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Regular Article

Phospholipid Scramblase 1 Localizes Proximal to Sphingomyelin Synthase Isoforms but Is Not Involved in Sphingomyelin Synthesis

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Ceramide (Cer) is synthesized *de novo* in the bilayer of the endoplasmic reticulum and transported to the cytosolic leaflet of the *trans*-Golgi apparatus for sphingomyelin (SM) synthesis. As the active site of SM synthase (SMS) is located on the luminal side of the Golgi membrane, Cer translocates to the lumen *via* transbilayer movement for SM synthesis. However, the mechanism of transbilayer movement is not fully understood. As the Cer-related translocases seem to localize near the SMS, the protein was identified using proximity-dependent biotin identification proteomics. Phospholipid scramblase 1 (PLSCR1), which is thought to act as a scramblase for phosphatidylserine and phosphatidylethanolamine, was identified as a protein proximal to the SMS isoforms SMS1 and SMS2. Although five isoforms of PLSCR have been reported in humans, only PLSCR1, PLSCR3, and PLSCR4 are expressed in HEK293T cells. Confocal microscopic analysis showed that PLSCR1 and PLSCR4 partially co-localized with p230, a *trans*-Golgi network marker, where SMS isoforms are localized. We established CRISPR/Cas9–mediated PLSCR1, PLSCR3, and PLSCR4 single-knockout cells and PLSCR1, 3, 4 triple knockout HEK293T cells. Liquid chromatography-tandem mass spectrometry revealed that the levels of species with distinct acyl chains in Cer and SM were not significantly different in single knockout cells or in the triple knockout cells compared to the wild-type cells. Our findings suggest that PLSCR1 is localized in the vicinity of SMS isoforms, however is not involved in the transbilayer movement of Cer for SM synthesis.

Key words sphingomyelin, phospholipid scramblase 1, protein using proximity-dependent biotin identification

INTRODUCTION

Sphingomyelin (SM) is a major component of cell membranes that are particularly enriched in lipid rafts and detergent-resistant sphingolipid-rich microdomains that play a central role in many cellular processes, including membrane sorting and trafficking, cell polarization, and signal transduction.[1)] SM is synthesized *de novo* from serine and palmitoyl-CoA by sequential reactions of various enzymes. The final step of its synthesis is catalyzed by SM synthase (SMS). In mammals, the SMS enzyme has two isoforms, SMS1 and SMS2.[2)] SMS transfers the phosphorylcholine moiety from phosphatidylcholine to the primary hydroxyl group of ceramide (Cer), resulting in SM synthesis. Cer is hypothesized to translocate to the Golgi lumen *via trans*-bilayer movement for SM synthesis. However, the mechanism of Cer *trans*-bilayer movement in the Golgi membrane is not fully understood. As the asymmetric increase in Cer on one side of the plasma membrane by the addition of sphingomyelinase promotes transbilayer Cer movement in cells,[3,4)] Cer may spontaneously translocate to the lumen without the need for membrane proteins. Although these experiments clearly demonstrate the possibility of sphingomyelinase-mediated Cer signaling in the plasma membrane, they do not mimic the physiological conditions required for Cer synthesis in the Golgi membrane. Alternatively, as ATP-binding cassette subfamily A member 12 (ABCA12) has been identified as a translocase for esterified omega-hydroxy-ultra-long-chain Cer and/or its metabolites,[5)] the related translocase for Cer may be important for the delivery of Cer to SMS isoforms. ABCA12 is a lipid transporter that is primarily expressed in keratinocytes and is not ubiquitously expressed. Therefore, the mechanism of the *trans*-bilayer movement of Cer for SM synthesis on the Golgi membrane is not fully understood. Recent development of a highly active biotin ligase named TurboID, has made it possible to effectively biotinylate the interacting proteins that interact weakly or dynamically.[6,7)] As the Cer-related translocases seem to localize near the SMS, the protein was identified using proximity-dependent biotin identification proteomics in this study.

MATERIALS AND METHODS

Plasmids The complete open reading frames for human SMS isoforms and PLSCR family were amplified from the cDNA library derived from human cell lines, HEK293T, Jurkat, and Molt4 cells. The expression vectors for TurboID-fused

SMS were constructed by PCR using overlapping primers as previously described.[8] C1(1–29)-TurboID-V5_pCDNA3 was a gift from Alice Ting (Addgene plasmid # 107173; http://n2t.net/addgene:107173; RRID: Addgene_107173)[6] and was used as the PCR template. A glycine linker $(GGGGS)_3$ was inserted between the SMS and TurboID to facilitate proper protein folding. TurboID-fused SMS was cloned into a pQCXIP retroviral vector (TaKaRa Bio, Shiga, Japan). Human phospholipid scramblase (PLSCR) 1, 2, 3, 4, and 5 expression vectors were constructed by PCR from the cDNA library derived from using specific primers containing sequences corresponding to the HA epitope in front of the stop codon. The primers used were 5′-GAATTGATCCGCGGCCGCCACCATGGACAAACAAAACTCACAG-3′/5′-GGGCGGAATTCCGGATCCTCAAGCGTAATCTGG-AACATCGTATGGGTACCACACTCCTGATTTTTGTTCC-3′ for PLSCR1, 5′-GAATTGATCCGCGGCCGCCACCATGAGATCATGGAACTCACTC-3′/5′-GGGCGGAATTCCGG-ATCCTCAAGCGTAATCTGGAACATCGTATGGGTACCTAGTTCTTTCAAAAAACATG-3′ for PLSCR2, 5′-GAATTGATCCGCGGCCGCCACCATGGCAGGCTACTTGCCCCCC-3′/5′-GGGCGGAATTCCGGATCCTCAAGCGTAATCTGGAACATCGTATGGGTAACTGGTGACGGCAGAGGGCCC-3′ for PLSCR3, 5′-GAATTGATCCGCGGCCGCCACCATG-TCAGGTGTGGTACCCACAG-3′/5′-GGGCGGAATTCCGGATCCTCAAGCGTAATCTGGAACATCGTATGGGTATCTTGAACGTTGTGGTGGAGATC-3′ for PLSCR4, and 5′-GAATTGATCCGCGGCCGCCACCATGGCCTCTAAAGATGCCCAG-3′/5′-GGGCGGAATTCCGGATCCTCAAGCGTAATCTGGAACATCGTATGGGTATAATCCAGCCAGTGAATGTTC-3′ for PLSCR5. PCR products were subcloned into the pQCXIP retroviral vector.

Cells The HEK293T cells were obtained from Abcam (Cambridge, U.K.). The cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg/mL of penicillin, and 100 μg/mL of streptomycin.

Generation of Stable Cell Lines HEK293T stable cell lines were generated by expressing PLSCR1, PLSCR3, PLSCR4, TurboID-fused SMS1, and TurboID-fused SMS2. Briefly, GP2-293 cells were simultaneously transfected with the pVSV-G envelope vector (TaKaRa Bio) and the pQCXIP vector encoding TurboID-fused SMS or PLSCR. The retroviral particles were then used to infect HEK293T cells. The stably transduced HEK293 cells were selected using 2 μg/mL puromycin.

Antibodies Mouse anti-V5 (Catalog No. 011-23591) and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Catalog No. 016-25523) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). The mouse anti-p230 antibody (Catalog No. 611280) was purchased from BD Biosciences (NJ, U.S.A.). Horseradish peroxidase (HRP)-conjugated streptavidin (Catalog No. N100), anti-mouse immunoglobulin G (IgG)- HRP (Catalog No. 62-6520), anti-mouse IgG-Alexa Fluor 488 (Catalog No. A11001), anti-rat IgG-Alexa Fluor 488 (Catalog No. A11006), anti-mouse IgG-Alexa Fluor 546 (Catalog No. A11030), anti-rabbit IgG-Alexa Fluor 546 (Catalog No. A11010) and Hoechst 33342 were obtained from Thermo Fisher Scientific (Waltham, MA, U.S.A.). Rat anti-V5 antibody (Catalog No. ab206571), rabbit anti-PLSCR1 (Catalog No. ab180518), rabbit anti-PLSCR3 (Catalog No. ab137128), and rabbit anti-PLSCR4 (Catalog No. ab233005) were purchased from Abcam. The anti-rabbit IgG-HRP antibody (Catalog No. 7074S) was obtained from Cell Signaling Technology (Danvers, MA, U.S.A.).

Immunocytochemistry and Fluorescence Microscopy Stable HEK293T cell lines expressing TurboID-fused SMS or PLSCR in 35 mm-diameter glass bottom dishes (MatTek, MA, U.S.A.) were fixed with 3% paraformaldehyde in phosphate buffered saline (PBS) for 10 min. The cells were permeabilized with 0.1% Triton X-100 in PBS at 25 °C for 10 min. After treatment with ImmunoBlock (KAC, Kyoto, Japan) for 30 min the samples were incubated with specific antibodies at 4 °C overnight followed by incubation with Alexa Fluor-conjugated secondary antibodies at 25 °C for 2 h. Images were captured using an Olympus FV10i confocal microscope equipped with a 60× water objective using the OLYMPUS FLUOVIEW Ver.3.1 Viewer (Olympus, Tokyo, Japan).

Streptavidin Immunoprecipitation HEK293T stable cell lines expressing TurboID-fused SMS were seeded in a 10-cm dish and cultured overnight. The cells were then treated with 500 μM biotin. After 3 h of incubation, the cells were washed three times with cold PBS and lysed in radio immunoprecipitation assay (RIPA) lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholate, and 1% Nonidet P40) supplemented with Protease Inhibitor Cocktail Set III and Phosphatase Inhibitor Cocktail Solution II (FUJIFILM Wako Pure Chemical Corporation), and then passed 15 times through a 22G needle for homogenization. After removal of cell debris by centrifugation at 17000×***g*** at 4 °C for 5 min, the free biotin present in the supernatant was removed using Amicon Ultra-4, 10 kDa (Merck Millipore, MA, U.S.A.). The concentrated supernatants were incubated with Sera-Mag™ SpeedBeads Neutravidin-Coated Magnetic Particles from Cytiva (MA, U.S.A.) at 4 °C, overnight. Beads were collected and washed twice in washing buffer 1 (2% SDS), once in washing buffer 2 (50 mM Tris–HCl [pH 7.5], 0.1% deoxycholate, 1% TritonX-100, 500 mM NaCl, and 1 mM ethylenediaminetetraacetic acid (EDTA)), once in washing buffer 3 (10 mM Tris–HCl [pH 8.0], 0.5% deoxycholate, 0.5% NP-40, 250 mM LiCl, and 1 mM EDTA), and twice in washing buffer 4 (50 mM Tris–HCl [pH 7.5] and 50 mM NaCl). The biotinylated proteins were eluted with 10 mM Tris–HCl (pH 7.5), 2% SDS, 5% β-mercaptoethanol, and 2 mM biotin for 5 min at 95 °C.

LC-Tandem Mass Spectrometry (LC-MS/MS) LC-MS/MS was used to detect biotinylated proteins. The analysis method was performed according to Nguyen-Tien *et al.*[9] Briefly, biotinylated proteins were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and stopped before protein separation. The protein mixture was detected as a single band and excised. The gel bands were reduced and alkylated with 50 mM DTT and 100 mM acrylamide. Gel slices were digested with trypsin (TPCK-treated; Worthington Biochemical) by incubating at 37 °C for 12 h. The resulting peptide mixture was acidified with 1% formic acid, and an aliquot was subjected to LC-MS/MS using Q Exactive HF-X (Thermo Fisher Scientific) and Easy-nLC1200 (Thermo Fisher Scientific). Liquid chromatography was performed using 0.1% formic acid as solvent A and 0.1% formic acid with 80% acetonitrile as solvent B. Peptides were separated using a nano-ESI spray column (NTCC-360, 0.075 mm internal diameter×150 mm length,

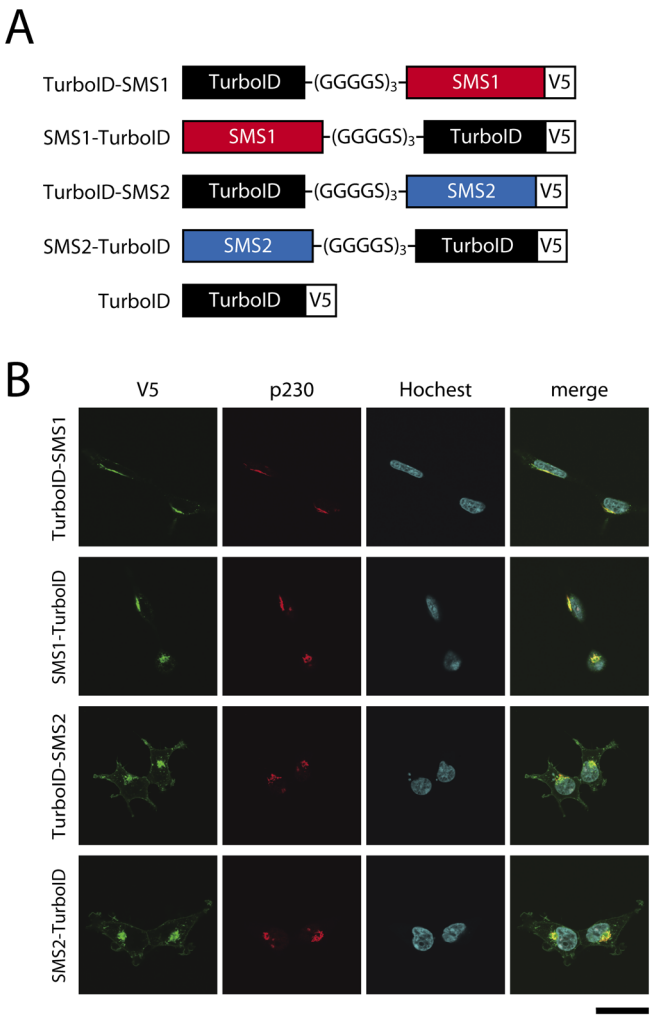

Fig. 1. Constructions of TurboID-Fused SMS Isoforms

(A) Schematic representation of the chimeric proteins used for the TurboID assay. The constructs were designed to contain the amino acid sequences of TurboID, fused to the N- or C-terminus of SMS. Location of the V5 epitope tag is also indicated. A spacer $(GGGGS)_3$ linker was inserted between SMS and TurboID to facilitate proper folding of the proteins. (B) Distributions of TurboID-fused SMS isoforms stably expressed in HEK293T cells. Cells were stained with anti-V5 antibody, followed by appropriate Alexa Fluor-conjugated secondary antibodies and analyzed by confocal microscopy. Localization was confirmed by co-staining with an antibody against the *trans*-Golgi network marker p230. TurboID-fused SMS, green; p230, red; Hochest, blue. Scale bar, 100 μm. The images are from one experiment representative of three independent experiments.

3 μm, Nikkyo Technos Co.) at a flow rate of 300 nL/min under a linear gradient for 30 min with a gradient of 0–64% solvent B. The mass spectrometer was operated in positive mode using the top 10 method. The MS/MS data obtained were searched against the NCBI nr database 20150601 using Mascot version 2.5 software (Matrix Science, U.K.) integrated with Proteome Discoverer 2.2 (Thermo Fisher Scientific). Mascot searches were performed using the following parameters (taxonomy, Homo sapiens [human] [326427 sequences]; search type, MS/MS ion search; enzyme, trypsin; fixed modification, none; variable modifications, acetyl [protein N-term], Gln→pyro-Glu [N-term Q], oxidation [M], propionamide [C]; mass values, monoisotopic; peptide mass tolerance, ±15 ppm; fragment mass tolerance, ±30 mmu; max missed cleavages, 3; instrument type, and ESI-TRAP). Proteins were quantified by label-free quantification using Proteome Discoverer.

SDS-PAGE Separation of Proteins The eluted proteins in RIPA lysis buffer and the biotinylated proteins were mixed with SDS sample buffer and loaded on a 10% SDS-PAGE gel. The separated proteins were detected with the Silver Stain Kit (FUJIFILM Wako Pure Chemical Corporation) and immunoblotting analysis.

Absolute Quantification of mRNA Copy Number in HEK293T Cells by Real-Time PCR Total RNA was isolated from the cells using ISOSPIN Cell & Tissue RNA (Nippon Gene, Tokyo, Japan) and reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Quantitative PCR was performed on a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific) using TB Green Premix Ex Taq II (TaKaRa Bio). The primers used were 5′-TGCCTTCAGGAGATAGAAATC-CA-3′/5′-GGTGCCAAGTCTGAATAACATAACC-3′ for PLSCR1, 5′-CGGGTGTTGATTTTGAGATTACAT-3′/5′-CCCAGA CCAGTGCTTAGAAATCC-3′ for PLSCR2, 5′-TGCCTT-CTG GCCTCGAATT-3′/5′-CTCAGCCTTCTGGTGAATCAAA-3′ for PLSCR3, 5′-CCCACTAGACCTGGATGTGAAGA-3′/5′-TGAAGTCAATGAGGAAGCAAGCT-3′ for PLSCR4, 5′-AGC TGCTTGGAATGATACTTGGTA-3′/5′-TGTCCCAAGCTG TTTTTAATCTCA-3′ for PLSCR5. Standard curves were generated using each expression vector as a template.

Generation of CRISPR/Cas9-Based Knockout (KO) Cells To establish PLSCR1-KO, PLSCR3-KO, PLSCR4-KO, and PLSCR1, 3, 4-triple knockout (TKO) cells, we designed guide RNAs (pSpCas9-PLSCR1gRNA-For, CACCGACAG AGGGTTTACTTTGCAG; pSpCas9-PLSCR1gRNA-Rev, AAA CCTGCAAAGTAAACCCTCTGTC; pSpCas9-PLSCR3gRNA-For, CACCGCGGAGCCGGCGCTACATCCT; pSpCas9-PLSCR3gRNA-Rev, AAACAGGATGTAGCGCCGGCTCCGC; pSpCas9-PLSCR4gRNA-For, CACCGTTGCCAGGCTGAT ACCGGAC; pSpCas9-PLSCR4gRNA-Rev, AAACGTCCGGTA TCAGCCTGGCAAC). Sequences corresponding to these sgRNAs were cloned into the pSpCas9(BB)-2A-GFP (PX458) vector. The vector pSpCas9(BB)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene plasmid #48138; http://n2t.net/addgene:48138; RRID:Addgene_48138).[10] The constructs were transfected into HEK293T cells using Lipofectamine 2000 (Thermo Fisher Scientific), according to the manufacturer's instructions. Transfected cells were cultured for 72 h and selected for the GFP marker using a FACS Aria II (BD Bioscience, NJ, U.S.A.). Using limiting dilution, clonal populations of the KO cells were isolated. Gene disruption in HEK293T cells was confirmed by DNA sequencing and Western blotting (WB).

Lipid Extraction and Quantification of Sphingolipids by LC-MS/MS Details of this procedure have been described previously.[11] The cells were washed once with cold PBS, collected in 100 μL of cold PBS, and then homogenized using sonication. Total lipids were extracted by adding 375 μL of chloroform : methanol (1 : 2, v/v). After sonicating the single-phase mixture, 100 μL of chloroform : methanol : 5N NaOH (1 : 2 : 0.8, v/v/v) was added and the solution was incubated for 1 h at 37 °C, followed by neutralization with acetic acid. Subsequently, 158 μL of chloroform and 158 μL of water were added and the mixture vigorously vortexed, before centrifuging for 1 min at 13000 × ***g*** at 4 °C. The lower phase was withdrawn and dried and then resuspended in acetonitrile : methanol (1 : 1, v/v), sonicated for 10 s, centrifuged at 14000 × ***g*** for 5 min, and

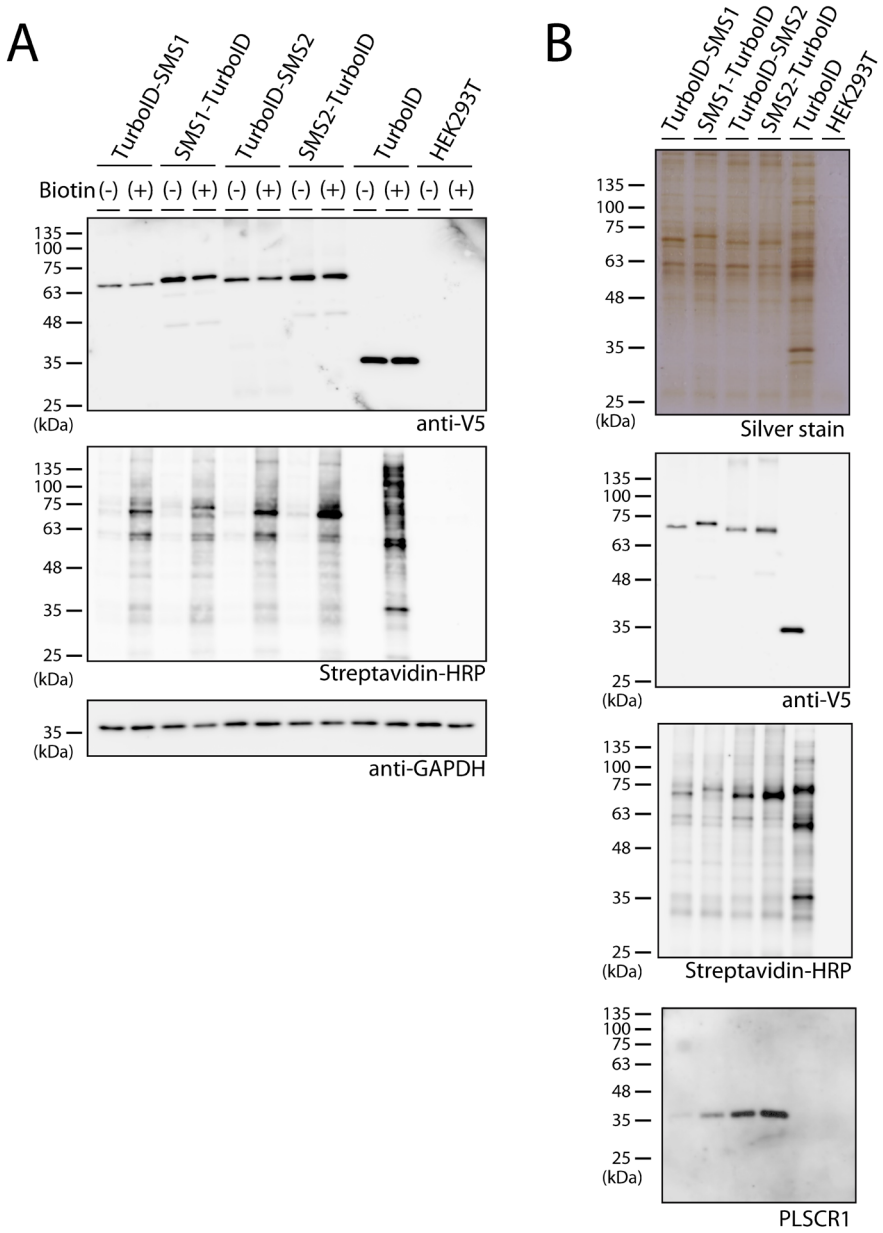

Fig. 2. Expression and Purification of TurboID-Fused SMS Isoforms

(A) TurboID-fused SMS stably expressing HEK293T cells were treated with or without biotin and were lysed and analyzed by immunoblotting with anti-V5 antibody and streptavidin-HRP. Anti-GAPDH was used as a loading control. Blots were obtained from one experiment representative of three independent experiments. (B) The biotinylated proteins were affinity-purified with streptavidin-conjugated beads and were analyzed by SDS-PAGE. The proteins were detected by silver staining and immunoblotting with anti-V5 antibody, streptavidin-HRP, and anti-PLSCR1 antibodies. Blots were obtaied from one experiment representative of three independent experiments.

the supernatant was transferred to vials. The concentrations of Cer and SM were analyzed using the QTRAP4500 instrument (SCIEX, Framingham, MA, U.S.A.). Sphingolipids containing C16:0, C18:0, C20:0, C22:0, C24:0, and C24:1 fatty acids were detected using a multiple reaction monitoring method.

Statistical Analysis All data were expressed as mean ± standard deviation (S.D.). Student's *t*-test was used for comparisons between the two groups. A p-value of <0.05 was set to denote statistically significant difference. All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, San Diego, CA, U.S.A.).

RESULTS

Establishment and Characterization of HEK293T Stable Cell Lines Expressing TurboID-Fused SMS Isoforms First, HEK293T stable cell lines were established expressing TurboID-fused SMS isoforms. Although the N- and C-termini of SMS isoforms face the same cytoplasmic side of the Golgi membrane, we have shown that the N- and C-termini of SMS have different functions in complex formation.[12,13] Therefore, we constructed chimeric protein expression vectors in which the biotinylation enzyme TurboID was connected to the N- and C-termini of the SMS isoforms (Fig. 1A). The localization of the chimeric proteins was then examined. Immunofluorescence staining and confocal microscopy demonstrated that TurboID-fused SMS1 was localized only in the perinuclear region, whereas TurboID-fused SMS2 was localized on the cell surface and concentrated in the perinuclear region (Fig. 1B). The perinuclear regions were colocalized with the *trans*-Golgi

network marker p230. These locations were consistent with the findings of a previous study, in which SMS1 was localized in the Golgi complex only, and SMS2 was localized in both the plasma membrane and Golgi complex.[12,13] These results show that the subcellular locations of SMS1 and SMS2 did not change by the addition of TurboID. TurboID alone showed cytoplasmic localization (data not shown).

Identification of Proteins in Close Proximity to SMS Isoforms To test TurboID-fused SMS isoforms in HEK293T cells, whole-cell lysates were analyzed by WB. The predicted molecular masses of the V5-tagged TurboID-fused SMS1 and SMS2 were 86 and 80 kDa, respectively, however, were detected at approximately 70 kDa, which was lower than expected (Fig. 2A). When TurboID-fused SMS isoforms with an HA tag at the N terminus and a V5 tag at the C terminus were expressed in HEK293T cells, chimeric proteins of approximately 70 kDa were detected using HA and V5 antibodies (Supplementary Fig. 1). These results indicate that the TurboID-fused SMS chimeric proteins detected at approximately 70 kDa were expressed as full-length proteins without being degraded in HEK293T cells. Streptavidin blotting was performed to evaluate the efficiency of TurboID-based proximity labeling (Fig. 2A). When biotin was added to the cell culture medium, biotinylated proteins increased, and the band pattern of biotinylated proteins was different in cells expressing TurboID-fused SMS isoforms compared to that in cells expressing only TurboID. These results indicate that TurboID-fused SMS isoforms function as intracellular biotinylation enzymes.

The biotinylated proteins were isolated using streptavidin-coated magnetic beads (Fig. 2B). We identified over 1700 biotinylated proteins isolated from TurboID-fused SMS, not from TurboID alone, by mass spectrometry. We focused on PLSCR1 among these proteins, since PLSCR1 was initially characterized as a transmembrane protein involved in the movement of phospholipids, such as phosphatidylserine and phosphatidylethanolamine in the bilayer[14–16]; however, its involvement in SM synthesis has not been investigated.

Silver staining and WB was used to visualize the isolated biotinylated proteins. Mass spectrometry revealed PLSCR1 in biotinylated proteins isolated from SMS isoforms with TurboID fused to the N- and C-termini; however, not from TurboID alone. These results were consistent with the WB results obtained using the PLSCR1 antibody (Fig. 2B).

Analysis of the PLSCR Family in HEK293T Cells The PLSCR family consists of five homologous proteins, named PLSCR1, PLSCR2, PLSCR3, PLSCR4, and PLSCR5. The mRNA expression levels of the PLSCR family members in the HEK293T cells were also determined. Absolute quantification using real-time PCR revealed that HEK293T cells expressed PLSCR1, 3, and 4, however, not PLSCR2 and 5 (Fig. 3A). To further examine the PLSCR family in HEK293T cells, we established HEK293T stable cell lines expressing PLSCR1, 3, or 4. Immunofluorescence staining using confocal microscopy demonstrated that PLSCR1 was localized on the cell surface and concentrated in the perinuclear region (Fig. 3B). The perinuclear regions were colocalized with the *trans*-Golgi network marker p230, suggesting that PLSCR1 was in close proximity to the Turbo-fused SMS isoforms in the Golgi apparatus. PLSCR4 showed a localization pattern similar to that of PLSCR1, whereas PLSCR3 was localized in the nucleus and cytoplasm.

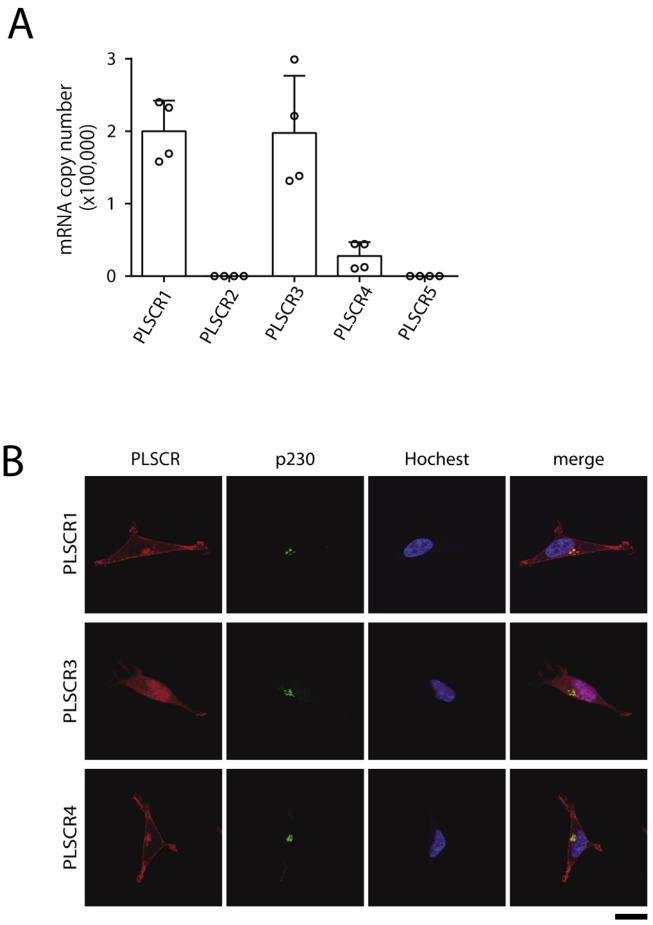

Fig. 3. Analysis of Expression Level and Expression Localization of PLSCR Gene Family

(A) Real-time PCR analysis for the absolute quantification of PLSCR gene family in HEK293T cells. Data are mean ± S.D. of four independent experiments. (B) HEK293T cell lines expressing stable PLSCR1, 3, or 4 were stained with each PLSCR antibody, followed by appropriate Alexa Fluor-conjugated secondary antibodies and analyzed by confocal microscopy. Localization was confirmed by co-staining with antibody against the *trans*-Golgi network marker p230. PLSCR, red; p230, green; Hochest, blue. Scale bar, 50 μm. Results shown are from one experiment representative of three independent experiments.

Establishment of PLSCR-KO HEK293T Cells To evaluate the involvement of the PLSCR family in the luminal transport of Cer during SM synthesis, we established PLSCR1-KO, PLSCR3-KO, and PLSCR4-KO HEK293T cells using the CRISPR/Cas9 system. We also prepared TKO cells by introducing a CRISPR/Cas9 vector containing PLSCR1 guide RNA followed by PLSCR3 guide RNA into PLSCR4-KO cells. Sequence analysis showed that deletions and insertions in the PLSCR family caused frameshift mutations (Fig. 4A). Furthermore, the loss of the PLSCR family was examined by WB analysis. As shown in Fig. 4B, immunoblotting revealed a loss of PLSCR1 and PLSCR3 expression in PLSCR1-KO and PLSCR3-KO cells, respectively. We also found that PLSCR1 and PLSCR3 expression was lost in PLSCR1-, 3-, and 4-TKO cells. However, the PLSCR4 antibody used in this study was able to detect overexpressed PLSCR4, but not endogenous levels in HEK293T cells.

Lipid Quantification of Sphingolipids by LC-MS/MS In mammalian cells, SM and Cer are composed of *N*-acyl chains of various lengths, including C16:0, C18:0, C20:0, C22:0, C24:0, and C24:1.[17] We quantified the SM and Cer levels in these cells using LC-MS/MS. The levels of all SM and Cer

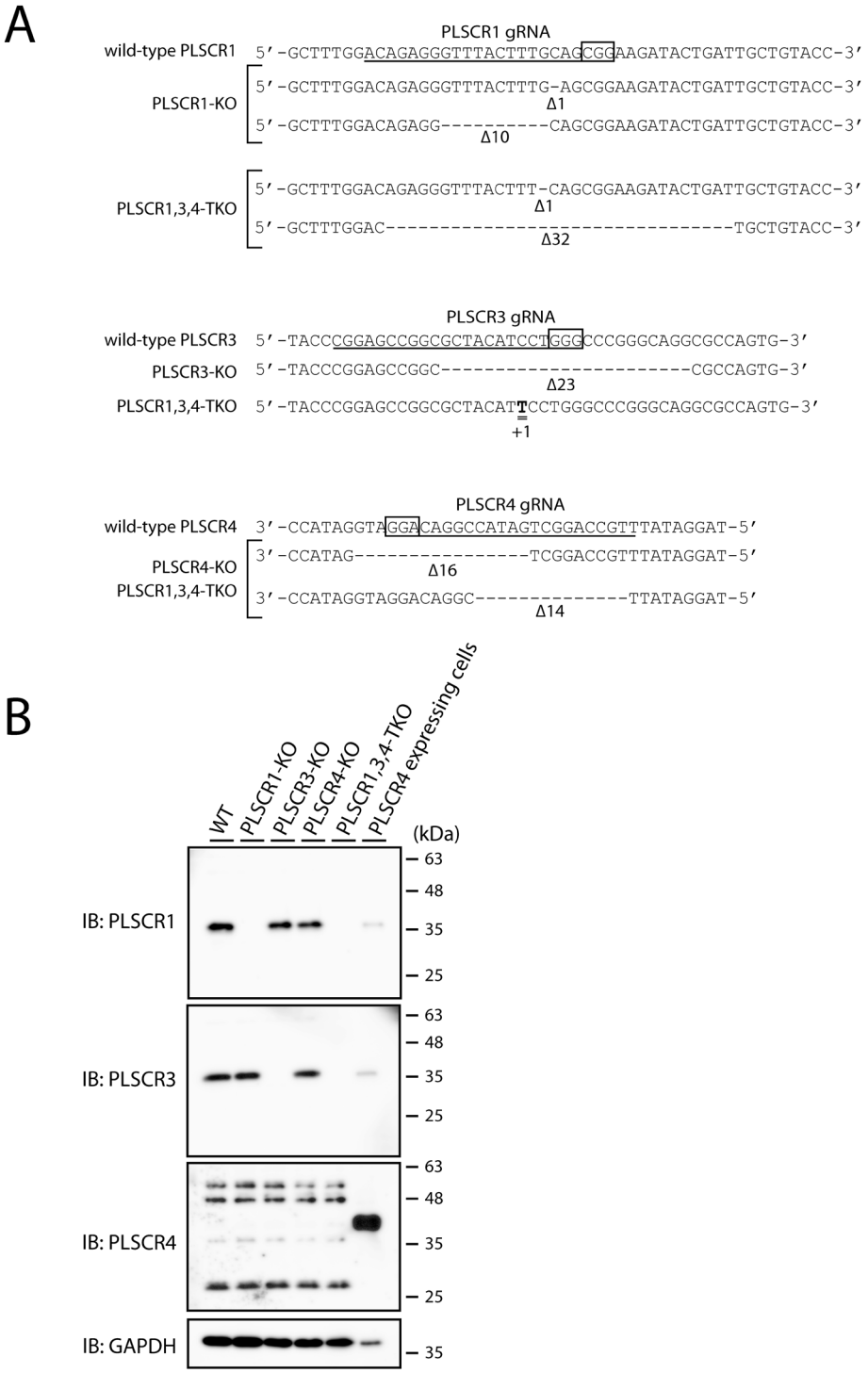

Fig. 4. Generation of CRISPR/Cas9-Based PLSCR1-KO, PLSCR3-KO, PLSCR4-KO, and PLSCR1,3,4-TKO Cells

(A) Genomic DNA were extracted from PLSCR1-KO, PLSCR3-KO, PLSCR4-KO, and PLSCR1,3,4-TKO cell lines, and a genomic region containing the target sites of the gRNAs was sequenced. The 20-bp target sequences and the PAM sequences are depicted. A bold letter and dashes depict the identified insertions and deletions, respectively. The numbers of insertions and deletions (+, insertions; Δ, deletions) are shown. (B) HEK293T (WT cells), PLSCR1-KO, PLSCR3-KO, PLSCR4-KO, PLSCR1,3,4-TKO, and HEK293T transfected with PLSCR4 cell lines were lysed and analyzed by immunoblotting with each PLSCR antibody. Anti-GAPDH was used as a loading control. Blots were obtained from one experiment representative of three independent experiments.

species with different acyl chains were not significantly different in the PLSCR1-KO, PLSCR3-KO, and PLSCR4-KO cells compared to those in the wild-type (WT) cells (Figs. 5A–L). Moreover, there was no difference in SM and Cer levels between PLSCR1, 3, and 4-TKO and WT cells. These results indicate that the PLSCR family is not involved in the luminal transport of Cer to the Golgi membrane for SM synthesis.

DISCUSSION

Because the active residues of the SMS isoforms are located on the lumen side of the Golgi membrane, Cer, on the surface of the Golgi membrane must be translocated to the lumen side for SM synthesis. In this study, PLSCR1 was identified as a proximal protein in the SMS isoforms

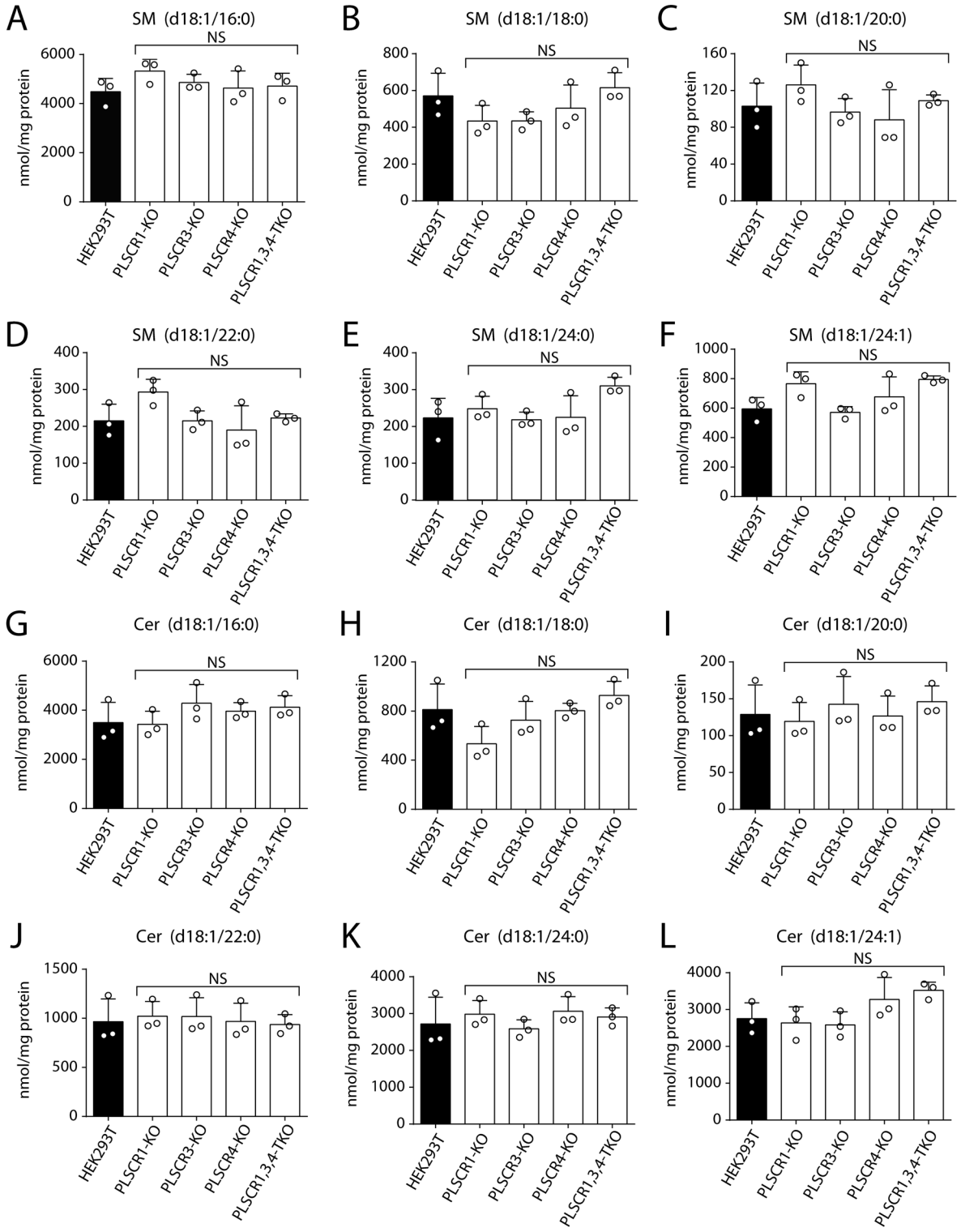

Fig. 5. Cellular SM and Cer Species in PLSCR1-KO, PLSCR3-KO, PLSCR4-KO, PLSCR1,3,4-TKO, and Wild-Type HEK293T Cells

The cellular levels of SM and Cer species with a distinct acyl chain were quantified using LC-MS/MS. Bar graphs show levels of SM (A–F) and Cer (G–L). Values represent the mean ± S.D. from three independent experiments. Statistical significance was determined using one-way ANOVA followed by Dunnett test for multiple comparisons; NS, not significant.

using proximity-dependent biotin identification proteomics. LC-MS/MS analysis showed no difference in the levels of Cer and SM between PLSCR1-KO and WT cells (Fig. 5). Furthermore, triple gene disruption of the PLSCR family members PLSCR1, PLSCR3, and PLSCR4 expressed endogenously in the HEK293T cells did not affect the levels of Cer and SM (Fig. 5). Since PLSCR1 was thought to be involved in the rapid Ca^{2+}-dependent redistribution of cell membrane phospholipids across the bilayer,[15] treatment of PLSCR1, 3, 4-TKO cells and stable PLSCR1-expressing cells with the calcium ionophore ionomycin did not change the levels of Cer and SM compared to WT cells (data not shown). Thus, the results indicate that the PLSCR family is not involved in Cer translocation during SM synthesis.

The physiological function of the proximity between SMS isoforms and PLSCR1 is unknown. Lipid rafts are detergent-resistant SM-rich microdomains of cellular membranes.[1] PLSCR1 is a component of the membrane lipid rafts that interact with activated epidermal growth factor (EGF) receptors.[18] Given that PLSCR1 promotes the EGF-dependent activation of c-Src through the EGF receptor,[19] interaction between PLSCR1 and SMS isoforms may be important for growth factor signaling. Further studies are required to elucidate the precise mechanisms underlying the interaction between SMS isoforms and PLSCR1.

Previously, we reported that homodimerization of SMS isoforms was responsible for their efficient export from the endoplasmic reticulum.[12] Consistent with results of a previous study, we detected SMS as an SMS proximal protein. Furthermore, using a biomolecular fluorescence complementation assay, we found that SMS1 and glucosylceramide synthase (GCS) formed a heteromeric complex in which the N-terminus of SMS1 and the C-terminus of GCS were in close proximity.[13] However, GCS was not detected as a biotinylated protein, similar to SMS1. Thus, there are two possible explanations for this discrepancy in the experimental results. The first is the absence of a lysine residue at the C-terminus of GCS, which is in close proximity to the N-terminus of SMS1. Since TurboID biotinylates the lysine residues of nearby proteins, it may not be able to efficiently biotinylate GCS. Second, heterocomplexes of SMS1 and GCS were not detected at endogenous levels of GCS expression in HEK293T cells. Further in-depth analysis will provide significant information on the formation mechanism of SMS1 and GCS heterocomplexes.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials This article contains supplementary materials.

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