described previously.

Data analyses were performed using MassLynx software (Waters). SPH ceramides were quantified by calculating the ratio of the peak area of each ceramide species to that of the internal standard. For SPD ceramide quantification, the SPH ceramide internal standard was used, based on the observation that the fragment ion patterns and their peak intensities for SPD ceramides and SPH ceramides were similar.

2.6 Immunoblotting

Immunoblotting was performed as described previously. Anti-FLAG polyclonal antibody (1/1000 dilution), anti-calnexin monoclonal antibody 4F10 (1 µg/mL; Medical & Biological Laboratories, Aichi, Japan), anti-GAPDH monoclonal antibody 5A12 (0.5 µg/mL; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), or anti-caveolin-1 monoclonal antibody D46G3 (1/1000 dilution; Cell Signaling Technology, Beverly, MA, USA) was used as the primary antibody. Anti-rabbit IgG, HRP-linked F(ab′)2 fragment (1/7500 dilution; GE Healthcare Life Sciences, Chicago, IL, USA), or anti-mouse IgG, HRP-linked F(ab′)2 fragment (1/7500 dilution; GE Healthcare Life Sciences) was used as the secondary antibody.

2.7 Indirect immunofluorescence microscopy

Cells were visualized by indirect immunofluorescence microscopy as described previously. Anti-FLAG polyclonal antibody (1/2000 dilution) and anti-calnexin monoclonal 4F10 antibody (2.5 µg/mL) were used as primary antibodies. Alexa Fluor 488-conjugated anti-rabbit IgG antibody and Alexa Fluor 594-conjugated anti-mouse IgG antibody (each at 5 µg/mL; Thermo Fisher Scientific) were used as secondary antibodies. Cover slips were mounted using the ProLong Gold Antifade reagent (Thermo Fisher Scientific). Images were acquired using a Leica DM5000B microscope (Leica Microsystems, Wetzlar, Germany).
2.8 | In vitro ceramide synthase assay

An in vitro ceramide synthase assay was performed as described previously with slight modification. Cells were suspended in lysis buffer A [50 mM HEPES-NaOH (pH 7.4), 150 mM NaCl, 1 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 × protease inhibitor cocktail (Complete, EDTA-free; Merck)] and lysed by sonication. After centrifugation (400 g, 4°C, 3 min) to remove cell debris, the supernatant was recovered and subjected to ultracentrifugation (100,000 g, 4°C, 35 min). The pellet (membrane fraction) was suspended in lysis buffer A. Membrane fractions (50 µg of membrane fractions from cells overexpressing CERS1, CERS2, CERS3, CERS4, or CERS6; 25 µg of membrane fractions from cells overexpressing CERS5) were mixed with 400 µM CoA, 20 mM ATP, 0.1 µCi [14C]FA [for CERS1, CERS3, and CERS4, [14C]C18:0 FA (American Radiolabeled Chemicals, St. Louis, MO, USA); for CERS2, [14C]C22:1 FA (American Radiolabeled Chemicals); or for CERS5 and CERS6, [14C]C16:0 FA (Moravek, Brea, CA, USA)], and 10 µM sphingoid base (DHS, SPH, PHS, or SPD; all from Avanti Polar Lipids), and incubated at 37°C for 15 minutes. After this reaction, samples (100 µL) were successively mixed with 375 µL of chloroform/methanol/12 M HCl (100:200:1, v/v), 125 µL of chloroform, and 125 µL of 1% KCl. After phases were separated by centrifugation (20,400 g, room temperature, 3 min), the organic phase was recovered, dried, dissolved in chloroform/methanol (1:2, v/v), and separated by normal-phase thin layer chromatography (TLC) using high-performance TLC silica gel 60 plates (Merck) and chloroform/methanol/2 M ammonium hydroxide (95:5:2) as a solvent system. [14C]Ceramides were detected and quantified using a BAS-2500 bio-imaging analyzer (GE Healthcare Life Sciences).

2.9 | In vitro SPH kinase assay

An in vitro SPH kinase assay was performed essentially as reported previously, using the buffer system optimized both for both SPHK1 and SPHK2. Cells were suspended in lysis buffer B [50 mM HEPES-NaOH (pH 7.5), 10 mM KCl, 0.05% Triton-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl...
fluoride, and 1 × protease inhibitor cocktail] and lysed by sonication. After centrifugation (400 g, 4°C, 3 min), the supernatants (total cell lysates) were recovered. The total cell lysates (10 μg) were incubated with 0.2 μCi [γ-33P]ATP (PerkinElmer Life Sciences, Waltham, MA, USA), 2 mg/mL of FA-free bovine serum albumin (Merck), 10 μM sphingoid base (DHS, SPH, PHS, or SPD), 0.5 μM ATP, and 1 mM MgCl2 in the presence of SPH1P phosphatase inhibitors (10 mM NaF and 5 mM Na3VO4) and a SPH1P lyase inhibitor (0.5 mM 4-deoxypyridoxine) at 37°C for 15 minutes. Lipids were extracted as described above, dissolved in 20 µL of chloroform/methanol (2:1, v/v), and separated by a normal-phase TLC using high-performance TLC silica gel 60 plates and 1-butanol/acetic acid/water (3:1:1, v/v) as the solvent system. [33P]Sphingoid base 1-phosphates were detected and quantified using the BAS-2500.

2.10 | In vitro sphingoid 1-phosphate phosphatase and lyase assays

In vitro sphingoid 1-phosphate phosphatase and lyase assays were performed essentially as described previously.34 [33P] Sphingoid base 1-phosphate substrates were prepared as follows. Total cell lysates (10 μg) prepared from HEK 293T cells overexpressing the SPH kinase SPHK2 were mixed with 2 μCi [γ-33P]ATP (PerkinElmer Life Sciences, Waltham, MA, USA), 2 mg/mL of FA-free bovine serum albumin (Merck), 10 μM sphingoid base (DHS, SPH, PHS, or SPD), 0.5 μM ATP, and 1 mM MgCl2 in the presence of SPH1P phosphatase inhibitors (10 mM NaF and 5 mM Na3VO4) and a SPH1P lyase inhibitor (0.5 mM 4-deoxypyridoxine) at 37°C for 15 minutes. Lipids were extracted as described above, dissolved in 20 μL of chloroform/methanol (2:1, v/v), and separated by a normal-phase TLC using high-performance TLC silica gel 60 plates and 1-butanol/acetic acid/water (3:1:1, v/v) as the solvent system. [33P]Sphingoid base 1-phosphates were detected and quantified using the BAS-2500.
then resubjected to lipid extraction by mixing with 200 µL of chloroform and subsequent centrifugation (20,400 g, room temperature, 3 min). The resulting organic phase was recovered and combined with the previous one. The [33P]sphingoid base 1-phosphates thus obtained were dried and dissolved in ethanol.

An in vitro sphingoid base 1-phosphate phosphatase assay was performed by incubating membrane fractions (2 µg) in lysis buffer A with one of the above prepared [33P]sphingoid base 1-phosphates (each 22.1 pCi; 1.5 pM), 2 mg/mL of FA-free bovine serum albumin, and 0.5 mM 4-deoxypyridoxine at 37°C for 10 minutes (50 µL of total volume). An in vitro sphingoid base 1-phosphate lyase assay was conducted by incubating the membrane fraction (2 µg) with [33P]sphingoid base 1-phosphate (22.1 pCi; 1.5 pM), 2 mg/mL of FA-free bovine serum albumin, 10 mM NaF, and 5 mM Na3VO4 at 37°C for 10 minutes (50 µL of total volume). After these reactions, aliquots of the samples (2.5 µL each) were directly spotted on high-performance TLC silica gel 60 plates, and the lipids were separated using 1-butanol/acetate acid/water (2:1:1, v/v) as the solvent system. [33P]Sphingoid base 1-phosphate, [33P]orthophosphate, and [33P]phosphoethanolamine were detected and quantified using the BAS-2500.

### Table 3

| Ceramide species | Precursor ion (Q1) [M – H2O + H]+ | Product ion (Q3) | Collision energy (eV) |
|------------------|-----------------------------------|----------------|-----------------------|
| $d_7$-d18:1/C16:0 | 527.5                             | 271.3          | 20                    |
| $d_7$-d18:1/C18:0 | 555.6                             | 271.3          | 20                    |
| $d_7$-d18:1/C20:0 | 583.6                             | 271.3          | 25                    |
| $d_7$-d18:1/C22:0 | 611.6                             | 271.3          | 25                    |
| $d_7$-d18:1/C24:2 | 635.6                             | 271.3          | 30                    |
| $d_7$-d18:1/C24:4 | 637.6                             | 271.3          | 30                    |
| $d_7$-d18:1/C24:6 | 639.6                             | 271.3          | 30                    |
| $d_7$-d18:2/C16:0 | 525.5                             | 269.3          | 20                    |
| $d_7$-d18:2/C18:0 | 553.6                             | 269.3          | 20                    |
| $d_7$-d18:2/C20:0 | 581.6                             | 269.3          | 25                    |
| $d_7$-d18:2/C22:0 | 609.6                             | 269.3          | 25                    |
| $d_7$-d18:2/C24:2 | 633.6                             | 269.3          | 30                    |
| $d_7$-d18:2/C24:4 | 635.6                             | 269.3          | 30                    |
| $d_7$-d18:2/C24:6 | 637.6                             | 269.3          | 30                    |
| $d_7$-d18:2/C26:0 | 665.7                             | 269.3          | 30                    |

*a$d_7$-d18:1 indicates a sphingoid base with two hydroxyl groups (d), a carbon-chain length of 18, and one double bond.

### Table 4

| Ceramide species | Precursor ion (Q1) [M – H2O + H]+ | Product ion (Q3) | Collision energy (eV) |
|------------------|-----------------------------------|----------------|-----------------------|
| d18:1/C12:0      | 644.4                             | 263.4          | 40                    |
| d18:1/C16:0      | 700.5                             | 263.4          | 40                    |
| d18:1/C18:0      | 728.6                             | 263.4          | 40                    |
| d18:1/C20:0      | 756.6                             | 263.4          | 40                    |
| d18:1/C22:0      | 784.6                             | 263.4          | 40                    |
| d18:1/C24:2      | 808.6                             | 263.4          | 40                    |
| d18:1/C24:4      | 810.6                             | 263.4          | 40                    |
| d18:1/C24:6      | 812.6                             | 263.4          | 40                    |
| d18:1/C26:0      | 840.7                             | 263.4          | 40                    |
| d18:2/C16:0      | 698.5                             | 262.3          | 40                    |
| d18:2/C18:0      | 726.6                             | 262.3          | 40                    |
| d18:2/C20:0      | 754.6                             | 262.3          | 40                    |
| d18:2/C22:0      | 782.6                             | 262.3          | 40                    |
| d18:2/C24:2      | 806.6                             | 262.3          | 40                    |
| d18:2/C24:4      | 810.6                             | 262.3          | 40                    |
| d18:2/C24:6      | 838.7                             | 262.3          | 40                    |

*a$d_7$-d18:1 indicates a sphingoid base with two hydroxyl groups (d), a carbon-chain length of 18, and one double bond.

*bInternal standard.

### 2.11 Preparation of detergent-resistant membrane (DRM) fraction

The DRM fraction was prepared, and lipids were extracted as described previously. Extracted lipids were dried, dissolved in 225 µL of chloroform/methanol (1:1, v/v), and subjected to alkaline treatment by incubating them with 71 µL of 0.5 M NaOH for 1 hour at 37°C. The samples were neutralized by the addition of 35.5 µL of 1 M formic acid, mixed with 106.5 µL of water, and centrifuged (20,400 g, room temperature, 3 min) to separate the phases. The organic phase was recovered and dried, and the lipids were dissolved in 500 µL of chloroform/methanol (1:2, v/v) and analyzed by LC-MS/MS (injection volume, 5 µL) as described above.

### 2.12 Statistical analyses

Data are presented as means ± SD. The significance of differences between groups was evaluated using nonpaired two-tailed Student’s t-test or Tukey’s test in Microsoft Excel (Microsoft, Redmond, WA, USA) or JMP13 (SAS Institute, Cary, NC, USA), respectively. A P-value of <.05 was considered significant.
3 | RESULTS

3.1 | Broad tissue distribution of SPD in mammals

First, we developed the detection conditions for LC-MS/MS that can specifically and quantitatively detect mammalian SPD (4,14-SPD) by distinguishing it from 4,8-sphingadiene (4,8-SPD; the sphingoid base in plants). Some of the 4,8-SPD absorbed from the diet are reported to be converted to 4,8-SPD ceramides and 4,8-SPD ceramides from commercial SPD and 4,8-SPD, respectively. When they were subjected to LC-MS/MS analyses in the product ion scanning mode, each SPD ceramide species was detected with a retention time different from the corresponding 4,8-SPD ceramide species in LC (Figure 2A; C16:0 ceramide species are shown). Collision-induced dissociation of SPD ceramides in MS/MS mainly generated a fragmentation ion of m/z 262, which corresponds to SPD (Figure 2B). However, ceramides containing SPH (the most abundant sphingoid base in mammals) produced a fragment ion of m/z 264, which corresponds to SPH, as previously reported.39 This difference in m/z is the result of the saturation (in SPH) of the double bond in SPD. The ion fragmentation patterns and intensities of SPD ceramides were similar to those of SPH ceramides. These results indicate that SPD ceramides can be specifically detected by combining separation by LC with MRM by MS/MS, in which the Q3 and Q1 mass filters are set to the m/z value 262 (corresponding to SPD, the product ion) and those of each SPD ceramide species (precursor ions) with a specific FA chain length, respectively (Table 1).

To investigate the tissue distribution of SPD, we measured the SPD ceramide levels in 11 mouse tissues (brain, lung, heart, skeletal muscle, spleen, kidney, liver, colon, small intestine, testis, and epidermis) by LC-MS/MS. For comparison, we also examined SPD ceramide levels. SPD ceramides were detected in almost all tissues, although their levels varied among tissues (Figure 3A). SPD ceramide levels were highest in kidney, followed by brain, lung, and colon in that order, and they were low in testis, epidermis, and skeletal muscle. The ratio of SPD ceramides to SPH ceramides was highest in kidney (68%), approximately 30% in brain, lung, and heart, 10%-20% in liver, colon, and small intestine, and <10% in skeletal muscle, spleen, small intestine, and epidermis. Although the plant-type 4,8-SPD ceramides were detected at low levels in some tissues, they were below the detection limit in most tissues (data not shown).

Various ceramide species differing in FA chain length and degree of unsaturation exist in mammalian tissues and the composition of these ceramide species differs among tissues.5,36 The seven most abundant SPH ceramide species in many tissues contain a C16:0, C18:0, C20:0, C22:0, C24:0, C24:1, or C24:2 FA36; however, some tissues have ceramide species with a unique FA moiety: C28-C34 polyunsaturated FAs (PUFAs) in testis4 and C26-C36 saturated or monounsaturated FAs in epidermis.40 Consistent with our previous report,36 the most abundant SPH ceramide species were a C18:0 ceramide in brain and skeletal muscle, C24 (C24:0, C24:1, and C24:2) ceramides in spleen and liver, and a C16:0 ceramide in kidney, colon, and small intestine (Figure 3B). The FA composition of the SPD ceramides was similar to that of the SPH ceramides in most tissues. The exception was testis, where the proportion of SPD ceramides containing ≥C28 PUFAs was much smaller than that of the corresponding SPH ceramides, whereas that containing the C24:0 FA was larger. Ceramides containing ≥C28 PUFAs are synthesized by the ceramide synthase CERS3.4 We speculate that it is difficult for CERS3 to synthesize ceramides that are polyunsaturated in both the sphingoid base and the FA moiety.

To examine whether SPD exists not only in mice but also in humans, we next measured SPD ceramide levels in some human cell lines (HEK 293T, HeLa, and HepG2). We found that SPD ceramides were present at ~17% of SPD ceramide levels in all three cell lines (Figure 3C) and that the FA composition of the SPD ceramides was similar to that of the SPH ceramides (Figure 3D). In summary, we revealed that SPD is present in a wide range of mammalian tissues and cells, and that their abundance and FA composition varies among tissues and cells.

3.2 | Substrate specificities of ceramide synthases toward sphingoid bases

Ceramide synthases catalyze amide bond formation between a sphingoidbaseand aacyl-CoA to generate ceramides (Figure 4A). Six ceramide synthases (CERS1-6) exist in mammals, and each exhibits characteristic substrate specificity toward acyl-CoAs with different chain lengths (CERS1, C18; CERS2, C22-C24; CERS3, ≥C18; CERS4, C18-C22; CERS5 and CERS6, C16).5,32,41,42 While the substrate specificities of CERS1-6 toward acyl-CoAs are known, those toward sphingoid bases remained unclear. To reveal this, we performed an in vitro ceramide synthase assay toward SPD, DHS, PHS, and SPD, using membrane fractions prepared from HEK 293T cells overexpressing each of the CERS enzymes.

The expression of the enzymes was confirmed by immunoblotting, although the expression levels of CERS1, CERS3, and CERS4 were slightly lower than those of the other ceramide synthases (Figure 4B). Overexpression of CERS1 increased ceramide synthase activity toward all sphingoid bases approximately 3-fold relative to the vector control (Figure 4C). CERS2 and CERS4 showed ceramide synthase activity toward SPD, DHS, and PHS (that toward SPD and DHS was 2- to 3-fold higher and that toward PHS was 3- to 5-fold higher, both relative
Activity in CERS2- or CERS4-expressing cells toward SPD tended to be higher than that of the control, although the differences were not statistically significant. Overexpression of CERS3 slightly increased the ceramide synthase activity toward PHS and showed a tendency to increase the activity toward SPH, DHS, and SPD. This low
activity of CERS3 may have been due to technical problems. Although CERS3 exhibits activity toward acyl-CoAs with ≥C18, its preferred substrates are ≥C26.42–44 However, such ≥C26 substrates are unsuitable for in vitro assay, due to low solubility in the reaction buffer. We, therefore, used a C18:0 substrate to measure the CERS3 activity.

Overexpression of CERS5 or CERS6 increased ceramide synthase activity toward all sphingoid bases relative to the vector control (CERS5, 8- to 12-fold; CERS6, ~3-fold). Combining these results, CERS1-6 did not show clear differences in substrate specificity toward sphingoid bases. The activity of CERS2 and CERS4 toward SPD seemed to be lower than that toward other sphingoid bases. However, considering that there was no difference in FA composition between the SPH ceramides and SPD ceramides in mouse tissues (Figure 3B), CERS2 and CERS4 may have sufficient activity to produce SPD ceramides, at least in vivo.

### 3.3 Different metabolism of sphingoid bases

Sphingoid bases are converted to sphingoid base 1-phosphates by SPH kinases (SPHK1 and SPHK2) and are then either dephosphorylated by SPH1P phosphatases (SGPP1 and SGPP2) or cleaved by SPH1P lyase (SGPL1)18,45 (Figure 5A). The activity of these sphingoid base- or sphingoid-base 1-phosphate-metabolizing enzymes toward SPD and SPD1P has not yet been determined. Their activity toward PHS and PHS 1-phosphate (PHS1P) has also not yet been examined, except for that of SPH kinases.11,12 Therefore, we investigated the activity of these enzymes toward SPH, DHS, PHS, SPD, and their phosphorylated forms.

Substrate specificity toward sphingoid bases differs between the SPH kinases SPHK1 and SPHK2. SPHK1 has narrow substrate specificity; it phosphorylates only SPH and DHS.11,33 On the other hand, SPHK2 phosphorylates a broad range of substrates, including SPH, DHS, PHS, and the immune-suppressing drug fingolimod, a sphingoid base analog.12,33 To investigate the activity of SPHK1 and SPHK2 toward SPD, we performed in vitro SPH kinase assays using total cell lysates prepared from HEK 293T cells overexpressing SPHK1 or SPHK2. Expression levels of SPHK1 were higher than those of SPHK2 (Figure 5B). SPHK1 exhibited more activity toward SPH, DHS, and SPD than SPHK2 (Figure 5C). However, given the discrepancy in the expression levels of SPHK1 and SPHK2, their kinase activity toward these substrates is probably comparable. Only SPHK2 phosphorylated PHS, and it did so weakly, which is consistent with previous reports.11,12,33

To examine the activity of the SPH1P phosphatases SGPP1 and SGPP2 toward sphingoid-base 1-phosphates, we conducted in vitro sphingoid-base 1-phosphate dephosphorylation assays using membrane fractions prepared from HEK 293T cells overexpressing SGPP1 or SGPP2. The expression levels of SGPP1 were higher than those of SGPP2 (Figure 5D). Both SGPP1 and SGPP2 exhibited high levels of dephosphorylation activity toward SPH1P, DHS 1-phosphate (DHS1P), and SPD1P, but their activity toward PHS1P was low, especially for SGPP1.

Next, we performed in vitro sphingoid-base 1-phosphate lyase assays. SGPL1 exhibited less activity toward PHS1P and SPD1P than toward SPH1P and DHS1P (Figure 5F). The lyase activity of SGPL1 toward SPD1P was 66% and 57% of that toward SPH1P and DHS1P, respectively. In summary, sphingoid bases can be classified into three groups in terms of metabolism: SPH and DHS; PHS; and SPD. SPH and DHS (and their phosphorylated forms) undergo phosphorylation, dephosphorylation, and cleavage with high efficiency, whereas PHS and PHS1P are less susceptible to these reactions. SPD and SPD1P are intermediate in that they are susceptible to phosphorylation/dephosphorylation but not to cleavage.

### 3.4 Involvement of FADS3 in SPD ceramide biosynthesis

SPD is thought to be synthesized by the desaturation of SPH between C14 and C15. To investigate the involvement of FADS family proteins in SPD biosynthesis, HEK 293T cells overexpressing each of the FADS family members (FADS1-8) were incubated with deuterium-labeled SPH (d7-SPH; d7 indicates seven deuteriums). Lipids were extracted from the cells and the d7-SPD ceramides were quantified by LC-MS/MS. The expression of all FADS members was confirmed by immunoblotting, although expression levels varied among the proteins (Figure 6A). Only overexpression of FADS3 caused an increase in d7-SPD ceramide levels (about 10-fold relative to the vector control) (Figure 6B), although it had almost no effect on...
FA composition and had no effect on $d_7$-SPH ceramide levels.

FADS3 is known to be expressed ubiquitously and to be most abundant in kidney tissue, but its function remained almost completely unknown. Although it has been reported to introduce a cis double bond between C13 and C14 of trans-vaccenic acid (Δ11-C18:1), there is also a contradictory report.

To further confirm the involvement of FADS3 in SPD biosynthesis, we constructed FADS3 KO HAP1 cells, a near-haploid cell line derived from human chronic myelogenous leukemia. We obtained two FADS3 KO clones (KO1 and KO2), which lack 480 bp and 28 bp, respectively, around exon 1 of the FADS3 gene (Figure 6C). After incubation of HAP1 and FADS3 KO cells with $d_7$-SPH, lipids were extracted and $d_7$-SPD ceramides were quantified by LC-MS/MS. The $d_7$-SPD ceramide levels were reduced to less than 1% of those in parental HAP1 cells in both types of FADS3 KO cells (Figure 6D). In contrast, $d_7$-SPH ceramide levels were increased in the KO cells, presumably due to the change in metabolic flow from SPD ceramides to SPH ceramides.

Combined, our results indicate that FADS3 is involved in the SPD ceramide production.

Sphingolipids are synthesized in the endoplasmic reticulum (ER) up to ceramide production, followed by complex sphingolipid syntheses in the Golgi apparatus. Since the intracellular localization of FADS3 had not yet been determined, we investigated its localization by indirect immunofluorescent microscopy. It showed a reticular staining pattern that merged with calnexin, an ER marker (Figure 6E), indicating that FADS3 localizes in the ER.

### 3.5 SPD ceramide production by desaturation of SPH ceramides

Although we had detected SPD ceramides as the products of FADS3 (Figure 6), it still remained unclear whether the substrate of FADS3 is SPH or SPH ceramides. To determine this, we compared the time course of the conversion of DHS and SPH to SPD ceramides. DHS is produced via the de novo sphingolipid synthesis pathway and most of it is rapidly metabolized to complex sphingolipids via SPH ceramides (Figure 7A). Moreover, SPH is produced only via the sphingolipid degradation pathway. Sphingolipids functioning as a component of cell membranes are gradually degraded, mainly in lysosomes, so it takes time to convert DHS to SPH. SPH generated via the degradation
pathway is reused for sphingolipid synthesis via the salvage pathway. If the FADS3 substrate is SPH ceramides, DHS and SPH should be converted to SPD ceramides at similar rates (Figure 7A, hypothesis 1). Moreover, if the FADS3 substrate is SPH, the conversion from SPH to SPD ceramides would be rapid, while that from DHS to SPD ceramides would be slow (Figure 7A, hypothesis 2).

HEK 293T cells were labeled with $d_7$-DHS or $d_7$-SPH, and the time-dependent production of $d_7$-SPD ceramides was examined by LC-MS/MS. The two substrates were converted to $d_7$-SPD ceramides at similar rates: in both cases, the $d_7$-SPD ceramide levels peaked at 3 hours and had decreased slightly at 6 hours (Figure 7B, left panel). This result suggests that the substrates of FADS3 are SPH ceramides. We also measured $d_7$-SPH ceramide levels and found that they peaked at 1-3 hours and decreased at 6 hours for both $d_7$-DHS and $d_7$-SPH (Figure 7B, right panel). This decrease at 6 hours may be due to the conversion of SPH ceramides into complex sphingolipids such as sphingomyelin and glycosphingolipids. The fact that the SPH ceramides reached their maximum levels more rapidly than SPD ceramides also suggests that SPD ceramides are produced from SPH ceramides.

![Diagram of ceramide synthesis](image_url)

**Figure 4** Substrate specificities of ceramide synthases toward sphingoid bases. A, The reaction of ceramide synthesis catalyzed by ceramide synthases (CERS1-6). B and C, HEK 293T cells were transfected with the pCE-puro 3 × FLAG-1 (vector), pYU525 (3 × FLAG-CERS1), pYU394 (3 × FLAG-CERS2), pNS9 (3 × FLAG-CERS3), pYU525 (3 × FLAG-CERS4), pYU526 (3 × FLAG-CERS5), or pYU527 (3 × FLAG-CERS6) plasmid. Twenty-four hours after transfection, cells were harvested and membrane fractions were prepared. B, The membrane fractions (5 µg) were separated by SDS-PAGE and subjected to immunoblotting using anti-FLAG antibody or anti-calnexin antibody (as the loading control for the membrane fraction). C, The membrane fractions (25-50 µg) were incubated with 10 µM sphingoid base (SPH, DHS, PHS, or SPD), 0.1 µCi $[^{14}C]$FA (for CERS5 and CERS6, $[^{14}C]$C16:0 FA; for CERS1, CERS3, and CERS4, $[^{14}C]$C18:0 FA; for CERS2, $[^{14}C]$C22:1 FA), 400 µM CoA, and 20 mM ATP at 37°C for 15 min. Lipids were extracted and separated by TLC. $[^{14}C]$Ceramides were detected and quantified using a BAS-2500. Values presented are means ± SD from three independent experiments (*$P < .05$; **$P < .01$; Student’s t-test)
Next, to confirm that SPH ceramides are the substrate of FADS3, HEK 293T cells overexpressing FADS3 were treated with the C6:0 SPH ceramide, which is membrane permeable, and the generation of C6:0 SPD ceramide was examined by LC-MS/MS. Overexpression of FADS3 caused a 52-fold increase in C6:0 SPD ceramide levels, concomitant with a decrease in C6:0 SPH ceramide levels to 33% of vector control levels (Figure 7C). Combining these results, we conclude that FADS3 produces SPD ceramides using SPH ceramides as substrates (Figure 7D).

### 3.6 Localization of SPD ceramide outside lipid microdomains

Most sphingolipids do not have a cis double bond with a bent structure, making it easy to form lipid microdomains. However, since SPD-containing sphingolipids do possess a cis double bond, it is possible that they are only poorly able to form lipid microdomains. To investigate this possibility, we examined the lipid microdomain localization of SPD sphingolipids. HeLa cells were treated with the nonionic detergent Triton X-100, and the DRM was prepared by sucrose density gradient centrifugation. The lipid microdomain marker caveolin-1 was present in fraction 3 of the 10 fractions collected (Figure 8A). Lipids were extracted from this fraction as well as from total cell lysates, and the sphingolipid monohexosylceramides (HexCers) containing SPD or SPH (for comparison) were quantified by LC-MS/MS. Although the amount of SPH HexCers in the DRM fraction was 9.8% of that in total cell lysates, the amount of SPD HexCers...
was much lower (2.1% of that in total cell lysates) (Figure 8B). These results indicate that SPD sphingolipids preferentially localize outside lipid microdomains.

4 | DISCUSSION

The existence of SPD in mammals was first reported in the 1960s. However, the quantitative tissue distribution of SPD has remained unknown, mainly due to the lack of an SPD standard. Its synthesis and degradation mechanisms were also completely unknown. In the present study, using a chemically synthesized SPD standard that has recently become commercially available, we established a method for specifically detecting SPD, and found that SPD ceramides occur widely in mammalian tissues and cells (Figure 3). They were particularly abundant in kidney tissue, although substantial quantities were also present in other tissues (5%-35% of SPH ceramide levels). SPD sphingolipids cannot, therefore, be ignored when elucidating the physiological functions of sphingolipids.

SPD is a unique sphingoid base in that it has a cis double bond. We identified FADS3, a protein whose function...
was unknown until now, as the enzyme that introduces this double bond (Figure 6). The FADS3 protein was detected in kidney, liver, and heart of rat and mouse, with the highest levels in kidney, consistent with our data that SPD ceramides are abundant in kidney (Figure 2). FADS3 has also been reported to introduce a double bond between the C13 and C14 positions of trans-vaccenic acid (Δ11-C18:1) to generate Δ11,13-C18:2 FA. In that report, overexpression of FADS3 in rat primary hepatocytes treated with trans-vaccenic acid resulted in an approximately 2-fold increase in Δ11,13-C18:2 FA levels, and Fads3 knockdown lead to a decrease in Δ11,13-C18:2 FA levels to about 40%. However, these effects of FADS3 are much smaller than the effects on SPD ceramides revealed in this study [a 10-fold increase in SPD ceramide production resulting from FADS3 overexpression (Figure 6B), and a decrease to less than 1% resulting from FADS3 KO (Figure 6D)]. In another report, Fads3 KO mice were created and various PUFA species were measured in the KO mouse brain and liver, based on the prediction that FADS3 would act as FA desaturase. However, differences in PUFA levels between wild-type and Fads3 KO mice were very small. In that report, Δ11,13-C18:2 FA was not detected in the liver or heart of either wild-type or Fads3 KO mice administered with trans-vaccenic acid. Thus, we conclude that the primary function of FADS3 is the generation of SPD ceramides.

In the present study, we concluded that the substrates of FADS3 are SPH ceramides rather than SPH, based on the results obtained from the kinetic analysis of DHS and SPH metabolism (Figure 7B) and C6:0 SPH ceramide labeling (Figure 7C). Regarding the SPD ceramide synthesis pathway, the possibility that the introduction of a cis double bond between C14 and C15 by FADS3 precedes the introduction of a trans double bond between C4 and C5 in DHS ceramides by DEGS1 (FADS7) had also been considered. However, no ceramides that contained a double bond only between C14 and C15 were detected by our LC-MS/MS analyses (data not shown). Similarly, no sphingoid base with a double bond

FIGURE 7  SPD ceramide production by desaturation of SPH ceramide. A, Two hypotheses about the FADS3 substrate (hypothesis 1, SPH ceramide; hypothesis 2, SPH) and SPD ceramide synthesis pathway are illustrated. Hypothesis 1: DHS generated in de novo sphingolipid biosynthesis pathway and SPH generated in the salvage pathway are converted to DHS ceramides and SPH ceramides, respectively, by ceramide synthases (CERS1-6). DHS ceramides are rapidly metabolized to SPD ceramides by the DHS ceramide desaturase DEGS1. Finally, SPD ceramides are converted to SPD ceramides by FADS3. All these reactions take place in the ER. The conversion rates from DHS and SPH to SPD ceramides are expected to be similar. Hypothesis 2: DHS is metabolized to DHS ceramides and then to SPH ceramides in the ER, converted to complex sphingolipids in the Golgi apparatus, and transported to the plasma membrane. Complex sphingolipids in the plasma membrane are gradually subjected to degradation, mainly in lysosomes, to generate SPH. SPH is desaturated to SPD by FADS3 and then acylated by ceramide synthases to generate SPD ceramides. It is expected that the conversion from DHS to SPD ceramides would take much longer than that from SPH to SPD ceramides, since the conversion from DHS to SPH is time-consuming. Cer, ceramide. B, HEK 293T cells were cultured in the medium without serum or antibiotics for 30 min and incubated with 5 µM d7-DHS or d7-SPH at 37°C for the indicated times. After washing cells with PBS containing 1 mg/mL of FA-free bovine serum albumin twice, lipids were extracted from the cells, and the content of d7-SPD ceramides and d7-SPH ceramides was quantified by LC-MS/MS in MRM mode. Values presented are means ± SD from three independent experiments. C, HEK 293T cells were transfected with pCE-puro 3 × FLAG-1 (vector) or pJK22 (3 × FLAG-FADS3) plasmid. Twenty-four hours after transfection, cells were incubated with 2 µM C6:0 SPH ceramide for 4 h. Lipids were extracted from the cells, and C6:0 SPD ceramide and C6:0 SPH ceramide were quantified by LC-MS/MS in MRM mode. Values presented are means ± SD from three independent experiments (**P < .01; Student’s t-test). D, The FADS3-catalyzed reaction
only between C14 and C15 is detected in healthy individuals, whereas it is found in patients with a DEGS1 mutation. We, therefore, speculate that C4 desaturation reaction by DEGS1 occurs earlier than the C14 desaturation reaction by FADS3 and that under physiological conditions ceramide synthesis proceeds in the order DHS ceramides, SPH ceramides, and finally SPD ceramides. Lipid microdomains are enriched with cholesterol and sphingolipids. Most sphingolipids do not contain a bent, cis double bond and are thus suitable for lipid-lipid interactions. In contrast, SPD sphingolipids do have a cis double bond and they preferentially localize outside lipid microdomains (Figure 8). Similar results have been observed for sphingolipids with a cis double bond in the FA moiety. We hypothesize that FADS3 negatively regulates lipid microdomain formation by increasing the levels of anti-lipid-microdomain sphingolipids (SPD sphingolipids) with concomitant decreases in those of pro-lipid-microdomain sphingolipids (sphingolipids containing other sphingoid bases).

In summary, we found that SPD exists in a wide range of tissues, with especially high levels in kidney, and identified FADS3 as the ceramide desaturase involved in SPD production. Due to their unusual structure containing a cis double bond, SPD sphingolipids may have an opposite, negative role in lipid microdomain formation relative to conventional sphingolipids. Although Fads3 KO mice have previously been generated, only results of limited phenotypic analyses have been reported. In that report, no differences were observed between wild-type and Fads3 KO mice in body weight up to Day 60 after birth, or in brain weight up to Day 30 after birth. However, the comprehensive knockout mouse phenotypic analysis project conducted by the International Mouse Phenotyping Consortium (https://www.mousephenotype.org) has shown some phenotypes of Fads3 KO mice, such as decreased circulating phosphate levels, absence of pinna reflex, and auditory abnormalities. SPD levels are highest in the kidney, followed by the brain (Figure 3A). Therefore, it is possible that the phenotypes observed in Fads3 KO are caused by the dysfunction of these tissues (kidney, decreased circulating phosphate levels; brain, absence of pinna reflex, and auditory abnormality). However, more detailed analysis of Fads3 KO mice will be necessary, especially with regards to kidney and brain function, to elucidate the pathophysiological functions of SPD.

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CONFLICT OF INTEREST
The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS
K. Jojima and M. Edagawa designed and performed the experiments and analyzed the data. M. Sawai analyzed the data. Y. Ohno analyzed the data and wrote the manuscript. A. Kihara planned the project, designed the experiments, and wrote the manuscript.

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