PNPLA1 has a crucial role in skin barrier function by directing acylceramide biosynthesis

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Mutations in patatin-like phospholipase domain-containing 1 (PNPLA1) cause autosomal recessive congenital ichthyosis, but the mechanism involved remains unclear. Here we show that PNPLA1, an enzyme expressed in differentiated keratinocytes, plays a crucial role in the biosynthesis of $\omega$-O-acylceramide, a lipid component essential for skin barrier. Global or keratinocyte-specific $Pnpla1$-deficient neonates die due to epidermal permeability barrier defects with severe transepidermal water loss, decreased intercellular lipid lamellae in the stratum corneum, and aberrant keratinocyte differentiation. In $Pnpla1^{-/-}$ epidermis, unique linoleate-containing lipids including acylceramides, acylglucosylceramides and (O-acyl)-$\omega$-hydroxy fatty acids are almost absent with reciprocal increases in their putative precursors, indicating that PNPLA1 catalyses the $\omega$-O-esterification with linoleic acid to form acylceramides. Moreover, acylceramide supplementation partially rescues the altered differentiation of $Pnpla1^{-/-}$ keratinocytes. Our findings provide valuable insight into the skin barrier formation and ichthyosis development, and may contribute to novel therapeutic strategies for treatment of epidermal barrier defects.
The skin barrier of terrestrial mammals is essential for prevention of water and electrolyte loss, as well as protection from the penetration of harmful substances and pathogenic microbes. Impairment of skin barrier function can cause or aggravate skin disorders, including dry skin, ichthyosis, psoriasis, and atopic dermatitis. Although the epidermis is a highly organized stratified epithelium consisting of four distinctive layers, the innermost stratum basale (SB), the stratum spinosum (SS), the stratum granulosum (SG) and the uppermost stratum corneum (SC), its barrier function is provided mainly by specialized structures in the SC and tight junctions in the SG. The unique SC components include cross-linked, insoluble proteins of corneocytes forming the cornified envelope (CE) and its associated, external membrane monolayer, called the cornified lipid envelope (CLE), as well as the intercellular lipid lamellae, which are mainly composed of ceramides, cholesterol and free fatty acids (FFAs) and are secreted as lamellar body lipids by differentiated keratinocytes at the SG/SC interface. Among the epidermal ceramides with marked molecular heterogeneity (at least 12 classes in humans), acylceramide is essential for physical and functional organization of lipids in the SC interstices, and thereby the barrier function of the skin. Impaired biosynthesis or processing of acylceramide causes ichthyosis, characterized by dry, scaly and thickened skin. Acylceramide is an unusual ceramide species whose N-acyl chain is composed of ω-hydroxylated ultra-long chain FAs (ULCFAs) esterified at the ω-position with linoleic acid (LA; C18:2). It has been suggested that the ULCFA portion of acylceramide spans a bilayer while the LA tail inserts into a closely apposed section of bilayer, thus serving as a molecular rivet to link two membranes together in the lipid lamellae. In addition, acylceramides containing ω-Ω-esterified fatty acids other than LA cannot be converted to covalently protein-bound ω-hydroxyacylceramide (Cer OS), which forms CLE and functions as a template on the surfaces of corneocytes for the organization of lipid layers in the SC interstices. Indeed, in essential FA deficiency, LA in acylceramide is replaced by oleic acid, which fails to support skin barrier function properly. Until now, several important steps for acylceramide biosynthesis and processing in the epidermis have been identified from studies of autosomal recessive congenital ichthyosis (ARCI) in humans and corresponding mouse models with genetic knockouts: the synthesis of ULCFAs by the FA elongase ELOVL4, ω-hydroxylation of ULCFAs by the FA ω-hydroxylase CYP4F22 (or CYP4F39 in mice), and formation of ceramides with ULCFAs by the ceramide synthase CERS3. However, the mechanism underlying the formation of acylceramide with LA in the epidermis is still under debate.

The current model for ω-Ω-esterification of ULCFA-ceramides with LA involves the hydrolysis of triacylglycerol (TG) in lipid droplets to provide LA via a yet unknown lipase, followed by its transfer (via acyl-CoA form) to the ω-hydroxy group of ULCFA in ceramides or glucosylceramides (GlCer) by a putative ω-O-acyltransferase. Alternatively, LA can be directly transferred from TG to ω-Ω-Cer and/or ω-Ω-GlcCer by an LA-specific transacylase. Recent studies of patients with neutral lipid storage disease with ichthyosis (NLSDI or Chanarin-Dorfman syndrome) and in mice with Abhd5 deletion suggest that TG accumulation due to loss-of-function of ABHD5 (also known as CGI-58) reduces the availability of LA for acylceramide production. ABHD5 is an essential co-factor for stimulation of ATGL (adipose triglyceride lipase, also known as PNPLA2 or iPLA2), which plays a major role in TG hydrolysis in most tissues. ATGL is a member of the patatin-like phospholipase domain-containing protein (PNPLA) or Ca2+-independent phospholipase A2 (iPLA2) family, which comprises 9 enzymes in humans acting as lipid hydrolases, acyltransferases or transacylases with diverse substrate specificities including phospholipids and neutral lipids. Interestingly, ichthyosis features and decreased acylceramide levels in the skin have been observed in patients and mice with defective ABHD5 function, but not in those with ATGL mutations or deletion, leading to the proposal that ABHD5 could activate a different lipase that regulates epidermal TG hydrolysis. Nonetheless, the molecular entity of ω-O-acyltransferase or transacylase responsible for the linoleoyl ω-O-esterification of ULCFA-ceramides has not yet been identified.

Loss-of-function mutations in PNPLA1, a paralog of ATGL/PNPLA2, have recently been identified in humans or dogs with ARCI (refs 27–29). PNPLA1 fails to hydrolyse TG, however, even in the presence of ABHD5 (ref. 29), raising the question of the role of this functionally orphan enzyme. How lipid metabolism regulated by PNPLA1 contributes to epidermal homeostasis is a fundamental issue which remains to be addressed. To this end, we herein generated Pnpla1-deficient mice in our ongoing efforts to decipher the biological roles of PLA2-related enzymes by gene targeting. We provide evidence that absence of PNPLA1 causes a severe skin permeability barrier defect by perturbing the linoleoyl ω-O-esterification of ceramides to yield acylceramides, along with abnormal differentiation of keratinocytes, thus demonstrating that PNPLA1 is a long-sought enzyme that plays a critical role in acylceramide synthesis in the skin.

Results

Expression of PNPLA1 in highly differentiated keratinocytes. Among adult mouse tissues, Pnpla1 messenger RNA (mRNA) was expressed most abundantly in the skin, followed by the stomach (Supplementary Fig. 1a). Immunohistochemistry of newborn mouse skin revealed localization of PNPLA1 protein in the boundary area between the nucleated SG and the denucleated SC, just above the location of the granular layer marker loricrin, in the epidermis (Supplementary Fig. 1b). In agreement with a previous report, PNPLA1 was partially colocalized with filaggrin (a SG marker), but not with keratin 1 and 5 (SS and markers, respectively) (Supplementary Fig. 1b). In adult mouse skin, the localization of PNPLA1 in the epidermis was essentially the same as that in newborn skin (Supplementary Fig. 1c). In a monolayer culture of mouse keratinocytes, Ca2+-induced treatment resulted in marked induction of keratinoctye differentiation markers (Krt10 and Loricrin), as well as Pnpla1 (Supplementary Fig. 1d). Likewise, a marked increase of PNPLA1 expression was observed in human keratinocytes after Ca2+-induced differentiation, an event that occurred in parallel with induced expression of the keratinoctye differentiation markers KRT1 and FLG, but not with constitutive expression of the SB marker KRT5 (Supplementary Fig. 1e). These results suggest that PNPLA1 has a specific role in highly differentiated keratinocytes in the uppermost layer of the SG, where lipids required for epidermal barrier function are processed and secreted into the intercellular space to form lipid lamellae and CLE.

Impaired epidermal permeability barrier in Pnpla1−/− mice. To gain insight into the function of PNPLA1 in vivo, we generated mice with targeted disruption of the Pnpla1 gene on a C57BL/6 background (Supplementary Fig. 2a,b). The absence of mRNA and protein for PNPLA1 in the skin of Pnpla1−/− mice was confirmed by quantitative PCR (qPCR) (Supplementary Fig. 2c) and immunohistochemistry (Fig. 1a), respectively. Offspring from heterozygote intercrosses were born at the
expected Mendelian ratio (Supplementary Fig. 2d). Although Pnpla1+/– mice were healthy and indistinguishable from Pnpla1+/+ mice, newborn Pnpla1–/– pups had shiny and taut skin, often with a necrotic tail tip (Fig. 1b; Supplementary Fig. 2e), and died within 24 h after birth. We hypothesized that the cause of death in these Pnpla1-deficient mice might be dehydration, and therefore we assessed their skin permeability barrier function. Pnpla1+/– pups delivered by Caesarean section at E18.5 had normal body weight as compared with littermate wild-type (WT) and heterozygous mice at birth, but rapidly lost as much as 20% of their weight within 16 h (Fig. 1c). In accordance with this steep weight loss, trans-epidermal water loss (TEWL) was markedly higher in Pnpla1–/– newborns than in Pnpla1+/+ and Pnpla1+/– newborns (Fig. 1d), indicating a severe defect of the inside-out barrier in the null mice. In the toluidine blue exclusion assay to assess the outside-in permeability barrier, WT littermates excluded dye, whereas Pnpla1–/– pups showed robust dye penetration into the skin (Fig. 1e). These phenotypes, which have been commonly observed in mutant mice with disruption of genes associated with ARCI (ref. 1), suggest that PNPLA1 is required for epidermal permeability barrier function.

Histological analysis revealed that control mice had a clear basket weave-like structure segregated by interspaces, indicative of the presence of lipid lamellae (Fig. 1f, left). In contrast, Pnpla1–/– mice exhibited a tightly packed structure in the SC, a reduced number of keratohyaline granules in the uppermost SG,
and epidermal hyperplasia (Fig. 1f, right), which is considered to be an adaptive response to barrier disruption. Nile red staining of the Pnpla1-/-/+ epidermis showed wavy lipid multilayers characteristic of SC intercellular lipid lamellae, whereas granular-like lipid aggregates were present within increased number of densely packed lamellar sheets in the Pnpla1-/-/- epidermis (Fig. 1g), suggesting that keratinocytes are hyperproliferative and defective in the secretion and/or composition of SC lipids in mutant mice. Ultrastructural examination of Pnpla1-/-/- mice by transmission electron microscopy confirmed the tightly stacked layers of corneocytes with a substantially decreased amount of intercellular lipid lamellae, as evidenced by narrowed SC interstices, in comparison with control mice (Fig. 1h). At the SC–SC interface of Pnpla1-/-/+ and Pnpla1-/-/- epidermis, lipid lamellae were released into the intercellular spaces from lamellar bodies (Fig. 1h). In contrast, the secretion of lipid granule contents was hampered and abnormal vesicular structures, which were thought to represent defective lamellar bodies, were retained within corneocytes in Pnpla1-/-/- epidermis (Fig. 1h). Moreover, Pnpla1-/-/- mice exhibited either less or abnormalities of the CLE (Fig. 1i). These results indicate that PNP1A1 plays a critical role in the proper formation of intercellular lipid lamellae and CLE in the SC, which are important for the permeability barrier function of the skin.

### Aberrant keratinocyte differentiation in Pnpla1-/-/- mice.

To further address the skin abnormalities in Pnpla1-/-/- mice, we performed microarray gene profiling using skins of newborn Pnpla1-/-/+ and Pnpla1-/-/- mice. Heat map visualization of selected genes indicated down-regulation of genes for late keratinocyte differentiation and CÉ constituents (for example, Lor, Flg, Flg2, Pnpla1, Cldn4, Cldn7, Dsc1, Dsc2, Ocln, Tjp1 and Tjp2), lipid metabolism (for example, Fasn, Scd1, Pla2g4d and Pla2g4e) and skin-associated immune responses (for example, Il1b, Il12a, Il13, Il20, Il12, Il23a, Tnf, Ifng and Cxcl1) (Supplementary Fig. 3a). It is likely that the enhanced expression of inflammatory cytokines and chemokines is a secondary effect resulting from impaired barrier function, since similar changes have also been observed in several genetically distinct mouse models with barrier defects and patients with skin diseases such as ichthyosis, atopic dermatitis and psoriasis. Interestingly, expression levels of genes associated with synthesis and processing of epidermal acylceramide were consistently elevated in Pnpla1-/-/- mice relative to Pnpla1-/-/+ and Pnpla1-/-/- mice (Supplementary Fig. 3a,b). These genes included Elov4, Abhd5, Cers3, Gypf39 (a mouse ortholog of human CYP4F22), Uggc, Abca12 and Gba, mutation or deletion of which has been shown to cause ARCI in humans and neonatal death in mice due to severe skin barrier defects.

Immunofluorescence staining and qPCR confirmed the diminished expression of terminal differentiation markers, such as filaggrin (Flg) and loricrin (Lor), in Pnpla1-/-/- skin relative to Pnpla1-/-/+ skin, whereas mRNA and protein expression levels of the basal and early suprabasal keratinocyte differentiation markers, keratin 5 (Krt5) and 1 (Krt1), were similar between the two genotypes (Fig. 2a,b). In contrast, keratin 6 (Krt6a and Krt6b) was expressed in the lower suprabasal layer in Pnpla1-deficient but not in control skin, reflecting the hyperproliferative state of the mutant epidermis. Abnormal differentiation of keratinocytes has also been observed in several mouse lines with targeted disruption of genes implicated in epidermal ceramide metabolism and altered keratinocyte differentiation in the mutant mice.

Moreover, expression of PPARδ (Ppard) and its potential target genes such as Fabp5 and Sprr1b (ref. 35) was markedly increased in Pnpla1-/-/- skin relative to WT skin (Fig. 2c, Supplementary Fig. 3a), indicating that PNP1A1 deficiency leads to hyperactivation of PPARδ. Activation of EGF receptors has been shown to control keratinocyte proliferation and differentiation with decreased expression of differentiation-related genes including filaggrin and loricrin. Indeed, heparin-binding EGF-like growth factor (HB-EGF), a potent autocrine growth factor for keratinocytes and putative target gene of PPARδ (ref. 43), was robustly upregulated in Pnpla1-/-/- epidermis (Fig. 2c; Supplementary Fig. 3a), suggesting that EGF receptor signalling contributes, at least in part, to epidermal hyperplasia and altered keratinocyte differentiation in the mutant mice.

### Defective acylceramide biosynthesis in Pnpla1-/-/- skin.

To identify the endogenous lipid metabolism regulated by PNP1A1, we performed thin-layer chromatography (TLC) and quantitative liquid chromatography mass spectrometry (LC–MS/MS) using epidermal lipids extracted from neonatal WT and mutant mice. TLC analysis revealed that the bands for acylceramide (esterified omega-hydroxyacyl-sphingosine; EOS), which is a key determinant of skin permeability barrier function, and its derivative acylglycosylceramide (GlEcOS) were markedly reduced or almost undetectable in Pnpla1-/-/- mice relative to WT and heterozygous mice (Fig. 3a). We also noticed that another lipid species, with a TLC motility slightly faster than that of FA, was nearly absent in mutant mice, and LC–MS/MS analysis with collision-induced fragmentation of this lipid extracted from the TLC plate identified it as (O-acetyl)-omega-hydroxy FA (OAHFA), particularly (O-linoleoyl)-omega-hydroxy FA (OLHFA) (see below). In contrast, O-omega-hydroxy FA (O-FA) and O-omega-Cer and GlcCer were present in substantially greater amounts in Pnpla1-/-/- mice than in control Pnpla1-/+ and Pnpla1-/-/+ mice (Fig. 3a).

To determine the changes in ceramide molecular species in terms of the length and saturation of their N-acyl chains, lipids extracted from Pnpla1-/+ and Pnpla1-/-/- epidermis were analysed quantitatively by LC–MS and LC–MS/MS. Epidermal ceramide species are grouped into non-hydroxylated ceramides (NS, NDS, NH and NP), omega-hydroxylated ceramides (for example, AS, ADS, AH and AP) and acylceramides (for example, EOS, EOH and EOP), where S, P, DS and H stand for sphingosine, phytosphingosine, dihydrophosphosine and 6-hydroxyphosphosine, respectively. EOS and EOP species with residues of (O-linoleoyl)-omega-hydroxy ULCFAs (C28–C38) were almost entirely lost in the epidermis of Pnpla1-/-/- mice (Fig. 3b; quantitative data for representative molecular species are depicted in Supplementary Fig. 4a,b). Correspondingly, there was marked accumulation of various molecular species of O-omega-Cer, a putative precursor of EOS, in mutant mice relative to WT mice (Fig. 3c). In addition, in mutant mice, the amounts of OLHFA species with C28–C36 ULCFAs were markedly decreased, with reciprocal increases in corresponding O-omega ULCFA species (Fig. 3d,e), confirming the results of TLC analysis (Fig. 3a).
Moreover, the amount of Cer OS covalently bound to the CE was robustly reduced in mutant mice relative to WT mice (Supplementary Fig. 4c). In contrast to the dramatic reductions of acylceramides and their downstream products, various ceramide molecular species (AS, AP, NS, NH and NP) were of acylceramides and their downstream products, various (Supplementary Fig. 4c). In contrast to the dramatic reductions of acylceramides and their downstream products, various ceramide molecular species (AS, AP, NS, NH and NP) were robustly reduced in mutant mice relative to WT mice (Fig. 3a; Supplementary Fig. 5a). Since a proper ratio of ceramide molecular species (AS, AP, NS, NH and NP) were required for linoleoyl ω-O-esterification of the free and/or ceramide-bound forms of ω-OH ULCFA residues. Interestingly, the linoleate residue of several, if not all, EOS and OAHFA species was partially replaced by the palmitate or oleate residue in the linoleate residue of several, if not all, EOS and OAHFA ceramide-bound forms of ω-OH ULCFA residues. Interestingly, the linoleate residue of several, if not all, EOS and OAHFA species was partially replaced by the palmitate or oleate residue in the linoleate residue of several, if not all, EOS and OAHFA ceramide-bound forms of ω-OH ULCFA residues. Interestingly, the linoleate residue of several, if not all, EOS and OAHFA species was partially replaced by the palmitate or oleate residue in the linoleate residue of several, if not all, EOS and OAHFA ceramide-bound forms of ω-OH ULCFA residues. Interestingly, the linoleate residue of several, if not all, EOS and OAHFA species was partially replaced by the palmitate or oleate residue in the linoleate residue of several, if not all, EOS and OAHFA ceramide-bound forms of ω-OH ULCFA residues. 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Figure 3 | Impaired acylceramide formation in Pnpla1−/− epidermis. (a) Representative TLC analysis of lipids extracted from Pnpla1+/+, Pnpla1+/− and Pnpla1−/− epidermis. In Pnpla1−/− mice, EOS, GlcEOS and OAHFA were almost completely depleted (blue), with reciprocal increases in ω-OH FA, ω-OH Cer and GlcCer (red), relative to Pnpla1+/+ and Pnpla1+/− mice. TG, triglyceride; FFA, free fatty acid; Chol, cholesterol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin. (b-e) LC-MS/MS analysis of epidermal ceramide and related lipid species showing marked reductions in EOS (b) and OAHFA (d) species with linoleic acid (18:2) and increases in corresponding ω-OH Cer (c) and ω-OH ULCFA (e) species in Pnpla1−/− mice in comparison with Pnpla1+/+ mice (mean ± s.e.m., n = 3 animals; *P<0.05, **P<0.01 and ***P<0.001 versus Pnpla1+/+ mice). In b-e, C18-sphingosine-based ceramide (d18:1) species are selected and shown. Results from one or two independent experiments are shown.
(Supplementary Fig. 5e), probably because of the perturbed LA metabolism resulting from impaired formation of OLHFA and acylceramide.

**Keratinocyte-specific Pnpla1 ablation impairs skin barrier.** To ascertain whether the skin barrier defects observed in global Pnpla1<sup>−/−</sup> mice were indeed intrinsic to skin, mice carrying the loxP-flanked Pnpla1 allele (Pnpla1<sup>fl/fl</sup>) were crossed with mice transgenic for Krt14 promoter-driven Cre recombinase to obtain mice lacking PNPLA1 selectively in epidermal keratinocytes (Pnpla1<sup>fl/fl</sup>K14-Cre). Expression of Pnpla1 in the skin was reduced by ~80% in Pnpla1<sup>fl/fl</sup>K14-Cre mice in comparison with control Pnpla1<sup>fl/fl</sup> mice (Fig. 4a), confirming that Cre mediated recombination efficiently ablated Pnpla1 in epidermal keratinocytes.

About half reduction of Pnpla1 expression was also evident in the stomach, in which the K14 promoter is active<sup>46</sup>, yet it is unlikely that this reduction could influence the skin phenotype since global heterozygous Pnpla1<sup>−/+</sup> mice showed no abnormality. Although Pnpla1<sup>fl/fl</sup>K14-Cre animals were indistinguishable from control littermates shortly after birth, the mutant mice died within 6 days (Fig. 4b). The death was accompanied by focal desquamation with a markedly elevated TEWL value, whereas the value in other unaffected skin region remained unchanged (Fig. 4c,d). Histologically, a lower zone of the SC layers became densely packed with lipid-poor interspaces in Pnpla1<sup>fl/fl</sup>K14-Cre mice at P5 (Fig. 4e), as was seen in global Pnpla1<sup>−/−</sup> mice (Fig. 1f). Immunostaining of Pnpla1<sup>fl/fl</sup>K14-Cre mice skin demonstrated diminished expression of filaggrin and loricrin in comparison to Pnpla1<sup>fl/fl</sup> mice (Fig. 4f). Furthermore, epidermal

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**Figure 4 | Phenotypes of keratinocyte-specific Pnpla1-deficient mice.** (a) qPCR analysis of Pnpla1 expression in various tissues of control (fl/fl) (n = 2) and Pnpla1<sup>fl/fl</sup>K14-Cre (fl/fl K14-Cre) (n = 4) mice at P5. (b) Postnatal death within 6 days due to epidermal-specific disruption of Pnpla1 (n = 5 per genotype). (c) Gross appearance of control (fl/fl) and mutant (fl/fl K14-Cre) mice at P5. Mutant animals showed smaller body size. Yellow circles labelled with a,b indicate regions without and with severe desquamation, respectively. (d) TEWL of control (n = 13) and mutant (n = 18) mice at P5. Labels a,b are as indicated in c. (e) Representative images of hematoxylin-eosin staining of skin sections from control and mutant mice at P5. (f) Impaired terminal differentiation of epidermal keratinocytes at P5 in Pnpla1<sup>fl/fl</sup>K14-Cre mice. Sections were stained with anti-filaggrin and anti-loricrin antibodies (red) and DAPI (blue). (g) Densitometric analysis of TLC separation of epidermal lipids extracted from control and mutant mice at P5. Individual lipid levels were normalized with SM content (n = 6 animals). In a,d,g, values are mean ± s.e.m.; *P < 0.05, **P < 0.01 and ***P < 0.001 versus control mice. Scale bars in e,f, 20 μm. Data are from at least two independent experiments.
levels of EOS and GlcEOS were markedly lower, while those of ω-OH Cer and GlcCer were conversely higher, in Pnpla1−/−K14-Cre mice (Fig. 4g). Taken together, these results suggest that PNPLA1 is required in a cell-autonomous manner for acylceramide formation and keratinocyte differentiation.

**Discussion**

It is generally known that PLA2 is a group of enzymes that hydrolyse the sn-2 position of glycerophospholipids to give rise to fatty acids and lysophospholipids. In fact, by hydrolyzing glycerophospholipids, cytosolic PLA2α plays a central role in arachidonic acid metabolism in a wide variety of cells, secreted PLA2-γ modulate tissue-specific homoeostasis or diseases in given extracellular microenvironments, and PNPLA9 (iPLA2β) and PNPLA8 (iPLA2γ) participate in energy metabolism and neurodegeneration46,47. However, it has recently become obvious that several members of the PNPLA/iPLA2 family catalyse forms of lipid metabolism other than the typical PLA2 reaction, as exemplified by PNPLA2/ATGL (iPLA2C) acting as a major TG lipase in lipolysis and PNPLA3 (iPLA2a) probably acting as an acyltransferase or transacylase leading to TG accumulation in non-alcoholic fatty liver disease24,48. Herein, as part of our ongoing attempts to clarify the biological roles of the PLA2 family using comprehensive gene targeting strategies, we have identified PNPLA1, which represents an ichthyosis-causative gene with unknown function49, as an enzyme essential for the biosynthesis of acylceramide, a unique lipid component, the presence of which has long been recognized as prerequisite for normal skin barrier function.

Three abundant lipid groups were almost completely absent in Pnpla1−/− epidermis. One of these groups is the acylceramide EOS (and EOP), a key lipid intermediate that is an absolute requirement for formation of the skin barrier and contains saturated, monounsaturated or diunsaturated ULCFA in the N-acyl chain and linoleate in the ω-O-acyl chain. The second group is GlcEOS, a glucosylated form of EOS, which can be stored in lamellar bodies to be secreted into the intercellular space of the SC and then converted back to EOS by the glucosidase GBA. The third group is linoleate-containing OAHFA (OLHFA), as described below. The corresponding accumulation of putative precursors of these three lipid groups, namely ω-OH Cer, ω-OH GlcCer and ω-OH ULCFA, in PNPLA1-deficient epidermis provides strong evidence that PNPLA1 acts as an ω-O-acyltransferase or transacylase required for acylceramide synthesis. In this regard, the accompanying study by Ohno et al.49 has clearly shown that exogenous overexpression of PNPLA1 in cells or PNPLA1-reconstituted proteoliposomes promotes acylceramide formation likely as a transacylase and that PNPLA1 mutations associated with ARCI inactivate this transacylase activity.

So far, the order and molecular mechanisms by which ULCFAs and specifically LA are hooked onto the ω-OH ULCFAs of (glucosyl)ceramides has not been fully clarified15,21. Our new proposed model for epidermal ceramide metabolism is as follows (Fig. 6d): LA is directly transferred from a linoleate-containing TG pool to the ω-OH ULCFA moiety by PNPLA1 as a
CoA-independent transacylase to form OAHFA, EOS and/or GlcEOS. Glucosylation of ceramide occurs in the cis-Golgi apparatus through the action of UDP-glucose ceramide glucosyltransferase, UGCG, and then the resulting GlcEOS is incorporated into lamellar bodies and secreted into the intercellular space of the SC. At the SC interstices, the glucosidase GBA deglycosylates GlcEOS to EOS, which form lipid lamellae together with cholesterol and FFA. Two lipoxygenases, ALOX12B and ALOXE3, sequentially oxygenate the linoleate moiety in EOS (ref. 50), which then allows a second

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**Figure 6 | Roles of PNPLA1 and ABHD5 in epidermal ceramide metabolism.** (a) Representative TLC analysis of lipids extracted from WT, Abhd5<sup>−/−</sup>, and Pnpla1<sup>−/−</sup> epidermis at E18.5. (b) Quantification of panel A by densitometric analysis (mean ± s.e.m., n = 6, 5 and 5 for WT, Abhd5<sup>−/−</sup> and Pnpla1<sup>−/−</sup> mice, respectively; **P < 0.01 and ***P < 0.001 versus WT mice). Cumulative results of three independent experiments are shown. (c) A summary profile for a,b. Up and down arrows represent an increase and a decrease, respectively, in the level of individual lipids in mutant mice relative to WT mice. The number of arrows indicates the relative degree of difference. (d) Schematic diagram showing the proposed role of PNPLA1 in epidermal ceramide metabolism in association with keratinocyte differentiation and skin barrier function. Significantly increased and decreased lipid metabolites in Pnpla1<sup>−/−</sup> epidermis are highlighted in red and blue, respectively. Upregulated enzymes are shown in italics. LA derived from TG appears to be esterified at the ω-position of ω-OH ULCFA, ω-OH Cer and/or ω-OH GlcCer (reactions 1, 2 and 3, respectively) by PNPLA1 transacylase. For details, see text.
as-yet-unidentified lipase to de-esterify acylceramides. The resulting pool of o-OH Cer can then be covalently linked to the outer surface of the CE, thus forming the CLE.

Although it has been reported that OAHFAs are abundant in the epidermis  51, the function and origin of this unique class of lipids in the epidermis have remained unknown. It is likely that OAHFAs share a biosynthetic reaction with acylceramides, which also contain an N-acyl chain composed of a particular type of OAHFA, namely linoleate-containing OLHFA. There are at least two possible pathways for OAHFA biosynthesis, either directly via \( \omega-O \)-esterification of \( \omega-O \)-ULCEFA with LA by PNPLA1 (Route 1 in Fig. 6d) or indirectly via synthesis of acyl(\( \omega \)-glucosyl)ceramides by PNPLA1 and subsequent hydrolysis by a ceramidase (Route 2 and 3 in Fig. 6d). Whether CERS3 could use OAHFA (or its -CoA form) as a substrate for acylceramide synthesis remains to be determined.

The marked alteration of epidermal structure and function along with impaired acylceramide synthesis in \( \text{Pnpla1}^{-/-} \) newborns, accompanied by down-regulation of CE proteins and up-regulation of EGF ligands, indicate that acylceramide biosynthesis is required for normal epidermal differentiation, the intercellular lipid lamellae in the SC, but also the proper transition from proliferation to terminal differentiation of keratinocytes. The delayed onset of skin phenotypes in \( \text{Pnpla1}^{V/K14-Cre} \) mice in comparison with global \( \text{Pnpla1}^{-/-} \) mice may be due to incomplete deletion of cutaneous \( \text{Pnpla1}^{-/-} \) expression at birth in the former. The increased expression of PPAR\( \delta \) in \( \text{Pnpla1}^{-/-} \) epidermis could explain, at least in part, the induction of a panel of lipid metabolism-related genes associated with ARC1. Indeed, PPAR\( \delta \) contributes to up-regulation of ABCA12 and GBA in keratinocytes, and PPAR\( \delta \) deficiency decreases lipid metabolism required for lamellar membrane formation and thereby skin barrier function 52–54. On the other hand, hyperactivation of PPAR\( \delta \) enhances keratinocyte proliferation through inducing HB-EGF (ref. 43), an event that is recapitulated in cutaneous \( \text{Pnpla1}^{-/-} \) keratinocytes.

It is tempting to speculate that the increased extracellular levels of acylceramide or its derivative(s) at the SG/SC border could provide a critical signal for keratinocyte maturation to corneocytes. In our study using cultured \( \text{Pnpla1}^{-/-} \) keratinocytes, the supplementation with SOS reversed the decreased expression of filaggrin and increased expression of HB-EGF towards normal levels. In support of this observation, application of synthetic pseudo-acylceramide or GlcEOS recovers diminished barrier function in \( \text{Pnpla1}^{-/-} \) corneocytes. In corneocytes, the supplementation with epidermal lipids from WT mice to keratinocytes with \( \text{Pnpla1}^{-/-} \) keratinocytes with competent permeability barrier function, although full understanding of the underlying mechanism needs further elucidation.

Overall, our analyses of epidermal lipids, morphology and permeability barrier function lend strong support to the contention that PNPLA1 is essential for acylceramide synthesis and skin barrier function. Our genetic approach using knockout mice and the biochemical approach by Ohno et al. 59 complement each other by providing different lines of evidence that prove that PNPLA1 catalyses the \( \omega-O \)-esterification in acylceramide biosynthesis. While our manuscript was under final review, Grond et al. 60 also reported that acylceramide biosynthesis was impaired in the skin of another \( \text{Pnpla1}^{-/-} \) mouse strain and in human keratinocytes with PNPLA1 mutation, and that topical application of epidermal lipids from WT mice to \( \text{Pnpla1}^{-/-} \) skin promoted rebuilding of the CLE. Herein, by means of comprehensive lipidomics, global gene profiling and conditional targeting, we have provided additional insights that the action of PNPLA1 is highly linoleate-selective and keratinocyte-intrinsic. Indeed, \( \omega \)-acyl linoleate in acylceramides and OAHFAs is largely abolished with only partial replacement by other fatty acids (the \( \text{Pnpla1}^{V/K14-Cre} \) example), but the way that PNPLA1 selectively utilizes linoleic acid for acylceramide biosynthesis and that the loss of this lineolate specificity causes epidermal barrier defect. Although the catalytic mechanism, subcellular localization and functional regulation of PNPLA1 still remain to be elucidated, the findings obtained from these three complementary studies altogether contribute to a better understanding of the skin barrier function and ichthyosis development, and should be useful in providing novel therapeutic strategies for treatment of patients with skin barrier disorders.

**Methods**

**Keratinocyte culture.** Mouse primary keratinocytes were isolated as described previously 51. Briefly, skins of newborn mice were treated with 5 mg mL\(^{-1}\) Dispase (Thermo Fisher) overnight at 4 °C. The epidermis was then mechanically separated from the dermis and incubated with Accutase (Nacalai Tesque) for 20 min at room temperature to collect keratinocytes. Human and mouse progenitors for epidermal keratinocytes were purchased from CELLSNTEC and have been tested for mycoplasma by the distributor. These cells were cultured in CrT-Prime medium containing 1% (v/v) antibiotic-antimycotic solution (Thermo Fisher). After reaching confluency, the cells were cultured for appropriate periods in CrT-Prime 2D Diff medium supplemented with 1.2 mM CaCl\(_2\) to induce keratinocyte differentiation. As required for experiments, Cer EOS (N-(30-Linoleoyloxy-triacontanoyl)-sphingosine; Matreya LLC) was dispensed by sonication for 1 min and then added to the culture. All media were from CELLSNTEC.

**Mice.** \( \text{Pnpla1}^{-/-} \) deficient mice, containing a ‘knockout-first’ allele targeted to the \( \text{Pnpla1} \) genomic locus named \( \text{Pnpla1}^{m1(\text{KOMP})/\text{Wld}} \), were generated from a conditional targeting vector obtained from the Knockout Mouse Project resource (KOMP-CD ID:79620) (ref. 62). Briefly, mouse embryonic stem cells derived from C57BL/6N mice (BENKA) containing the correctly targeted \( \text{Pnpla1} \) locus were injected into blastocysts and transplanted in pseudopregnant mouse to generate chimaera mice. Highly (80–90%) chimeric males were mated with C57BL/6N female mice, and germ line transmission of the targeted allele was confirmed by PCR. The IRES-LacZ and Neo cassettes were removed by flippase-mediated excision. The excised cassette containing the homozygous mice were intercrossed to obtain homozygous null mice, and littermate WT mice were used as controls. Mice with a floxed allele of \( \text{Pnpla1} \) were crossed with transgenic mice for K14 promoter-driven Cre recombinase 68 to obtain skin-specific \( \text{Pnpla1}^{-/-} \) mice (\( \text{Pnpla1}^{V/K14-Cre} \)).

To generate \( \text{Abhd5}^{-/-} \) mice, genomic \( \text{Abhd5} \) clones were isolated from mouse 129Sv/Ev genomic library. A 6.7-kb fragment of an \( \text{Abhd5} \) clone was subcloned into a targeting vector with exon 1 being replaced by the PGK-Neo cassette. The targeting vector was introduced into 129Sv/Ev embryonic stem cells and a correctly targeted embryonic stem cell line was injected into blastocysts, resulting in the gene-targeted mouse strain. Heterozygotes were backcrossed onto C57BL/6J background for at least five generations and then intercrossed to obtain homozygous null mice.

Genotyping of offspring was performed by PCR of tail-snip DNA using genotyping primers (Supplementary Table 1). Animals were fed ad libitum (CE2, Clea Japan), had free access to water, and were kept on a 12:12-h light-dark cycle in single cages. All experimental procedures involving animals in this study were approved by the Institutional Animal Care and Use Committees of Tokyo Metropolitan Institute of Medical Science and Nagoya University and were...
conducted in accordance with the Japanese Guide for the Care and Use of Laboratory Animals.

Skin permeability assays. Toluidine blue staining of newborn mice was described previously22. In brief, newborns were anesthetized, dehydrated in methanol, washed in PBS and stained for 30 min in 0.1% (w/v) toluidine blue/PBS. After washing in PBS, the pups were photographed. TEWL was measured using a Tewameter TM300 with a 2-mm-diameter adaptor (Courage-Khazaka Electronics, Germany). Measurements were performed after calibration of the device at room temperature with minimized influence of air turbulence, and the results were recorded when the TEWL values were stabilized 30–45 s after probe placement.

Histology and immunohistochemistry. Skin samples were frozen and embedded in OCT compound, or fixed overnight with buffered 4% (w/v) parafomaldehyde at 4 °C and embedded in paraffin. Skin sections (5 μm) were stained with hematoxylin and eosin or stained for immunofluorescence. For the deparaffinized sections were blocked with PBS containing 10% (w/v) Block Ace (Sumitomo Dainippon Pharma), incubated with primary antibodies overnight at 4 °C and with secondary Alexa Fluor antibodies at room temperature for 1 h. Rabbit polyclonal antibody against PNPLA1 was raised against a synthetic peptide GPPVeldonPRTAPGSP as an immunogen and used at 1:2,000 dilution. Other primary antibodies used at 1:1,000 dilution were rabbit antibodies against flaggrian, keratin 1, keratin 5, keratin 6 and loricrin (PRB-417P, PRB-149P, PRB-160P, PRB-169P and PRB-145P; Covance). Secondary antibodies used at 1:1,000 dilution were Alexa Fluor 546 goat anti-rabbit IgG and Alexa Fluor 633 goat anti-rabbit IgG (Thermo Scientific). All the immunofluorescence reactions were pre-labelled with Alexa Fluor 488 or Alexa Fluor 555 using the Zenon Rabbit IgG Labeling Kits (Thermo Fisher). After immunoreactions and counterstaining with DAPI (Vector Laboratories), the slides were visualized using an LSM710 confocal microscope (Zeiss). For immunostaining of adult mouse skin, the sections were incubated with a rabbit anti-PNPLA1 antibody, treated with biotinylated anti-rabbit antibody (BA-1000, Vector Laboratories), and then processed with an avidin–biotin–peroxidase system (Vectastain Elite ABC kit, Vector Laboratories) and diaminobenzidine, followed by counterstaining with hematoxylin. Lipids were visualized by cryosections by staining with Nile Red (5 mg mL−1 in 75% (v/v) glycerol, Wako).

Electron microscopy. Neonatal mouse skin samples were fixed in 5% (w/v) glutaraldehyde solution, post-fixed in 0.1% (w/v) ruthenium tetroxide (RuO4), dehydrated and embedded in Epon12 (TAAB Laboratories). All the samples were ultra-thin sectioned at a thickness of 70 nm, and stained with uranyl acetate and lead citrate. Photographs were taken using a JEM1400 transmission electron microscope (JEOL Ltd.).

Quantitative PCR. Total RNA was extracted with TRIzol (Thermo Fisher) and was reverse-transcribed into cDNA using ReverTra Ace qPCR RT Master Mix (Toyobio) in accordance with the manufacturer’s instructions. qPCR reactions were performed on a LightCycler480-II (Roche) using THUNDERBIRD Probe qPCR Mix (Toyobo). The sequences of primers designed to be compatible with the Roche Universal Probe Library (UPL) are provided in Supplementary Tables 2 and 3. The mRNA levels. Expression levels were normalized to 18S and analysed using Thermo Fisher’s 2-ΔΔCt method.

LC–MS/MS and LC–MS were used for identification and quantification of the lipids. Non-targeted lipidomics analysis65,66 were performed with minor modifications. Briefly, dried total lipid extracts were re-dissolved in 50 μl of chloroform/methanol (2:1, v/v) and 2 μl of samples were separated by an ACQUITY UPLC BEH C18 column (50 × 2.1 mm i.d., particle size 1.7 μm, Waters) at a flow rate of 300 μl min−1 at 45 °C using an ACQUITY UPLC system (Waters) equipped with a binary pump and automatic sample injector. Solvent A consisted of chloroform/methanol/water (20:20:60, v/v) and solvent B was isopropanol, both containing 5 mM ammonium acetate. The solvent composition started at 100% A for the first min and was changed linearly to 64% B at 7.5 min, where it was held for 4.5 min. The gradient was increased linearly to 82.5% B at 12.5 min, following a 5 min equilibration, ramping 85% B at 15 min and 95% B at 20 min before re-equilibrating the column with 100% A for 5 min. Qualitative and quantitative analysis of lipids was performed by MS and data-dependent MS/MS acquisition with a scan range of m/z 70–1250 using a Triple TOF 5600+ System (AB SCIEX) in the negative and positive ion mode. Raw data files from the TOF-MS were converted to MGF files using OpenMS (openms.io) and converted for submission to data analysis software with 2DICAL (Mitsui Knowledge Industry). Identification of molecular species was accomplished by comparison with retention times and MS/MS spectra with commercially available standards or reference samples.

Ceramides, VCLFAs, and cholesterol were quantified by LC–MS using an Acquity UPLC Series LCMS/MS. Samples were equilibrated with a multi-ion-source, ChemStation software, an autosampler and an Agilent C18 column (150 × 2.1 mm i.d.; Waters) for 3 min before injection. The lipids were separated by LC–MS/MS analysis. The intensities of bands were quantified by densitometry using the Genesis software (AFCB, Sweden). Lipids were visualized by cryosections by staining with Nile Red (5 mg mL−1 in 75% (v/v) glycerol, Wako).

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between the groups that were statistically compared. A P value of <0.05 was considered statistically significant. All the data were presented as mean ± s.e.m.

Data availability. The data that support the findings of this study are available from the corresponding author on request. The microarray data can be accessed at the GEO repository under the accession number GSE87682.

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Author contributions
T.H. and M.M. designed experiments. T.H., T.A., A.K., Y.S., H.T., K.Y., Y.N. and A.M.-G., performed the experiments. T.H., T.A., Y.S., K.M., K.F., A.R.B., K.I. Makoto Arita and Masashi Akiyama analysed the data. T.O. and C.T. designed and constructed mutant mice. T.H. and M.M. wrote the manuscript with input from all the other authors.

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