PNPLA1 is a transacylase essential for the generation of the skin barrier lipid \(\omega\)-O-acylceramide

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Lipids are the primary components of the skin permeability barrier, which is the body’s most powerful defensive mechanism against pathogens. Acylceramide (\(\omega\)-O-acylceramide) is a specialized lipid essential for skin barrier formation. Here, we identify PNPLA1 as the long-sought gene involved in the final step of acylceramide synthesis, esterification of \(\omega\)-hydroxyceramide with linoleic acid, by cell-based assays. We show that increasing triglyceride levels by overproduction of the diacylglycerol acyltransferase DGAT2 stimulates acylceramide production, suggesting that triglyceride may act as a linoleic acid donor. Indeed, the \textit{in vitro} analyses confirm that PNPLA1 catalyses acylceramide synthesis using triglyceride as a substrate. Mutant forms of PNPLA1 found in patients with ichthyosis exhibit reduced or no enzyme activity in either cell-based or \textit{in vitro} assays. Altogether, our results indicate that PNPLA1 is directly involved in acylceramide synthesis as a transacylase, and provide important insights into the molecular mechanisms of skin barrier formation and of ichthyosis pathogenesis.

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The body surface epidermis forms a permeability barrier, which has essential roles in protecting terrestrial animals from invasion of pathogens and harmful substances such as allergens and pollutants as well as from internal water loss. Accordingly, several cutaneous disorders—such as ichthyosis, atopic dermatitis, infectious diseases and dry skin—are characterized by alterations or defects of this skin barrier. The principal compound family in the skin permeability barrier is lipids. Lipids form multi-layered structures (lipid lamellae) extracellularly in the stratum corneum, the outermost layer of the epidermis, and their high hydrophobicity inhibits the invasion of external materials and water loss from inside the body.

To carry out this special, barrier-creation function, lipid lamellae contain unusual lipids. Approximately half of stratum corneum lipids are ceramide, which is the backbone of sphingolipids, and epidermis-specific ceramide species such as acylceramide (ω-acylc polaramide) exist. Acylceramide is especially important for skin barrier formation. Loss of acylceramide due to mutations in acylceramide synthesis genes leads to autosomal recessive congenital ichthyosis (ARCI) in humans and neonatal lethality in mouse models, where gene loss causes similar skin barrier defects as in humans. Ichthyosis is characterized by dry, thickened and scaly skin, and the skin barrier defect in ARCI is the most severe among several types of ichthyoses.

The structure of acylceramide is quite unique. Although normal ceramides contain two hydrophobic chains, a long-chain base and a fatty acid (FA) with carbon chain-length of C16–24 (refs 6,14), acylceramide has an additional hydrophobic chain, linoleic acid (Fig. 1a). Furthermore, the chain-length of the FA moiety is extremely long (C28–C36) (refs 15,16). Therefore, acylceramide is one of the most hydrophobic lipids in mammalian bodies. The characteristic structure of acylceramide plays a pivotal role in organizing lipid lamellae. In addition, acylceramide is also important as a precursor of protein-bound ceramide.

Despite the physiological and pathological importance of acylceramide, the elucidation of the molecular mechanism by which it is created has not been completely resolved. Although recent studies have identified the genes involved in the acylceramide synthesis-specific reactions—such as the FA elongases ELOVL1 and ELOVL4, which are involved in the elongation of very-long-chain (VLC) FAs (VLCFAs; ≥C21) to ultra-long-chain (ULC) FAs (ULCFAs; ≥C26), the FA ω-hydroxylase CYP4F22, which hydroxylates the ω-carbon of ULCFAs, and the ceramide synthase CERS3, which catalyses an amide bond formation between a long-chain base and a ULCFA (refs 7,9,11,13,19,20) (Fig. 1a)—the gene involved in the final step of acylceramide production, that is, ester bond formation between ω-hydroxyceramide and linoleic acid, has not been identified. This means the molecular mechanism by which the skin barrier is created is still unclear.

Human cannot synthesize the acylceramide component linoleic acid. Therefore, linoleic acid is an essential FA that must be supplied from diet. Essential FA deficiency causes several skin symptoms including ichthyosis due to impairment of normal acylceramide production. Large decreases in or loss of acylceramide due to mutations in the genes involved in acylceramide synthesis (such as CERS3, CYP4F22 and ELOVL4) causes non-syndromic ARCI (CERS3 and CYP4F22) as described above, or a syndromic form of ichthyosis (ELOVL4) (refs 5,8,10,12,21). Furthermore, decreases in acylceramide levels are also observed in atopic dermatitis patients.

Many ichthyosis-causing genes have been identified, and some of them have been shown to be involved in acylceramide synthesis (as described above) or protein-bound ceramide production. However, there still remain several genes whose functions or pathogenic roles have not been revealed. For example, the functions of the ARCI-causative genes NIPAL4 (NIPA-like domain-containing protein 4) and PNPLA1 (patatin-like phospholipase domain-containing protein 1) are currently unclear.

In the present study, we aim to identify the missing gene responsible for the final step of acylceramide production (esterification)
bond formation between ω-hydroxyceramide and linoleic acid). Our results indicate that PNPLA1 encodes the transacylase that catalyzes acylceramide production using a triglyceride (TG) as the donor of the substrate linoleic acid. Thus, our findings provide important insights into the molecular mechanism of acylceramide production and into the function and pathogenic role of the ichthyosis-causative gene PNPLA1.

**Results**

**PNPLA1 is involved in acylceramide production.** To identify the acyltransferase or transacylase involved in acylceramide production, a proper assay system that can detect its activity or product is necessary. However, the prior lack of such assay systems meant there was no way of identifying the responsible acyltransferase/transacylase for a long time. Cell-based assays had been unsuccessful, since most mammalian cells cannot produce ULC ω-hydroxyceramides, the precursors of acylceramides. However, we recently established a cell system that produces ULC ω-hydroxyceramides by overexpressing the FA elongase ELOVL4, the ceramide synthase CERS3 and the FA ω-hydroxylase CYP4F22 in HEK 293T cells13, opening the door to identify the acyltransferase/transacylase of interest.

In this cell system, when lipids prepared from HEK 293T cells labelled with [3H]sphingosine, the sphingolipid precursor, were separated by normal phase thin layer chromatography (TLC), only long-chain (LC, C11–C20) and VLC ceramides were detected as ceramide species (Fig. 1b). Overexpression of CERS3 and ELOVL4 caused cells to produce ULC ceramides, and further co-overexpression of CYP4F22 leads to production of ULC ω-hydroxyceramides, as we have described previously13 (Fig. 1b). Using this cell system, we examined the involvement of several candidate genes in acylceramide production. The selected candidate genes were ABHD5 (ζ/β hydrolase domain containing 5)/CG5-8 (comparative gene identification-58), LIPN (lipase, family member N), PNPLA1, PLA2G15 (phospholipase A2, group XV)/LPL (lecithin:cholesterol acyltransferase-like lysophospholipase), LCAT (lecithin:cholesterol acyltransferase) and DGA2T (diacylglycerol O-acyltransferase 2). ABHD5 is a causative gene of Chanarin–Dorfman syndrome (also known as neutral lipid storage disease with ichthyosis (NLSD-I)), an ichthyosis-causative gene of Chanarin–Dorfman syndrome (also known as DGAT2 mice exhibit a skin-barrier-defect phenotype 30. To confirm that PNPLA1 is indeed involved in acylceramide production, we performed knockdown analysis using human keratinocytes and a lentiviral vector-encoding shRNA system.

Figure 2 | Knockdown of PNPLA1 causes impairment of acylceramide production. Keratinocytes were infected with lentivirus harbouring control shRNA or shPNPLA1 and were differentiated for 7 days. (a) Total RNAs prepared from differentiated keratinocytes were subjected to RT-PCR using primers specific for PNPLA1 and GAPDH. Uncropped scanned photographs of the electrocataphoresis gels are provided as Supplementary Fig. 2. (b) Cells were labelled with [3H]sphingosine for 6 h at 37 °C in the presence of 10 μM linoleic acid. Lipids were extracted, separated by normal-phase TLC and detected by autoradiography. AcylCer, acylceramide; Cer, ceramide; Acyl-GlcCer, acyl-glucosylceramide; GlcCer, glucosylceramide; SPH, sphingosine; SM, sphingomyelin. Two independent experiments gave similar results, and a representative result is shown. (c) Lipids were extracted and subjected to LC-MS/MS analysis. Acylceramides were detected in the MRM mode and quantified using MassLynx software. The graph depicts the amount of each acylceramide species with the indicated FA chain-length. The inset represents the total acylceramide levels. Values represent the means ± s.d.s of three independent experiments. Statistically significant differences compared to control cells are indicated (two-tailed Student’s t-test; *P < 0.05; **P < 0.01).
PNPLA1 is not involved in TG metabolism. Stimulation of acylceramide synthesis by DGAT2 suggests that TGs are involved in acylceramide production. We next examined the possibility that PNPLA1 also stimulates acylceramide synthesis through an increase in TG levels by \([^{14}\text{C}]\)linoleic acid labelling assay. DGAT2 expression increased TG levels as expected, whereas PNPLA1 had no effect (Fig. 3a). We next measured linoleic acid-containing TG levels by LC-MS/MS. The amounts of all of the TGs examined were increased by DGAT2 overexpression (Fig. 3b). In contrast, PNPLA1 again had no effect, whereas slight increases were observed for some TG species with shorter chain-lengths for unknown reasons. However, these slight changes might not be able to cause the acylceramide increase. Thus, these results suggest that PNPLA1 does not affect acylceramide production through increasing TG levels.

PNPLA1 belongs to the patatin-like phospholipase domain-containing protein (PNPLA) family. The PNPLA family members are known to exhibit phospholipase, TG hydrolase or transacylase activity, or a combination\(^{28}\). Considering these functions of the PNPLA family members, PNPLA1 was expected to be involved in acylceramide production as a TG hydrolase/phospholipase supplying linoleic acid from TG/phospholipids to an unknown acyltransferase, or as a transacylase catalysing acylceramide production directly using the linoleic acid in TGs as a substrate. If the former possibility were true, an increase in cellular linoleic acid levels caused by adding linoleic acid exogenously might bypass the otherwise required step of PNPLA1 in acylceramide production. However, addition of linoleic acid in the medium did not cause an increase in acylceramide in the absence of PNPLA1 (Fig. 4). On the other hand, linoleic acid stimulated acylceramide synthesis in a dose-dependent manner in cells overproducing PNPLA1 (Fig. 4). These results suggest that the role of PNPLA1 in acylceramide synthesis is not to supply linoleic acid as a TG hydrolase/phospholipase, and instead that PNPLA1 may be directly involved in acylceramide synthesis. The conclusion—that it is unlikely PNPLA1 acts as a TG hydrolase—is consistent with the MS data, which showed that PNPLA1 expression did not reduce TG levels (Fig. 3b).

PNPLA1 is a transacylase using TG as a substrate. To prove that PNPLA1 directly catalyses acylceramide production as a transacylase using TG as a substrate, we performed \textit{in vitro} assays. For this purpose, PNPLA1 was translated using a wheat germ cell-free translation system. Since PNPLA1 is a membrane protein\(^{32}\), we added liposomes to the translation reaction mixture. Recently, several membrane proteins have successfully been inserted directly into the lipid bilayer of liposomes by similar cell-free translation systems\(^{33,34}\). After translation of \textit{PNPLA1} mRNA, the resulting proteoliposomes were recovered by centrifugation and...
transacylase using TG as a substrate rather than an acyltransferase using linoleoyl-CoA.

Conclusion between PNPLA1 activity and ichthyosis pathology. PNPLA1 is one of the genes known to cause ARCI (refs 24,25). Two missense mutations, which cause amino acid substitution (A34T or A59V), and one nonsense mutation (E131X) have been found in the PNPLA1 of ichthyosis patients. The mutated residues (Ala34 and Ala59) are located in the patatin domain. We expressed wild type and mutant forms of PNPLA1 in HEK 293T cells together with ELOVL4, CERS3 and CYP4F22 and examined the expression and acylceramide production activities of these mutant PNPLA1 proteins. The point mutants PNPLA1 A34T and A59V were expressed at equivalent levels to the wild-type protein (Fig. 6a). [3H]Sphingosine labelling assay revealed that their activities were decreased to ~20% of wild-type protein activity (Fig. 6b). The nonsense mutant protein PNPLA1 E131X was detected as a truncated protein of 14 kDa (Fig. 6a). The acylceramide levels in PNPLA1 E131X-producing cells were indistinguishable from those in vector-transfected cells (Fig. 6b), indicating that the truncated PNPLA1 protein (E131X) had no acylceramide production activity.

Next, we directly measured the transacylase activities of the ichthyosis mutants of PNPLA1 in vitro. PNPLA1 mutants were properly expressed by the cell-free translation system in a manner equivalent to the wild-type protein (Fig. 6c). An acylceramide synthesis assay in the presence of TG and ω-hydroxyceramide revealed that both of the point mutants (PNPLA1 A34T and A59V) exhibited reduced activities compared to wild type. The truncated mutant E131X had no activity (Fig. 6d), consistent with the results obtained from the cell-based assay (Fig. 6b). These results show a clear relationship between PNPLA1 activity and ichthyosis pathology. Although it has been unclear why PNPLA1 mutations cause ichthyosis, our results suggest that decreases in acylceramide levels are the cause of skin barrier defects and lead to ichthyosis.

Discussion
Over 30 years have passed since acylceramide and acyl-glucosylceramide were discovered and their structures determined. However, the synthetic genes of acylceramide remained unclear for a long time. There had long been only limited knowledge about candidate genes involved in acylceramide production. However, recent determination of ichthyosis-causative genes gave a clue to the identification of acylceramide synthetic genes. For example, the ichthyosis-causative genes CERS3, ELOVL4 and CYP4F22 are all involved in acylceramide production. Over 30 years have passed since acylceramide and acyl-glucosylceramide were discovered and their structures determined. However, the synthetic genes of acylceramide remained unclear for a long time. There had long been only limited knowledge about candidate genes involved in acylceramide production. However, recent determination of ichthyosis-causative genes gave a clue to the identification of acylceramide synthetic genes. For example, the ichthyosis-causative genes CERS3, ELOVL4 and CYP4F22 are all involved in acylceramide production. In addition, we recently succeeded in establishing a cell system to produce ULC ω-hydroxyceramide, the substrate of acylceramide, by overproducing CERS3, ELOVL4 and CYP4F22 in HEK 293T cells. Using this system again here, we revealed that the ARCI-causative gene PNPLA1 is involved in acylceramide production (Fig. 1b–d). Furthermore, we demonstrated that PNPLA1 catalyses the transacylation of the linoleic acid portion of TG to ULC ω-hydroxyceramide for acylceramide production in vitro (Fig. 5). Murakami and his colleagues reached the same conclusion using Pnpla1 knockout mice. Their mice exhibited neonatal lethality due to skin barrier defects. Acylceramide was not produced in Pnpla1 knockout mice, but its substrate ULC ω-hydroxyceramide accumulated. During the preparation of our manuscript, similar in vivo results were also reported by another group. In that study, decreases in acylceramide levels in the differentiated keratinocytes from an ARCI patient were also reported. Taking our cell-assay-based and biochemical in vitro
results with the in vivo results from the two groups, we conclude that PNPLA1 plays an essential role in the final step of acylceramide production, esterification of ULC ω-hydroxy-acylglycerol and linoleic acid. Since acylceramide is essential to maintain skin barrier integrity, our findings constitute important information by which we can understand the molecular mechanisms behind skin barrier formation. At present, there are no therapeutic agents for the causal treatment of ichthyosis or atopic dermatitis. Elucidation of the molecular mechanisms behind skin barrier formation may lead to the development of such new therapeutic medicines.

Methods
Cell culture and transfection. HEK 293T cells and Lenti-X 293T cells (Takara Bio, Shiga, Japan; catalogue number, 632180) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin, and were grown in dishes pre-coated with 0.1 mg ml⁻¹ collagen (Cellmatrix type I-P; Nitta Gelatin, Osaka, Japan). Transfections were performed using ViaFect Transfection Reagent (Promega, Madison, WI, USA), according to the manufacturer’s instructions. Human keratinocytes (CELLnTEC, Bern, Switzerland; catalogue number, HPEKp) were cultured in CnT prime Epidermal Keratinocyte Medium (CELLnTEC). Differentiation was induced by incubating the cells with GmPt-Prime 3D Barrier Medium (CELLnTEC).

Plasmids. The mammalian expression vector pCE-puro 3xFLAG−1 is derived from the mammalian expression vector pCE-3xFLAG-ABHD5 and designed for N-terminal 3xFLAG-tagged protein production41. Human ABHD5, LIPN, PNPLA1, PLA2G15, LCAT and DGAT2 genes were amplified by reverse transcription (RT)-PCR using their respective forward (F) and reverse (R) primers listed in Supplementary Table 1. The PCR fragments were first cloned into pGEM-T Easy Vector (Promega) and then transferred to the pCE-puro 3xFLAG−1 vector, producing the pCE-puro 3xFLAG−ABHD5, pCE-puro 3xFLAG−LIPN, pCE-puro 3xFLAG−PLA2G15, pCE-puro 3xFLAG−LCAT and pCE-puro 3xFLAG−DGAT2 plasmids. The pCE-puro 3xFLAG−ELOVL4, pCE-puro 3xFLAG−CERS3 and pCE-puro 3xFLAG−CYP4F22 plasmids encoding 5α-15α-hydroxyceramide, ULC−OH−Cer and 5α-15α-hydroxyceramide production, respectively, were described previously11,13. Ichthyosis mutations were introduced into the PNPLA1 gene using the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA), using the primers described in Supplementary Table 1. For in vitro transcription, wild-type and mutant versions of PNPLA1 tagged with 3xFLAG at their 5′-termini were cloned into the pEUK−1 (1x3-FLAG-PNPLA1, pEUK−1x3-FLAG-PNPLA1(A34T), pEUK−1x3-FLAG-PNPLA1(A59V) and pEUK−1x3-FLAG-PNPLA1(E131X)) plasmids.

Lipid labelling assay. HEK 293T cells were transfected with plasmids according to test group. Twenty-three hours and thirty minutes after transfection, medium was changed to DMEM (without FBS). After 30 min incubation, cells were labelled with 0.2 µCi [14C]Linoleic acid (55 mCi mmol⁻¹) (PerkinElmer Life Sciences, Waltham, MA, USA) for 6 h at 37 °C or 0.5 µCi [14C]Linoleic acid (55 mCi mmol⁻¹), American Radiolabeled Chemical, St Louis, MO, USA) for 4 h at 37 °C. In the case that cold linoleic acid (Sigma) was added to the medium, the addition was done at the same time as labelling. After washing with 1 ml of PBS, cells were suspended in 150 µl of chloroform/methanol (2:1, vol/vol), 75 µl of chloroform and 125 µl of 1% KCl successively. Phases were then separated by centrifugation (20,000g, room temperature, 3 min).

PNPLA1 activity is impaired in ichthyosis-causing mutants.

(a) HEK 293T cells were transfected with the plasmids encoding 3xFLAG−ELOVL4, 3xFLAG−CERS3, 3xFLAG−CYP4F22 and 3xFLAG−PNPLA1 (wild type (WT) or mutant) as indicated. (b) Total cell lysates were separated by SDS-PAGE and subjected to immunoblotting with anti-FLAG antibody. (c) Cells were labelled with [3H]inositol in the presence of 25 µM linoleic acid for 6 h at 37 °C. Lipids were extracted, separated by normal-phase TLC and detected by autoradiography. AcylCer, acylceramide; Cer, ceramide; GlcCer, glucosylceramide; SM, sphingomyelin; SPH, sphingosine; ω-OH−Cer, ULC ω-hydroxy-acylglycerol. Three independent experiments gave similar results, and a representative result is shown. (d) Genes on control plasmid (pEU-E01-T1R1) or plasmid encoding wild type (WT) were amplified by reverse transcription (RT)-PCR using their respective forward (F) and reverse (R) primers listed in Supplementary Table 1. The PCR fragments were first cloned into pGEM-T Easy Vector (Promega) and then transferred to the pCE-puro 3xFLAG−1 vector, producing the pCE-puro 3xFLAG−ABHD5, pCE-puro 3xFLAG−LIPN, pCE-puro 3xFLAG−PLA2G15, pCE-puro 3xFLAG−LCAT and pCE-puro 3xFLAG−DGAT2 plasmids. The pCE-puro 3xFLAG−ELOVL4, pCE-puro 3xFLAG−CERS3 and pCE-puro 3xFLAG−CYP4F22 plasmids, encoding 5α-15α-hydroxyceramide, ULC−OH−Cer and 5α-15α-hydroxyceramide production, respectively, were described previously11,13. Ichthyosis mutations were introduced into the PNPLA1 gene using the QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA), using the primers described in Supplementary Table 1. For in vitro transcription, wild-type and mutant versions of PNPLA1 tagged with 3xFLAG at their 5′-termini were cloned into the pEUK−1 (1x3-FLAG-PNPLA1, pEUK−1x3-FLAG-PNPLA1(A34T), pEUK−1x3-FLAG-PNPLA1(A59V) and pEUK−1x3-FLAG-PNPLA1(E131X)) plasmids.

PNPLA1, by overproduction of DGAT2 (Fig. 3) indirectly enhances acylceramide production. Dgat2 knockout mice exhibit a skin-barrier-defect phenotype22, although the mechanism remains unclear. From our results, we speculate that the decreased TG causes impairment of acylceramide synthesis, leading to the skin barrier defect.

PNPLA1 is known as an ARCI-causing gene24,25. However, it was previously unclear by what mechanism PNPLA1 mutations cause ARCI. In the present study, we revealed that the PNPLA1 mutant proteins exhibited weak or no acylceramide production activity (Fig. 6). Thus, our results suggest that the pathology of ARCI associated with PNPLA1 mutation is caused by reduced acylceramide levels. Similar correlations among ichthyosis pathology, enzyme activities and acylceramide levels have been observed for ARCI caused by CERS3 and CYP4F22 mutations as well10,13.

Our results indicate that PNPLA1 is the transacylase that acts at the final step of acylceramide production, esterification between ULC ω-hydroxy-acylglycerol and linoleic acid. Since acylceramide is essential to maintain skin barrier integrity, our findings constitute important information by which we can understand the molecular mechanisms behind skin barrier formation. At present, there are no therapeutic agents for the causal treatment of ichthyosis or atopic dermatitis. Elucidation of the molecular mechanisms behind skin barrier formation may lead to the development of such new therapeutic medicines.

Figure 6 | PNPLA1 activity is impaired in ichthyosis-causing mutants.
The resulting organic (lower) phase was recovered, dried and dissolved in chloroform/methanol (2:1, vol/vol). Lipids were separated by normal-phase TLC (Silica Gel 60 TLC plates; Merck Millipore, Darmstadt, Germany). The following three TLC solvent systems were used sequentially: (i) chloroform/methanol/water (40:10:1, vol/vol/vol), developed to 2 cm from the bottom, dried and developed to 5 cm from the bottom; (ii) chloroform/methanol/acetic acid (47:20:5, vol/vol/vol), developed to the top; and (iii) hexane/diethylether/acetic acid (65:35:1, vol/vol/vol), developed to the top. Labelled lipids were visualized by spraying a fluorographic reagent (4 mg ml\(^{-1}\) 2.5-diphenyl-oxazole in 2-methyl-naphthalene) toluene (9,1 vol, vol) and exposing to X-ray film at \(80^\circ\) C.

Lipid analysis by LC-MS/MS. Acylceramides containing sphingosine (EoS; esterified omega-hydroxy-acyl-sphingosine) and TGs containing linoleic acid were quantified by LC-MS/MS. After washing with PBS, cells were suspended in 100 \(\mu\)l of PBS and subjected by successive addition and mixing of chloroform/methanol/12 M formic acid (100:200:1, vol/vol/vol). Lipids were separated by normal-phase TLC (N-octane/ethyl acetate/125 M formic acid (30:100:1, vol/vol/vol)) and exposing to X-ray film at \(80^\circ\) C. The culture medium was replaced with 0.5 ml of the same medium but containing 10 \(\mu\)g ml\(^{-1}\) polybrene (Nacalai tesque, Kyoto, Japan). Six hours after incubation, the medium was changed to the one without the virus solution and polybrene, and incubated for 3 days. Differentiation was induced by incubating the proteoliposomes for 1 h at 37 \(^\circ\) C. The culture medium was replaced with 0.5 ml of the same medium but containing 10 \(\mu\)g/ml of 2,5-diphenyl-oxazole in 2-methyl-naphthalene. The pNS72 plasmid encoding shRNA target sequence (shPNPLA1) was constructed as follows. Oligo DNAs containing the PNPLA1 shRNA target sequence (shPNPLA1-F and -R) were annealed and cloned into pAK1072, the vector for shRNA production under the U6 promoter, generating the pNS56 plasmid. The U6-shPNPLA1 region in the pNS56 plasmid was digested and two copies of the digested fragment were tandemly inserted into the pNS56 plasmid, generating pNS68 plasmid.

**Gene knockdown.** The lentiviral vector pNS64 was constructed by modifying the restriction sites of pGFP-C3-hShLent ( OriGene Technologies, Rockville, MD, USA). The pNS64 plasmid was extracted and cloned into pShP1A (Supplementary Table 1) and annealed and cloned into pAK1072, the vector for shRNA production under the U6 promoter, generating the pNS64 plasmid. Lipid extraction and quantification of the C30:0 acylceramide EOS by LC-MS/MS were performed as follows. Oligo DNAs containing the PNPLA1 shRNA target sequence (shPNPLA1-F and -R) were annealed and cloned into pAK1072, the vector for shRNA production under the U6 promoter, generating the pNS64 plasmid. The pNS64 plasmid was digested by sequential digestion of the C30:0 acylceramide EOS (N-[30-linoleoyltriacyl-sn-glycerol)-3-phosphocholine; Avanti Polar Lipids) with 20 \(\mu\)g TG (trilinolein; Sigma), 20 \(\mu\)g linoleoyl-CoA (Avanti Polar Lipids), and 20 \(\mu\)g C0:0 co-acyl-diacetylglycerol (N-octyl-hydroxyoctanoyl-d-ceramidoyl-sphingosine; Matreya) as necessary] were dried in glass tubes. The dried lipid films were suspended in 80 \(\mu\)l of 1xSUB-AMIX SGC, incubated for 1 h at room temperature, and sonicated for 5 min.

**Immunoblotting.** After separation by SDS-PAGE, proteins were electrotransferred to an Immobilon PVDF membrane (Millipore, Billerica, MA, USA). The membrane was incubated with an HRP-conjugated anti-mouse IgG (Bio-Rad), developed with 75,000 volume dilution; GE Healthcare Life Sciences were detected on an X-ray film at \(80^\circ\) C for 1 h at room temperature, followed by washing with TBS-T three times. Labeling was performed using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific).

**RT-PCR.** Total RNAs were isolated from cells using Nucleospin RNA (Takara Bio). RT-PCR was performed using a PrimeScript One Step RT-PCR Kit (Takara Bio). Specific primer sets for PNPLA1, PNPLA1-H and -R; for GAPDH, GAPDH-F and -R; Supplementary Table 1), according to the manufacturer's protocols.
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