Supplementary Information

MATERIALS AND METHODS

Reagents

Lysophospholipids including LPA (1-palmitoyl (16:0), 1-stearoyl (18:0), 1-oleoyl (18:1) and 1-arachidonoyl (20:4) species), 1-18:1-LPC, 1-18:1-LPE, 1-18:1-LPG, 1-18:1-LPI, 1-18:1-LPS and S1P were purchased from Avanti Polar Lipids. 1-linoleoyl (18:2)-LPA was from Echelon Biosciences. Stabilized LPA analogs were synthesized by Drs. Y. Xu and L. Qian (University of Utah, USA). Lysophospholipids, except for S1P, were dried under nitrogen gas and dissolved in 0.1% fatty acid-free BSA (Sigma-Aldrich)-PBS using water bath sonication and stocked in -20ºC. S1P was dissolved at 10 mM in 300 mM NaOH solution. NF449, PTX and Ro31-8425 were from Calbiochem. Phospholipase D from Actinomadura sp., SDZ 242-484 (Kottirsch et al, 2002) and YM254890 (Takasaki et al, 2004) were kindly provided by Meito Sangyo (Japan), Novartis Pharma AG and Astellas Pharma (Japan), respectively. Recombinant murine EGF was obtained from PeproTech. EGFR inhibitor, AG1478, was obtained from BIOMOL and dissolved in DMSO. Stealth™ siRNA duplex against human P2Y5 (targeting sequence, 5’- UGGCCAUUUGGAGAUUUACUUUGUA -3’), human TACE (5’- CAGAAUCGUGUUGACAGCAAAGAAA -3’) and Stealth™ negative control were purchased from Invitrogen. Other chemicals were purchased from Wako Pure Chemical Industries unless otherwise indicated.

Antibodies

An anti-mouse PA-PLA₁α monoclonal antibody (mAb, clone mask1) made in rat was established according to the previously described method (Sakagami et al, 2005) with minor modifications (also see supplementary methods). Biotinylation of anti-PA-PLA₁α antibody was performed using EZ-Link Sulfo-NHS-Biotin reagents (Pierce). Anti-trichohyalin (AHF) mAb made in rat and polyclonal
antibody (pAb) made in rabbit (dilution 1:1000) were kind gifts from Dr. Hirai (Kyoto University, Japan). The following antibodies were purchased from distributors: anti-ERK1/2 and anti-pERK1/2 pAbs made in rabbit (Cell Signaling Technologies); anti-EGFR (EP38Y, 1:2000) and anti-pEGFR (EP774Y, recognizes phosphotyrosine 1068) monoclonal antibodies (mAbs) made in rabbit (Abcam); anti-K71, anti-K72, anti-K75 and anti-K85 pAbs made in guinea pig (Progen); anti-α-tubulin (DM1a) and anti-actin (AC-40) mAbs made in mouse (Sigma-Aldrich); anti-phosphotyrosine (PY20) mAb conjugated with agarose beads (Santa Cruz Biotechnology); anti-mouse IgG, anti-rabbit IgG (Amersham) and anti-rat IgG (American Qualex) secondary antibodies conjugated with HRP; anti-rat IgG, anti-rabbit and anti-guinea pig IG secondary antibodies conjugated with Alexa Fluor 488, 568, 594 and 647 (Invitrogen). For immunoblot analysis, the dilutions of primary antibodies and secondary antibodies were 1:1000 and 1:2000, respectively, unless otherwise noted. For immunofluorescent staining, the dilutions of primary antibodies and secondary antibodies were 1:100 and 1:1000, respectively, unless otherwise noted.

Plasmids

A full-length cDNA encoding PA-PLA₁α was cloned from C57BL6/J mouse intestine cDNA and inserted into mammalian expression vector pCAGGS (Niwa et al, 1991) (a gift from Dr. Junichi Miyazaki, Osaka University, Japan). A catalytically inactive mutant harboring a serine-to-alanine substitution at residue 154 (S154A) was generated by the two-step PCR method as described previously (Sonoda et al, 2002). The single-exon ORFs encoding human and mouse P2Y5 were cloned from genomic DNA isolated from human umbilical vein endothelial cells (HUVEC) and C57BL6/J mouse tail, respectively. P2Y5 expression vectors were constructed by inserting a FLAG epitope tag (DYKDDDDK) between the first and the second codon positions of P2Y5. Alkaline phosphatase (AP)-tagged TGFα (AP-TGFα) (Tokumaru et al, 2000) was kindly provided by Dr. Hideki
Higashiyama (Ehime University, Japan). All clones were verified by sequencing (ABI Prism 3700, Applied Biosystems). Dominant negative (DN) forms of G proteins including DN Gq (Q209L and D277N), G12 (G228A), G13 (G225A) and RhoA (T19N) were kindly provided by Drs. Yutaro Obata and Norimichi Nakahata (Tohoku University, Japan).

**Generation of PA-PLA$_{1\alpha}^{-/-}$ mice**

A 13-kb fragment of the mouse PA-PLA$_{1\alpha}$ gene containing exons 2, 3 and 4 was isolated from a lambda FIXII genomic library (129/SvJ; Stratagene) and subcloned into pBluescript SK (-) (Stratagene). A targeting vector was created by inserting a 7.5 kb fragment of the 5’ homologous region and a 1.2-kb fragment of the 3’ homologous region into PGKneolox2DTA vector (kindly provided by Dr. Soriano, Fred Hutchinson Cancer Research Center, USA) (see Supplementary data in detail). The targeting vector was introduced into 129/SvE ES cells by electroporation. Two independent clones (1D-A1 and 4C-D3) were confirmed for successful targeted homologous recombination by Southern blot analysis using probes A, B and C. These clones were used for blastocyst injection to generate chimaeric mice. Subsequently, heterozygous (PA-PLA$_{1\alpha}^{+/-}$) mice were obtained from chimaeric male mice and were backcrossed to C57BL6/J mice (CLEA Japan) 8 times. Homozygous (PA-PLA$_{1\alpha}^{-/-}$) mice were obtained from intercrosses of heterozygotes. In all experiments using homozygous mice, littermates were used for a control.

**Histology and in situ hybridization analyses**

Dorsal skins were excised and fixed in 4% formaldehyde in phosphate-buffered saline (PBS) at 4°C overnight. Specimens were dehydrated with an increasing concentration of ethanol followed by xylene and embedded in paraffin. Five-μm-thick paraffin sections were placed on MAS-coated glass slides (Matsunami Glass) and deparaffined with xylene followed by decreasing concentration of ethanol. For
histological analysis, sections were stained with hematoxylin and eosin (Mutoh Industries) and mounted in Entellan (Merck) with coverslips. For *in situ* hybridization, rehydrated sections were treated with Proteinase K and hybridized with digoxigenin labeled-RNA probes corresponding to 190-721 nt of PA-PLA<sub>1</sub>α cDNA (GenBank AK134352). After incubation with anti-digoxigenin antibody conjugated with alkaline phosphatase, coloring reaction was performed with BM purple AP (Roche Diagnostics). The sections were counterstained with Kernechtrot stain solution (Mutoh Industries). All microscopic images were acquired with a Zeiss Axio Imager (Carl Zeiss MicroImaging).

**Quantitative RT-PCR analysis**

Total RNA from dorsal skin was prepared using an RNeasy Fibrous Tissue Mini Kit (QIAGEN). Total RNA from other tissues and cultured cells were isolated using a GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). Total RNA was reverse-transcribed using High-Capacity cDNA RT Kits (Applied Biosystems) according to the manufacturer’s instructions. PCR reactions were performed with SYBR Premix Ex Taq (Takara Bio) and were monitored by ABI Prism 7300 (Applied Biosystems). Standard plasmids ranging from 10<sup>2</sup> to 10<sup>8</sup> copies per well were used to quantify the absolute number of transcripts of cDNA samples. The numbers of transcripts were normalized to the number of a house-keeping gene, *Gapdh* or *Actb* (encoding β-actin), in the same sample.

Primers used in mouse gene expressions are listed below:

- **Actb**, 5’-GCCTTCCTTTCTTGGATGTT-3’ and 5’-CAATGCCTGGGTACATGGTG-3’;
- **Adam9**, 5’-ACCCTGGAGAGTGTGACTG-3’ and 5’-GGAAGGAACTGGCAATCTTTACAAC-3’;
- **Adam10**, 5’-CATTGCTGAGTGGATGTGG-3’ and 5’-AGTCCTGGAAGTGGTTTAGG-3’;
- **Adam12**, 5’-TGTTACTGCTACAACGGCATC-3’ and 5’-TGACACTGGATTCCCACAC-3’;
Adam15, 5’-CTCAGCCTCCTGTTGTTATG-3’ and 5’-TTCTTGGGCGACGGGTGTAG-3’;
Adam17 (also known as Tace), 5’-AACTTGAGAGTCTGGTGTTG-3’ and 5’-ATTCTGGCCCATTCTGTGGT-3’;
Adam19, 5’-CCTATGGAAAAGTGGCAAGG-3’ and 5’-GTACAGGTGATGTTGCGTC-3’;
Adam33, 5’-GCTGACCACACCCTGGTCTTG-3’ and 5’-CGTCTGAGTGTGATCGACTG-3’;
Adamdec1, 5’-ATAACCACAGCACGCTTCTC-3’ and 5’-TGCAACAAGACCACATATTCTEGFR-3’;
EGFR, 5’-CGTGGCAGAACAAAGCAAC-3’ and 5’-GATTGGGTGTCCCGAAGAG-3’;
Gapdh, 5’-AGGAGCGAGACCCCACTA-3’ and 5’-CGGAGATGATGACCCTTTTG-3’;
Gata3, 5’-CCACCACCCATTACCCACCT-3’ and 5’-TTGCCCGCAGTTTAC-3’;
Krt17, 5’-TCTGCTGGAGGAGGAGT-3’ and 5’-CCGGGTAGATGACTTG-3’;
Krt 71, 5’-GCTGAGAAGACGGTGGATG-3’ and 5’-ATGTGCTGTGATGCGACTG-3’;
Krt 72, 5’-GAGTTCCGTTGGTGCTGAAGAG-3’ and 5’-GGATGTGTCGCTGATGCG-3’;
Krt 75, 5’-CTGAGCGAGGAGGGATTTC-3’ and 5’-ACTGGTGCTGAAGGAGTGC-3’;
Krt 85, 5’-AGTGGGAGTGTGGTGATG-3’ and 5’-TAAGGGAAGGGTTGGGAATG-3’;
LPA1, 5’-AGGAGGAATCGGAGGCAC-3’ and 5’-AGCAGACTCCGCGAATAAC-3’;
LPA2, 5’-TGCCCGCTTGGACTGAGT-3’ and 5’-GCTCCTGTCCCGCTGGATT-3’;
LPA3, 5’-ACCAACGTCCTATCTCCACACAC-3’ and 5’-CAGCAGCAGAACCCACCAACAG-3’;
LPA4, 5’-TCTCTACTGCTGTCCTTCA-3’ and 5’-GTGCGAGGCTGTCACCTCTT-3’;
LPA5, 5’-CTGTAAGGGTGGTGTCAGG-3’ and 5’-AGACCTGTAGTCGTTGCT-3’;
P2Y5, 5’-TGTGCCCTAAACATCAACT-3’ and 5’-CAAAGCAGCAGTTGGAAAC-3’;
PA-PLA1α (exon 3 and 4), 5’-TGGAATTTGGTGCGAGTCGTATG-3’ and 5’-TGGAATCGTCCACGAACAGT-3’;

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PA-PLA$_1$$\alpha$ (exon 6 and 7), 5’-AATGGCAAGTGTGTCAGCTGTGG-3’ and 5’-AAGAACGCTTCTCGTCATCGGAG-3’;
PA-PLA$_1$$\beta$, 5’-GGGGCACATTGATTTTTATCC-3’ and 5’-GCTCTCTGATGGTCGCATT-3’;
TGF$\alpha$, 5’-AAGTGCCCAGATTCCCCACA-3’ and 5’-GCAGTGATGGCTTGCATT-3’;
Tchh (encoding Trichohyalin), 5’-TGGAGCATCACTTAGCAAGAAAG-3’ and 5’-ATCGTCTCAGGGTCATGGA-3’;
Vim (encoding Vimentin), 5’-GATTTCTCTGCTCTGGACA-3’ and 5’-CCTGTCCATCTCTGGTCTCAAC-3’.

Primers used in human gene expressions are listed below:

ACTB, 5’-ATGAAGATCAAGATCATTGCTCCTC-3’ and 5’-ACATCTGCTGGGAAGGTGGAC-3’;
ADAM17 (also known as TACE), 5’-CTTTCTCTGAGAGGGAACAG-3’ and 5’-AGCATCGACATAGGGCACAC-3’;
GAPDH, 5’-GCCAAGGTCATCCCAGAAGAct-3’ and 5’-GAGGGGCCATCCACAGTCTT-3’;
LPA$_1$, 5’-GAGGAGATCGGACACCATGAT-3’ and 5’-ACATCCAGCAATAACGACAGCAATC-3’;
LPA$_2$, 5’-GACCACACTCAGCCTAGTCAAGAC-3’ and 5’-CTTACAGTCCAGGCCATCCA-3’;
LPA$_3$, 5’-GCTCCCATGAAGCTAATGAAGACA-3’ and 5’-AGGCCGTCCAGCAGCAGA-3’;
LPA$_4$, 5’-CAGTGCCTCCCTTGTTTGTCTTC-3’ and 5’-GAGAGGGCCAGGTTGGAT-3’;
LPA$_5$, 5’-AGCAACACGGGACAGCAGGTC-3’ and 5’-CCCAAAACAGCGAGGGAGGT-3’;
P2Y5, 5’-CCGCCGTTTTTTGTTGCAGTC-3’ and 5’-GAGATATGTTTTCCATGTTGCTTC-3’.

Note that primer sets including LPA$_1$-$4$, ACTB and GAPDH were same as previously described (Hama et al, 2004).

**Preparation of anti-PA-PLA$_1$$\alpha$ antibody**
Recombinant baculovirus encoding mouse PA-PLA₁α was produced as described previously. Briefly, bacmid shuttle vector encoding mouse PA-PLA₁α was prepared using BAC-TO-BAC system (Invitrogen) according manufacture’s instructions. Recombinant baculovirus was obtained from conditioned media of Sf9 insect cells transfected with the recombinant bacmid using Cellfectin reagent (Invitrogen). Sf9 cells were infected with the recombinant baculovirus and membrane fraction containing PA-PLA₁α was prepared by ultra centrifugation. The membrane fraction was used to raise rat monoclonal antibody as previously described (Sonoda et al, 2002). Briefly, membrane fraction was injected into hind foot pads of WKY/Izm rats (Japan SLC) in combination with Freund’s complete adjuvant, followed by second injection with Freund’s incomplete adjuvant. Iliac lymph node cells from immunized rats were fused with mouse myeloma PAI cells. Over two thousands hybridoma clones screened by ELISA, immunofluorescence and Immunoblotting, one hybridoma clone (mask1) was selected to prepare rat monoclonal antibody against mouse PA-PLA₁α.

Cell culture and transfection
HEK293 cells and human keratinocyte HaCaT cells were maintained in DMEM (Nissui Pharmaceutical) supplemented with 10% fetal bovine serum (GIBCO), 100 U/mL penicillin (Sigma-Aldrich) and 100 µg/mL streptomycin (GIBCO) in a 37 °C incubator with 5 % CO₂. HaCaT cells were seeded at 1.3 x 10⁵ per well in 24-well plate and cultured for 1 day, followed by 3 days with 3 mM of CaCl₂ to induce differentiation and used for TGFα release experiment. Primary mouse epidermal keratinocytes were seeded at 1.5 x 10⁶ cells and 6.0 x 10⁶ cells per well in 24-well plate (for TGFα release experiment) and 6 well-plate (for immunoblot experiment), respectively, coated with collagen type I (Nitta Gelatin) in K-SFM (GIBCO) supplemented with 250 ng/mL amphotericin B (GIBCO), 100 U/mL penicillin and 100 µg/mL streptomycin. Primary keratinocytes were cultured for 3 days at 35°C followed by 2 days at 37°C with medium renewal of every other day and used for assays.
Transient transfections for expression vectors and siRNA were performed using Lipofectamine™ 2000 and Lipofectamine™ RNAiMAX reagents (Invitrogen), respectively, according to the manufacturer’s instructions. Concentration of siRNAs was 10 nM in HEK293 cells and 50 nM in HaCaT cells. siRNAs were transfected two days and three days prior to the assay in HEK293 cells and HaCaT cells, respectively.

Isolation of epidermal keratinocytes and dorsal hair follicles

Mouse Primary keratinocytes were prepared as described previously with some modifications (Hama et al, 2004). Briefly, skins of newborn C57BL/6J mice were peeled and digested in 1000 U/mL of Dispase II (Nitta Gelatin) at 4 °C overnight. Epidermis was separated and treated with TrypLE Express (GIBCO) for 30 min at room temperature. Cell suspensions were filtered through 210 μm nylon mesh filter (Nippon Rikagaku Kikai). Mouse dorsal hair follicles were isolated according to the method described in another report (Takahashi, 2001). Briefly, dorsal skins of P4 C57BL6/J mice were excised and digested with 1000 U/mL of Dispase II at 4 °C overnight. Dermis was separated and further digested in 2.5 mg/mL collagenase (Nitta Gelatin) for 1 h at 37 °C. Hair follicle suspensions were filtered through 210 μm nylon mesh filters and the filterate was centrifuged at 30 x g. The pellet was resuspended in PBS. These filtration and centrifugation processes were repeated three times. Resulting pellets were recovered and used for total RNA extraction.

Quantification of lysophospholipids by LC-MS/MS

Vibrissa hair follicles were isolated as described above. Ten vibrissa hair follicles per a single mouse were extracted in 100 μL of methanol containing 100 nM 17:0-LPA and 1 μM 17:0-LPC using water bath sonication. The extracts were sequentially centrifuged at 1,000 x g and 21,500 x g and the resulting supernatant was used for LC-MS/MS analysis. Specimens of dorsal skins from P15 mice were minced and lipids were extracted in methanol with a MS-100R beads cell disrupter. The extracts were
sequentially centrifuged at 1,000 x g and 21,500 x g and the resulting supernatant was collected. Phospholipid concentration was measured with a Wako Phospholipids C assay kit (Wako Diagnostics) after resuspension of dried supernatant in PBS. The supernatant was diluted at a final concentration of 40 mg phospholipid/mL with methanol and mixed with internal standards at final concentrations of 50 nM 17:0-LPA and 500 nM 17:0-LPC standards. After filtration through a 0.2 µm acetyl cellulose filter (YMC), 20 µL of sample was injected into liquid chromatography (LC) and analyzed by tandem mass spectrometry (MS/MS). Briefly, lysophospholipids were separated by Nanospace LC (Shiseido) with a 1.5 mm x 250 mm C18 CAPCELL PAK ACR column using a gradient of solvent A (5 mM ammonium formate in water) and solvent B (5 mM ammonium formate in 95% (v/v) acetonitrile). MS/MS was carried out on a Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher Scientific). LPA, LPE, LPG, LPI and LPS were monitored in the negative ion mode and LPC and S1P were monitored in the positive ion mode. The product ions were as follows: m/z 153.0 (LPA), m/z 184.0 (LPC), m/z 264.2 (S1P), m/z parent ion - 87.0 (LPS) and corresponding fatty acids (LPE, LPG and LPI). The retention time for each lysophospholipids was verified using standard lysophospholipids or incubated plasma, which contains abundant LPA species. The ratio between analyte and internal standard peak area was used for quantification.

**Immunoblot and immunoprecipitation**

Primary mouse epidermal keratinocytes were prepared as described in Supplementary data. After starving overnight, cells were treated with vehicle control, 1-oleoyl LPA or 100 ng/mL EGF for 5 min (detection of p-EGFR) and 15 min (detection of p-ERK1/2). For inhibition of MMP activity and EGFR activation, cells were pretreated with 10 µM SDZ 242-484 and 1 µM AG1478 for 30 min, respectively. After incubation, cells were extracted in lysis buffer (10 mM HEPES (pH 7.3), 10% Glycerol, 1% Triton X-100, 1 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 10 µg/mL PMSF, 20 µg/mL leupeptin and 2.5 mM p-NPP) with a Smurt ultrasonic homogenizer (Microtech). Cell lysates were centrifuged at
1,000 x g and the resulting supernatants were subjected to immunoblot using standard procedures. Chemiluminescence signals were detected by LAS-4000 (FujiFilm, Japan).

Lysates of dorsal skins were prepared by removing hairs, followed by homogenizing specimens in lysis buffer with a beads cell disrupter (Micro Smash MS-100R, TOMY). The lysate was centrifuged at 1,000 x g and the resulting supernatant was collected. The total protein concentration was measured by the BCA Protein Assay (Pierce). Equal amounts of the supernatants were subjected to immunoblot according to standard procedures. For detection of tyrosine-phosphorylated EGFR, tyrosine-phosphorylated proteins were immunoprecipitated from the supernatant of dorsal skin using PY20-conjugated agarose beads. The beads were washed three times with lysis buffer and boiled in SDS-PAGE sample buffer (62.5 mM Tris-HCl (pH 6.8), 5% 2-mercaptoethanol, 2% SDS and 10% Glycerol). The phosphorylated EGFR in the dissolved immunoprecipitants was measured by immunoblot using anti-EGFR antibody. The signal intensity of the chemiluminescent image was analyzed by Multi Gauge ver. 3.0 (FujiFilm).
REFERENCES


FIGURE LEGENDS

Figure S1 Generation and phenotypes of PA-PLA₁α⁻/⁻ mice.

(A) Representations of the wild-type PA-PLA₁α gene locus, targeting vector and targeted mutant allele. Filled boxes and numbers below the boxes represent exons and corresponding exon numbers, respectively. Targeting vector was designed to replace the exon 3, which contains the active Ser 154 of the catalytic triad, by neomycin resistant cassette (PGK-Neo) in reverse orientation to PA-PLA₁α gene. Negative selection marker encoding diphtheria toxin A-fragment (PGK-DT-A) was inserted in the upstream of 5’ homology arm. Probe A and B correspond 5’ and 3’ flanking sequences of the homology arms, respectively. Probe C is located within PGK-Neo to examine random integration of the targeting vector. E, EcoRV; B, BamHI. (B) Southern blot analysis of wild-type (+/+), PA-PLA₁α heterozygous (+/-) and PA-PLA₁α homozygous (−/−) mice. Tail genomic DNA was digested with EcoRV for probe A, EcoRV and BamHI for probe B, and EcoRV for probe C and analyzed by Southern blot using ³²P-labeled probes. (C) Immunoblot analysis of protein extracts from stomachs showing that the targeted allele does not express mature PA-PLA₁α protein. Actin was monitored for a loading control. (D and E) Quantitative real-time reverse transcription-PCR (qRT-PCR) analysis of PA-PLA₁α mRNA. Total RNA from anagen dorsal skin on postnatal day (P) 10 was reverse-transcribed and assessed by qRT-PCR. The number of the transcripts was normalized to Gapdh in the same sample. The mean value of wild-type expression was set as 1 (n = 4). Primers in D and E are located in exon 3 and 4, and exon 6 and 7, respectively.

Figure S2 LPA is specifically reduced in PA-PLA₁α⁻/⁻ hair follicles.

(A) Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of anagen vibrissa hair follicles. Methanol extract of anagen vibrissa hair follicles from P15 WT (+/+) and PA-PLA₁α⁻/⁻ (−/−) mice were analyzed for nine LPA species by LC-MS/MS. IS, internal standard of 17:0-LPA. 16:0,
palmitoyl; 18:2, linoleoyl; 18:1, oleoyl; 18:0, stearoyl; 20:5, eicosapentanoyl; 20:4, arachidonoyl; 22:6, docosahexanoyl; 22:5, docosapentaenoyl. (B) Quantification of peak areas in A (for LPA) and other lysophospholipids. Peak areas of 1-acyl lysophospholipid and 2-acyl lysophospholipid were combined and normalized to the internal standard (17:0-LPA or 17:0-LPC). The mean value in +/+ sample was set at 1 ($n = 8$; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus +/+). 17:0-LPA and 17:0-LPC was used as the internal standard in the negative ion mode (LPA, LPE, LPG, LPI and LPS) and the positive ion mode (LPC), respectively. The sum of eight acyl moieties was evaluated for total lysophospholipid and the relative value was shown in Figure 2C. LPC, lysophosphatidylcholine; LPE, lysophosphatidyethanolamine, LPG; lysophosphatidylglycerol; LPI, lysophosphatidylinositol; LPS, lysophosphatidylserine. Besides LPA, no significant differences were observed between +/+ and -/-.

(C) Same as B, but monitoring sphingosine 1-phosphate (S1P) and dihydrosphingosine 1-phosphate (dhS1P). 17:0-LPC was used as the internal standard.

Figure S3 PA-PLA$_1$$\alpha^+$ hair follicles show apparently normal IRS keratinization.

(A) A representative image of an anagen vibrissa hair follicle. After removing a collagen capsule, a vibrissa hair follicle from a P15 mouse was photographed under microscope. The arrow and dotted arrow indicate length of IRS and total hair follicle, respectively. (B) Quantification of IRS length shown in D. In each vibrissa hair follicle, IRS length was normalized to total hair follicle length and relative IRS length is shown ($n = 8$). (C) Representative images of hematoxylin and eosin (HE) staining of anagen vibrissa hair follicle from a P15 mouse. The arrows and arrowhead denote keratinizing (eosin-positive) IRS and fully keratinized (eosin-negative) IRS, respectively. (D) Quantification of eosin-positive IRS length shown in C. In each vibrissa hair follicle, 5 µm of serial cross sections at interval of 20 µm were stained with HE and eosin staining in IRS was evaluated. The length of eosin-positive IRS was calculated and normalized to total hair follicle length. Relative eosin-positive IRS length is shown ($n = 5$). ns, no significant difference between two groups.
Figure S4 Hair cycle-dependent mRNA expression of PA-PLA₁α, LPA receptors, TGFα and EGFR.

(A) Specimens of dorsal skins of C57BL/6J mice at the indicated developmental ages were dissected and mRNA expression was analyzed by qRT-PCR. Results are depicted as the number of transcripts per one thousands of copies of Gapdh (n = 3-6). R², squared correlation coefficient between expression level of PA-PLA₁α and that of each gene (n = 58). Cat, catagen; Tel, telogen; Ana, anagen. Note that the first hair cycle starts with catagen (P17) and murine hair cycles are synchronized during the first few cycles (Muller-Rover et al, 2001). PA-PLA₁β (also known as Lipi) was below the detectable limit and thus data is not shown. (B) Hair depilation of adolescent C57BL/6J mice on P56 in the telogen stage was performed by application of wax to induce synchronized hair cycle (Muller-Rover et al, 2001). Total RNA from dorsal skins at the indicated days post-depilation was reverse transcribed and analyzed by qRT-PCR. Results are depicted as the number of transcripts per one thousands of copies of Gapdh (n = 4-6). R², squared correlation coefficient between expression level of PA-PLA₁α and that of each gene (n = 34).

Figure S5 Expression of TGFα and ADAMs in vibrissa hair follicles.

(A) qRT-PCR analysis of TGFα mRNA expression in vibrissa hair follicles. The number of transcripts was normalized to the housekeeping gene, Gapdh, in the same sample and the mean value in +/+ sample was set at 1 (n = 4). ; ns, no significant difference. (B) Membrane-bound TGFα level in vibrissa hair follicles. Lysates of anagen vibrissa hair follicles were ultracentrifuged and the resulting pellets (membrane fraction) were dissolved and subjected to TGFα sandwich ELISA. Concentration of TGFα was normalized to total protein in the same sample determined by the BCA method (mean ± s.e.m.; n = 4; * P < 0.05 versus +/+). Note that soluble TGFα level was also determined and shown in Figure 5A. ns, no significant difference between two groups. (C) Expression of catalytically active ADAM
members (Edwards et al, 2008) in vibrissa hair follicles. Total RNA from anagen vibrissa hair follicles from WT (+/+) and PA-PLA₁α⁻/⁻ (-/-) mice was reverse transcribed and analyzed by qRT-PCR. Results are depicted as the number of transcripts per one thousands of copies of Gapdh (n = 4). Note that there was no significant difference in expression of eight ADAM members between +/+ and -/-.

Figure S6 Immunofluorescent staining in hair follicles.
(A-C) Dorsal hair follicles from WT (+/+) and PA-PLA₁α⁻/⁻ (-/-) mice were immunostained with K72 (IRS marker) and one of TGFα-related proteins (A, TACE; B, TGFα; C, EGFR). Nuclei were stained with DAPI. Note that overall patterns of TGFα-related proteins were not significantly different between WT and PA-PLA₁α⁻/⁻ hair follicles. Also note that K72 fluorescent signal was faint in PA-PLA₁α⁻/⁻ hair follicles. (D and E) Dorsal hair follicles were immunostained with Trichohyalin (IRS marker) and K75 (companion layer marker, D) or K85 (hair shaft marker, E). Note that overall patterns of K75 and K85 were not significantly different between WT and PA-PLA₁α⁻/⁻ hair follicles. Also note that Trichohyalin fluorescent signal was faint in PA-PLA₁α⁻/⁻ hair follicles. Scale bars represent 20 µm.

Figure S7 Expression of LPA receptors in HEK293 cells.
Expression of LPA receptor mRNA in HEK293 cells was analyzed by qRT-PCR. Results are depicted as the number of transcripts per one thousands of copies of Gapdh (n = 4).

Figure S8 Ligand preference of P2Y5
(A) Activity of various lysophospholipids to induce AP-TGFα release from P2Y5-expressing cells. HEK293 cells that were transfected with mouse P2Y5 (mP2Y5), human P2Y5 (hP2Y5) or control vector were stimulated with the indicated lysophospholipids (concentration range, 3-1000 nM; * P < 0.05, ** P < 0.01 versus control cells). LPC, lysophosphatidylcholine; LPE,
lysophosphatidyethanolamine, LPG; lysophosphatidylglycerol; LPI, lysophosphatidylinositol; LPS, lysophosphatidylserine; S1P, sphingosine 1-phosphate. Note that data of LPA stimulation is identical to Figure 6B and data points at 1 µM of seven lysophospholipids are shown in Figure 6C. (B) Sensitivity of a phorbol ester (TPA)-induced AP-TGFα release. Note that transient expression of P2Y5 did not affect TPA-induced AP-TGFα release. Also note that data points at 100 nM are shown in Figure 6C. (C) Ligand preference of P2Y5 for LPA with various fatty acids. LPA species with various fatty acids were evaluated in the AP-TGFα shedding assay using mP2Y5-expressing HEK293 cells. LPA species examined here were 1-palmitoyl (16:0), 1-stearoyl (18:0), 1-oleoyl (18:1), 1-linoleoyl (18:2) and 1-arachidonoyl (20:4) species and EC50 values were 250, 1500, 35, 37 and 120 nM, respectively. Error bars in A-C represent s.d. (n = 4).

Figure S9 Agonistic activities of LPA analogs and LPA with various fatty acids to induce LPA receptor-mediated AP-TGFα release.

(A) Structures of LPA analogs used in this study. For comparison, 1-oleoyl is shown in the top. (B) Activity of LPA analogs to induce LPA receptor-mediated AP-TGFα release in HEK293 cells. Using the AP-TGFα shedding assay, P2Y5 agonists were screened from 120 LPA analogs we have developed so far (Kano et al, 2008; Prestwich et al, 2008). Compound XY31, XY36 and OMPT, were capable of inducing AP-TGFα release from P2Y5-expressing HEK293 cells. These LPA analogs were further tested for activity to induce AP-TGFα release from HEK293 cells transfected with other LPA receptor vectors (LPA1-5). AP-TGFα release at the concentration of 100 nM of LPA analogs was shown (n = 4). Rank orders are; for LPA1, LPA > XY36 > XY28, XY31, T10, OMPT; for LPA2, LPA > XY36 > XY28, XY31, T10, OMPT; for LPA3, OMPT > LPA > T10 > XY28, XY31, XY36; for LPA4, almost no response; for LPA5, XY28, XY31 > X36, T10, OMPT > LPA; for P2Y5, OMPT > LPA, XY36 > XY31 > XY28, T10.
Figure S10 Characterization of release of EGFR ligands in HEK293 cells.

(A) LPA/P2Y5-induced release of EGFR ligands is specific to TGFα. HEK293 cells transiently expressing P2Y5 and one of alkaline phosphatase (AP)-tagged EGFR ligands (TGFα, heparin-binding EGF-like growth factor (HB-EGF) and Amphiregulin (Amphireg)) (Tokumaru et al, 2000) were treated with 1 µM LPA or 100 nM TPA for 1 h. Release of AP-tagged EGFR ligands was quantified by measuring AP activities in both conditioned media and cell surface using a colorimetric reaction of AP substrate, p-NPP (see also Figure 7A). AP activity detected in vehicle treatment was used as the baseline (n = 4). Note that compared with TGFα, neither HB-EGF nor Amphiregulin was released from P2Y5-expressing HEK293 cells upon LPA or TPA stimulation. (B) LPA1, LPA3 and P2Y5 are capable of inducing AP-TGFα release. HEK293 cells expressing AP-TGFα and one of LPA receptors (mouse LPA1-5 or P2Y5) were treated with 18:1-LPA (1 µM) and AP-TGFα release was determined. (C) LPA3 and P2Y5, but not LPA1, are activated by co-expression of PA-PLA1α and induce spontaneous AP-TGFα release. Similar to the method shown in Figure 9A, HEK293 cells were transfected with combination of three vectors encoding AP-TGFα (20 ng per each well in a 96-well plate), PA-PLA1α (catalytically active form (WT) or catalytically inactive mutant (S154A); 2 ng per each well) and each LPA receptor (mouse LPA1-5 or P2Y5; 20 ng per each well). Spontaneous AP-TGFα release during 24 h after transfection in serum-free media was determined. For each LPA receptor-expressing condition, AP-TGFα release from S154A-expressing cells was determined and set as the baseline (i.e., bars depict differences between AP-TGFα release from WT-expressing cells and S154A-expressing cells) (B and C; n = 4; * P < 0.05, ** P < 0.01 versus control vector-transfected cells).
A

Inoue et al. Supplementary Figure S2

B

C

Relative peak area

Relative signal intensity

Retention time (min)

Acyl chain

Relative peak area

Relative peak area

Retention time (min)

Relative peak area

Relative peak area

2-acyl-LPA

1-acyl-LPA

16:0

18:2

18:1

18:0

20:5

20:4

22:6

22:5

17:0 (IS)

S1P

dhS1P

LPA

LPC

LPE

LPG

LPI

LPS

/++

-/-

/++

-/-
Inoue et al. Supplementary Figure S3

**A**

Bulb (lower)

Isthmus (middle)

Infundibulum (upper)

**B**

Relative IRS length (%)

+/

-/

ns

**C**

+/

-/

ns

**D**
**Inoue et al. Supplementary Figure S4**

**A**

- **PA-PLA\textsubscript{1,\alpha}**
- **LPA\textsubscript{1}** \(R^2 = 0.081\)
- **LPA\textsubscript{2}** \(R^2 = 0.769\)
- **LPA\textsubscript{3}** \(R^2 = 0.859\)
- **LPA\textsubscript{4}** \(R^2 = 0.002\)
- **LPA\textsubscript{5}**
- **LPA\textsubscript{6}** \(R^2 = 0.290\)
- **P2Y\textsubscript{5}** \(R^2 = 0.814\)
- **TGF\alpha** \(R^2 = 0.831\)
- **EGFR** \(R^2 = 0.905\)

**B**

- **PA-PLA\textsubscript{1,\alpha}**
- **LPA\textsubscript{1}** \(R^2 = 0.229\)
- **LPA\textsubscript{2}** \(R^2 = 0.755\)
- **LPA\textsubscript{3}** \(R^2 = 0.913\)
- **LPA\textsubscript{4}** \(R^2 = 0.046\)
- **LPA\textsubscript{5}**
- **LPA\textsubscript{6}** \(R^2 = 0.282\)
- **P2Y\textsubscript{5}** \(R^2 = 0.789\)
- **TGF\alpha** \(R^2 = 0.915\)
- **EGFR** \(R^2 = 0.840\)
Inoue et al. Supplementary Figure S5

A

B

C

Membrane fraction

+/-

-/-

Relative TGFα mRNA expression

TGFα (ng/mg protein)

mRNA expression (gene/Gadph x 10^-3)

ns
**Inoue et al. Supplementary Figure S6**

- **A**
  - DAPI, K72, TACE, Merge
  - +/-
  - +/-

- **B**
  - DAPI, K72, TGFα, Merge
  - +/-
  - +/-

- **C**
  - DAPI, K72, EGFR, Merge
  - +/-
  - +/-

- **D**
  - DAPI, K75, Trichohyalin, Merge
  - +/-
  - +/-

- **E**
  - DAPI, K85, Trichohyalin, Merge
  - +/-
  - +/-
mRNA expression (gene/Gadph x 10^-3)

Inoue et al. Supplementary Figure S7
Inoue et al. Supplementary Figure S9

A

1-oleoyl LPA

XY28

XY31

XY36

T10

OMPT

B

AP activity release (%)

control

LPA1

LPA2

LPA3

LPA4

LPA5

P2Y5
Inoue et al. Supplementary Figure S10

A

AP activity release (%)

AP-tagged EGFR ligand

TGFα  HB-EGF  Amphireg

vector  P2Y5  P2Y5  P2Y5

TGFα  HB-EGF  Amphireg

P2Y5  P2Y5  P2Y5

treatment  LPA  TPA

B

LPA

AP activity release (%)

control  LPA1  LPA2  LPA3  LPA4  LPA5  P2Y5

C

PA-PLA1α co-expression

AP activity release (%)

control  LPA1  LPA2  LPA3  LPA4  LPA5  P2Y5