Supporting Information

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SI Experimental Procedures

Lipin 1-Deficient Mouse Models. Mice harboring a *Lpin1* allele with exons 3 and 4 of the *Lpin1* gene flanked by *LoxP* sites have been described previously (1). To generate mice with the *Lpin1* gene selectively inactivated only in adipocytes or hepatocytes, the *Lpin1* floxed (*fl/fl*) mice were mated with *adiponectin-Cre* (Adn^{Cre}) (2) or *albumin-Cre* (Alb^{Cre}) (3) transgenic mice, respectively. The resulting offspring (Adn^{Cre+}-$Lpin1^{fl/wt}$ and Alb^{Cre+}-$Lpin1^{fl/wt}$) were mated with homozygous $Lpin1^{fl/fl}$ mice, leading to the generation of conditional knockout mice. The LpCond F and LpCond R primers were used for genotyping the mice generated (1). Mice constitutively deficient in lipin 1 (*fld* mice) were compared with WT ($^{+/+}$) littermate control mice (Balb/cByJ strain); 6-wk-old mice were used for all studies. All animal experiments were approved by Washington University School of Medicine's Animal Studies Committee.

Cloning of Truncated *Lpin1* cDNAs. A mouse lipin 1 cDNA lacking the first four exons of *Lpin1* (Δexon 4-lipin 1) was generated from mouse lipin 1 expression construct cDNA by PCR, using the following primers: sense, 5′-CGGTACCGAAATCATCCC-CATGTACCTGG-3′; antisense, 5′-GTCTAGATCAAGCTG-AGGCT GAATGCATGTCC-3. This cDNA encodes both the potential Δ100 and Δ115 start codons. The Δexon 4-lipin 1 cDNA was subcloned into a pCR-XL-TOPO vector (Invitrogen), and the entire insert was sequenced to verify its identity and integrity. For construction of an adenoviral expression vector, the truncated lipin 1 cDNA was subcloned into the AdTrack-CMV shuttle vector and recombined using the AdEasy-1 system (http://www.coloncancer.org/adeasy.htm). For the studies shown in Fig. 1, a triple-HA tag was added to the N terminus of lipin 1, and the Δ115 start codon was enforced. This HA-tagged Δ115-lipin 1 was subcloned into a pCDNA3 vector.

Western Blot Analyses. For Western blot studies, frozen tissues were homogenized using a sonicator in ice-cold RIPA buffer containing 1% Nonidet P-40, 0.1% SDS, and protease/phosphatase inhibitors, and then subjected to SDS/PAGE using Criterion XT precast gels (Bio-Rad Laboratories Inc.). Blots were probed using antibodies against lipin 1 (4), lipin 2 (5), hormone-sensitive lipase (Cell Signaling), adipose tissue TG lipase (Cell Signaling), perilipin 1 (a gift from Nathan Wolins, Washington University at St. Louis), phospho-perilipin (Vala Sciences), phospho-protein kinase A (PKA) substrate (Cell Signaling), phospho-cAMP response element binding protein (Cell Signaling), FLAG (Sigma-Aldrich), phospho-mammalian target of rapamycin (Cell Signaling), phospho-S6K (Sigma-Aldrich), t-S6K (Sigma-Aldrich), and α-tubulin (AA4.3; Developmental Studies Hybridoma Bank). Blots were visualized using the Odyssey Imaging System (LI-COR Biosciences).

For immunoprecipitation studies, 400 μg of whole-cell lysate was precleared with protein A plus agarose beads (Thermo Scientific) and then incubated with lipin 1 C terminus antibody overnight at 4 °C. Western blot analyses with total or phosphoserine lipin 1 antibodies (a gift from David Sabatini, Massachusetts Institute of Technology, Cambridge, MA) were conducted on immunopurified proteins (6).

Quantitative RT-PCR Analyses. For all analyses, total RNA from epididymal white adipose tissue (WAT) was isolated in RNAzol (Invitrogen) reagent. Total RNA (500 ng) was subjected to reverse transcription and SYBR Green RT-PCR (Applied Biosystems) following the manufacturer's instructions. Results were corrected to 36B4 expression. The sequence of gene-specific primers is available on request.

MS Quantification of Lipids. Lipids were extracted by the method of Bligh and Dyer (7) in the presence of internal standard [T21:0 triglyceride (TG), 10 nmol/mg protein], and separated on silica gel 60A plates (EMD Millipore). Spots corresponding to diacylglycerol (DAG), TG, and free fatty acids were visualized with 0.01% rhodamine 6G and identified with their respective standards. The bands were scraped and extracted with chloroform: methanol 3:1. Quantitative GC analysis was performed using a Hewlett-Packard 5890 gas chromatograph with a 30 m × 0.32 mm Omegawax 250 column (Sigma-Aldrich) and a flame ionization detector. For lysophosphatidic acid (LPA) and phosphatidic acid (PA) quantification, total lipid was extracted from epididymal fat pads and analyzed by LC/MS with a Shimadzu Prominence Ultra-Fast Liquid Chromatography System.

Phosphatidic Acid Phosphohydrolase Activity. For the data displayed in Fig. 1, type 1 Mg^{2+}-dependent phosphatidic acid phosphohydrolase (PAP) activity was measured by determining the rate of hydrolysis of 16:1_18:0 PA to form 16:1_18:0 DAG. Immunoprecipitated HA-tagged lipin 1 proteins were incubated with 0.5 mM 16:1_18:0 PA in buffer containing 0.25 mM $MgCl_2$ and 3.2 mM Triton-X100 in a total volume of 0.2 mL. The reaction was stopped by adding 0.3 mL of 0.1 M HCl. Lipids were extracted using acidified organic solvents an internal standard of d5 diglycerides added and 16:1_18:0 DAG, quantified by HPLC electrospray ionization MS/MS. Activity measurements were normalized to protein present in the starting lysate determined by the bicinchoninic acid assay.

PAP activity in adipose tissue was determined using a modification of the method of Lin and Carman (8) to measure phosphate release from Triton X-100/PA mixed micelles. ^{32}P-labeled PA was generated from DAG (Avanti, catalog no. 800811C) using recombinant DAG kinase. Mg^{2+}-dependent PAP activity was determined by subtracting activity obtained in the absence of $MgCl_2$ from activity measured in the presence of 2 mM $MgCl_2$ (lipid phosphate phosphatase activity).

WAT Explant Isolation and Metabolic Assays. WAT isolated from three or four *Adn-Lpin1*$^{-/-}$ or WT littermates were pooled and cut into small pieces (~10 mg each). The adipose tissue explants were immediately cultured in 24-well plates (20-30 mg per well) in a Krebs Ringer bicarbonate Hepes buffer supplemented with 6 mM glucose and 2% fatty acid-free BSA (assay buffer) using a metabolic incubator in a humidified atmosphere. All metabolic experiments were started after an initial culture period of 1 h and carried out in four replicates.

To determine the rate of lipolysis, the explants were cultured in fresh assay buffer alone or in the presence of 10 μM of isoproterenol for 90 min. An enzymatic kit (Sigma-Aldrich) was used to determine the amount of glycerol released into the media during this incubation period. Rates of lipolysis were expressed as nmol glycerol/μg protein/h.

To measure synthetic rates of TG, DAG, and phospholipids [phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL)], the adipose tissue explants were cultured in fresh Krebs Ringer bicarbonate Hepes buffer containing 0.3 mM BSA-bound oleic acid and 2.5 μCi of [9, 10-^{3}H]-oleic acid/mL for 1.5 h. Total lipids were extracted from the tissue explants and separated by TLC, and the

^{3}H-radioactivity in the labeled lipids was determined after TLC separation of PC, PE, PG, and CL, as described by Leray et al. (9). The synthetic rates of TG, DAG, and phospholipids PC, PE, PG, and CL were expressed as dpm/μg protein/h.

3T3-L1 Studies. 3T3-L1 cells were cultured to confluence in DMEM containing 20% (vol/vol) calf serum, with the medium changed every 2 d. At 2 d after cell confluence, differentiation was initiated by adding differentiation medium 1 [0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.25 μM dexamethasone, 1 μg/mL insulin in DMEM containing 10% (vol/vol) FBS]. Adipocytes were infected with previously described adenovirus expressing shRNA designed to knock down lipin 1 (10) or to overexpress glycerol-3-phosphate acyltransferase 1 (GPAT1; a generous gift from Rosalind Coleman, University of North Carolina, Chapel Hill, NC) (11). To overexpress phospholipase D (PLD), 3T3L1 cells were transfected with vector expressing mouse mPLD. Adenovirus expressing GFP and shRNA against LacZ was used as a control. At 72 h after infection, cells were treated with 10 nM isoproterenol, 250 nM Torin, 10 μM cilostamide, 10 μM rolipram, or 50 μM IBMX as indicated. After 4 h, cells were harvested, and protein was isolated for Western blot analysis. For experiments involving siRNA-mediated knockdown, siRNAs were transfected onto the differentiated 3T3 cells with Lipofectamin-2000 reagents (Invitrogen), and the cells were harvested for phosphodiesterase (PDE) assays at 44 h after transfection. Specific siRNAs for mouse Lpin-1, PDE4B, and PDE4D were purchased from Sigma-Aldrich.

Hepatocyte Isolation and Glucagon Stimulation. Hepatocytes from WT and *Alb-Lpin1*$^{-/-}$ mice were isolated as described previously (12) and cultured in DMEM containing 10% FBS for 16 h, followed by another 4-h incubation in serum-free DMEM. These hepatocytes were incubated in fresh DMEM containing 0, 10, or 100 ng/mL glucagon for 5 min and immediately washed twice with ice-cold PBS, and then harvested for Western blot analysis using an anti phospho-PKA substrate antibody.

PDE Activity and cAMP Concentration. For PDE activity assays, adipose tissue or 3T3-L1 lysates were homogenized in buffer containing 50 mM Tris (pH 7.4) and 3 mM $MgCl_2$ and centrifuged. The supernatant was used to quantify PDE activity using a kit (Mediomics) in accordance with the manufacturer's instructions. cAMP content was determined using a kit (Arbor Assays) in accordance with the manufacturer's instructions. Recombinant GST-PDE4 (BPS Bioscience) was used to determine the direct effect of PA, LPA, and PC on PDE enzymatic activity. Approximately 2 units of GST-PDE4 were mixed with vehicle or 200 μM PA, LPA, or PC emulsion, each derived from egg yolk, and the relative enzymatic activity was determined using a Mediomics kit.

Transcriptional Regulation by Dual-Luciferase Reporter Assay. The acyl CoA oxidase-thymidine kinase luciferase reporter construct has been described previously (13). Peroxisome proliferator-activated receptor-α (PPARα) was overexpressed using a pEFBOS-PPARα expression construct (14). Transient transfections were performed in Cos7 cells by the calcium phosphate coprecipitation method, and SV40-driven *Renilla* luciferase expression vector was cotransfected to control for transfection efficiency. Firefly and *Renilla* luciferase were assessed using the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions.

Studies in Human Subjects. Twenty-eight obese (mean body mass index, 35.8 ± 5.0 kg/m^2) men and women participated in this study. All subjects completed a comprehensive medical evaluation, which included a 2-h oral glucose tolerance test. No subject had any history or evidence of diabetes or took any medication that could affect metabolic function. All subjects provided written informed consent before participating in this study, which was approved by Washington University School of Medicine's Human Research Protection Office.

Subjects were admitted to the Clinical Research Unit at Washington University School of Medicine on the evening before the isotope infusion study. At 1900 hours, subjects were served a standard meal, after which they fasted until study completion the next day. At 0600 hours the next morning, one catheter was inserted into a forearm vein to infuse stable isotope-labeled palmitate (Cambridge Isotope Laboratories), and a second catheter was inserted into a radial artery in the contralateral arm to obtain blood samples. At 0800 hours, a continuous infusion of [U-^{13}C] palmitate (0.006 μmol/kg fat-free mass/min) was started and maintained for 90 min. Blood samples were obtained before the start of the tracer infusion and every 10 min during the final 30 min of the infusion to determine palmitate kinetics. Blood samples were collected in chilled tubes and placed on ice, and plasma was separated by centrifugation within 30 min of collection. Plasma samples were stored at −80 °C until final analysis.

Abdominal s.c. adipose tissue samples were obtained from the periumbilical area by aspiration. The biopsy sites were cleaned and draped, and the skin and underlying tissue were anesthetized with lidocaine. A small (~0.5 cm) skin incision was made with a scalpel, and adipose tissue was aspirated through a 4-mm liposuction cannula (Tulip Medical). Adipose tissue samples were immediately rinsed in ice-cold saline solution, frozen in liquid nitrogen, and stored at −80 °C until final analysis.

Plasma free fatty acid concentrations were determined by GC, and plasma palmitate tracer-to-tracee ratios were determined by electron impact ionization GC-MS, as described previously (15). Isotopic steady-state conditions were achieved during the final 30 min of isotope tracer infusion; thus, Steele's equation for steady-state conditions was used to calculate substrate kinetics (16).

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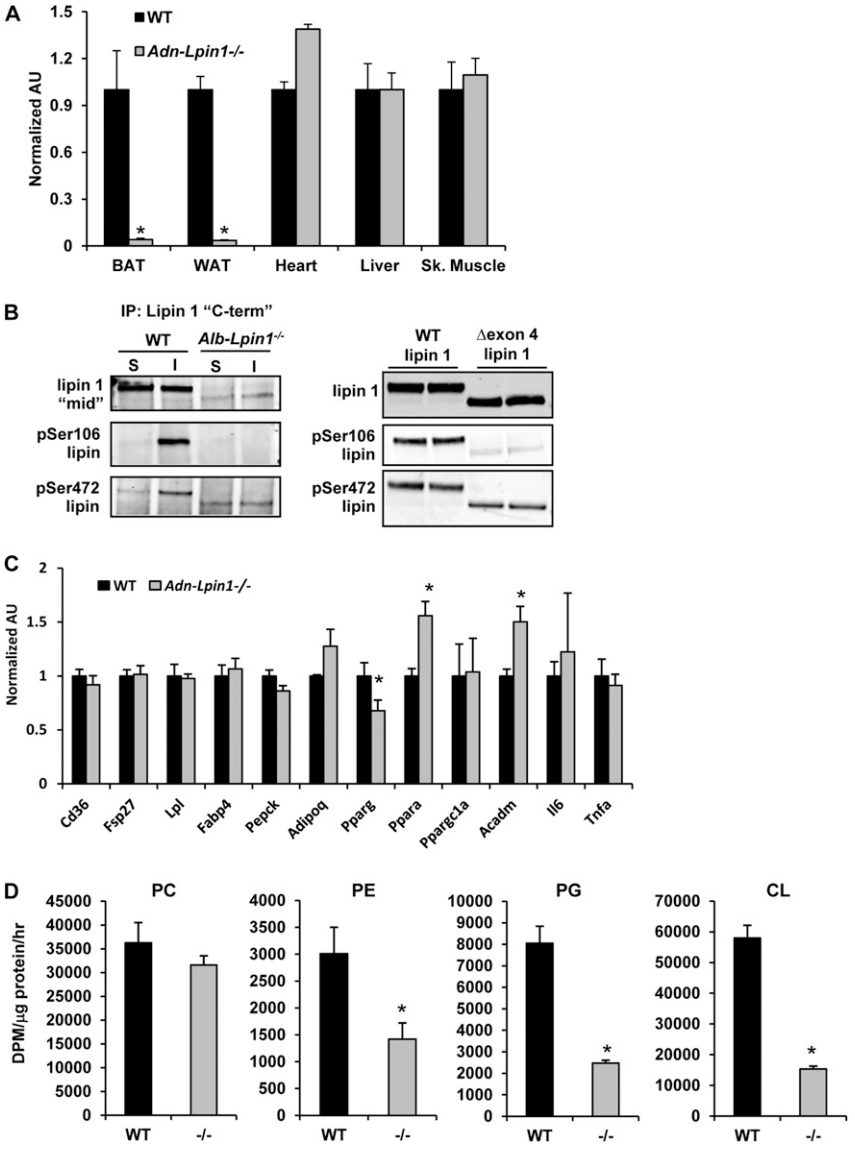

Fig. S1. Adipose tissue lipin 1 deficiency. (*A*) Graph showing lipin 1 mRNA expression in the brown adipose tissue (BAT), WAT, heart, liver, and skeletal muscle of 6- to 8-wk-old male WT and *Adn-Lpin1*$^{-/-}$ mice using primers annealing to the floxed exons. (*B*, *Left*) Representative Western blots for lipin 1 protein immunoprecipitated with an antibody against the C terminus of lipin 1 and blotted with a second antibody against the middle of the lipin 1 protein, phosphorylated serine 106 lipin 1, and phosphoserine 472 lipin 1. Lysates were obtained from 24-h fasted 6- to 8-wk-old male WT or *Alb-Lpin1*$^{-/-}$ mice injected with saline (S) or insulin (I). (*B*, *Right*) Western blots for total, phosphoserine 106, and phosphoserine 472 lipin 1 using lysates from hepatocytes overexpressing full-length or Δexon4-lipin 1 via adenovirus infection. (*C*) Graphs showing the expression of mRNA for a series of adipocyte markers and known lipin 1 target genes determined by quantitative RT-PCR using epididymal adipose tissue RNA from 6- to 8-wk-old male WT and Adn-*Lpin1*$^{-/-}$ mice ($n = 7$ per group). *$P < 0.05$ vs. WT mice. (*D*) Graph showing rates of PC, PE, PG, and CL synthesis from ^{3}H-oleate in epididymal adipose tissue explants isolated from 6- to 8-wk-old male WT and *Adn-Lpin1*$^{-/-}$ mice ($n = 4$ per group). *$P < 0.05$ vs. WT.

Exon 5 of the mouse Lpin1 gene

gaa atc atc ccc ATG tac ctg gcc acg tcc ccc atc ctg tca gaa gga gct gcg aga
ATG gaa agc cag ctg aag agg aac tct gta gac aga atc agg tgc ctg gat ccc act
aca gct gcc cag ggc ctg cct ccc agc gac acc cca tcc act ggt tct ctg ggg aag
aag aga agg aaa agg agg agg aag gcc cag ttg gac aat ctc aaa aga gat gac aat
gtc aac aca tct gag gat gag gac atg ttt ccc ata gag atg agc tcg gat gag gac
aca gca ccg atg gat gga agc

Δ115-lipin 1

MESQLKRNSVDRIRCLDPTTAAQGLPPSDTPSTGSLGKKRRKRRRKAQLDNLKRDDNVNTSEDEDMFPIEMSSDEDTA
PMDGSRTLPNDVPPFQDDIPKENFPSISTYPQSASYPSSDREWSPSPSPSGSRPSTPKSDSELVSKSADRLTPKNNLE
MLWLWGELPQAAKSSSPHKMKESSPLGSRKTPDKMNFQAIHSESSDTFSDQSPTMARGLLIHQSKAQTEMQFVNEEDL
ESLGAAAPPSPVAEELKAPYPNTAQSSSKTDSPSRKKDKRSRHLGADGVYLDDLTDMDPEVAALYFPKNGDPGGLPKQ
ASDNGARSANQSPQSVGGSGIDSGVESTSDSLRDLPSIAISLCGGLSDHREITKDAFLEQAVSYQQFADNPAIIDDPN
LVVKVGNKYYNWTTAAPLLLAMQAFQKPLPKATVESIMRDKMPKKGGRWWFSWRGRNATIKEESKPEQCLTGKGHNTG
EQPAQLGLATRIKHESSSSDEEHAAAKPSGSSHLSLLSNVSYKKTLRLTSEQLKSLKLKNGPNDVVFSVTTQYQGTCR
CEGTIYLWNWDDKVIISDIDGTITRSDTLGHILPTLGKDWTHQGIAKLYHKVSQNGYKFLYCSARAIGMADMTRGYLH
WVNERGTVLPQGPLLLSPSSLFSALHREVIEKKPEKFKVQCLTDIKNLFFPNTEPFYAAFGNRPADVYSYKQVGVSLN
RIFTVNPKGELVQEHAKTNISSYVRLCEVVDHVFPLLKRSHSCDFPCSDTFSNFTFWREPLPPFENQDMHSASA

Fig. S2. Peptide sequence of Δ115-lipin 1 protein. (*Upper*) DNA sequence of exon 5, the first intact exon after the deletion of exons 3 and 4. Two in-frame ATGs are underlined. The second ATG is flanked by a consensus Kozak sequence (also underlined) and corresponds to the translational start site for the Δ115-lipin 1 protein. (*Lower*) Peptide sequence of the deduced Δ115-lipin 1 protein. The polybasic motif that mediates nuclear import and the interaction with PA is shown in blue. The DXDXT and LXXIL motifs that compose the catalytic site for PAP activity and nuclear receptor interaction, respectively, are shown in green.

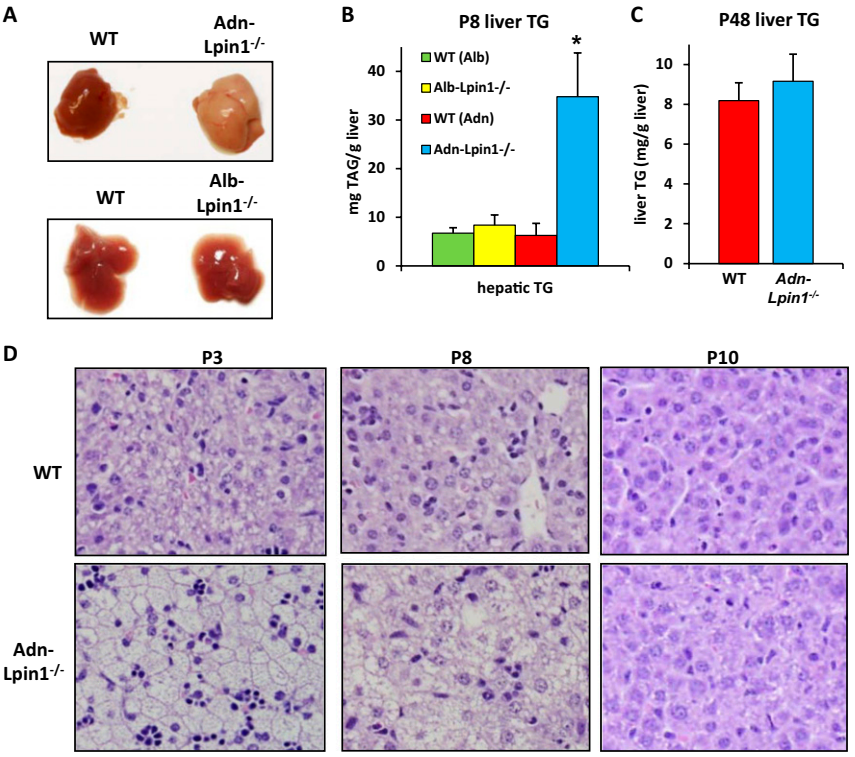

Fig. S3. Adipose tissue-specific lipin 1 deficiency leads to neonatal hepatic steatosis. (*A*) Livers from 8-d-old mice with adipose tissue-specific (Adn-Lpin1$^{-/-}$; *Upper*) and liver-specific (Alb-Lpin1$^{-/-}$; *Lower*) lipin 1 deficiency. (*B* and *C*) Graphs showing liver TG content of 8-d-old (P8; *Left*) and 48-d-old (P48; *Right*) mice with adipose tissue-specific (Adn-Lpin1$^{-/-}$) or liver-specific (Alb-Lpin1$^{-/-}$) lipin 1 deficiency. *$P < 0.01$ vs. control group ($n = 6$). (*D*) H&E-stained hepatic sections from 3-, 8-, and 10-d-old mice with adipose tissue-specific (Adn-Lpin1$^{-/-}$) lipin 1 deficiency.

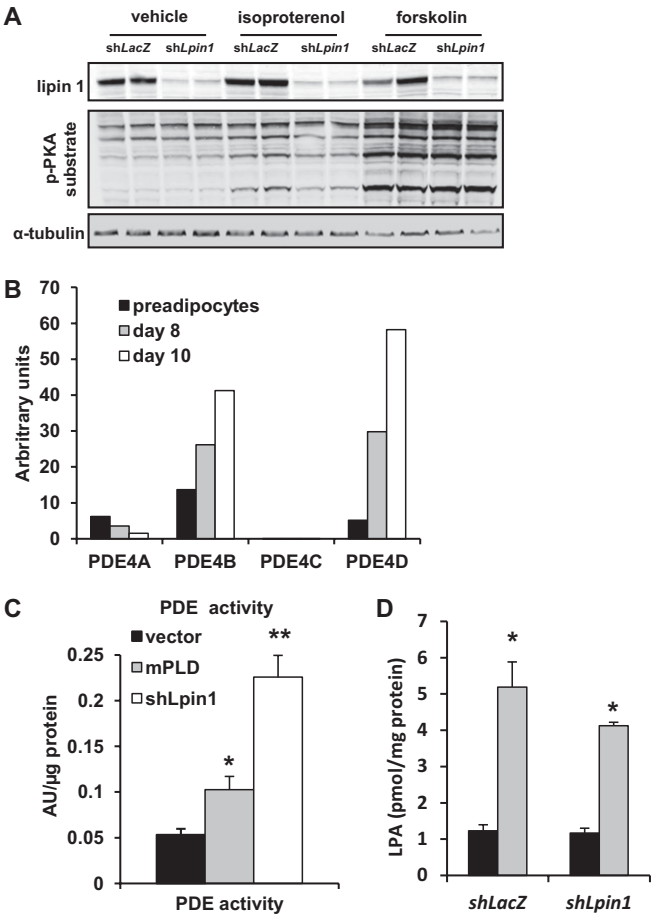

Fig. S4. PA activates PDE activity in 3T3-L1 adipocytes. (*A*) Representative Western blot images for the indicated proteins or phosphoproteins in 3T3-L1 adipocytes infected with adenovirus to knockdown lipin 1 or control shRNA and treated with vehicle, isoproterenol, or forskolin. (*B*) Graph showing PDE4 family gene expression in 3T3-L1 preadipocytes or after 8 d or 10 d of differentiation. (*C*) Graph showing PDE activity in lysates from 3T3-L1 adipocytes transfected with expression vectors to overexpress mouse phospholipase D (mPLD) or knockdown lipin 1 (sh*Lpin1*). *$P < 0.05$ vs. controls. (*D*) Graph showing LPA content of 3T3-L1 adipocytes infected with adenovirus to knockdown lipin 1 (sh*Lpin1*) and/or overexpressing GPAT1. Grey bars represent GPAT1-overexpressing cells and black bars represent GFP-expressing controls. *$P < 0.01$ vs. cells not overexpressing GPAT1.