Perilipin-5 is regulated by statins and controls triglyceride contents in the hepatocyte

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Supplemental Materials and Methods

RNA analysis

RNA was isolated from cells or livers using Trizol, and analyzed by real-time quantitative PCR (RT-qPCR). Primer sets are provided in Table S1. Values were normalized to 36B4 and relative expression calculated using the $\Delta\Delta C_T$ method.

Protein analysis

Fifty micrograms of protein were resolved in 4–12% Bis–Tris gels, transferred to PVDF membranes, and probed with antibodies for PLIN5 (1:500; sc-240627, Santa Cruz), PLIN2 (1:500; ab37516, Abcam), PLIN3 (1:500; 10694-1-AP, Proteintech), and β-ACTIN (1:1,000; A2066, Sigma), in TBS-Tween20 containing 5% non-fat dry milk. Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies (1:5,000; Bio-Rad).

Luciferase reporter assays

HEK293 cells were maintained in DMEM + 10% FBS. Transfection was performed in triplicate in 24-well plates using the calcium phosphate method, as described [1]. Luciferase activity was measured 48 h later using the Luciferase Assay System (Promega), and normalized to β-galactosidase activity to correct for small changes in transfection efficiency.

Oil Red O staining

Cells were fixed with 10% formalin, incubated with the Oil Red O, washed with 60% isopropanol, and counterstained with hematoxylin and saturated lithium carbonate.

Plasmids and adenovirus

A 3.5-kb fragment spanning the proximal promoter, first exon, and first intron of the mouse and human *PLIN5* genes was amplified from genomic DNA using Phusion polymerase

(Thermo Scientific), and subcloned into pGL3-basic (Promega). Mutagenesis was done using the QuickChange site-directed mutagenesis kit (Stratagene). Mouse mature SREBP-2 was amplified from genomic DNA, and cloned into pDsRed (ClonTech). A luciferase reporter construct containing the proximal promoter of the human LDLR was a kind gift from Dr. Thomas Vallim (UCLA). A plasmid containing the Myc-tagged *Plin5* was purchased from Origene (MR207402); *Plin5*-Myc was digested with KpnI and EcoRV and subcloned into pAdTrack-CMV. Replication-deficient adenovirus were produced using the AdEasy Adenoviral System (Stratagene), as described [1, 2], purified by CsCl gradient ultracentrifugation, and titrated in HEK293 cells.

Lipid analysis

Lipids in livers and primary hepatocytes were extracted into $CHCl_3$ by a modified Folch method and resolubilized in water as described [3], and quantitated enzymatically using kits for triglycerides and cholesterol (Wako Chemicals). Results were normalized to protein.

Plasma analysis

Lipids in plasma were quantitated enzymatically as above. Activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were quantified enzymatically using kits (BioVision).

Chromatin immunoprecipitation (ChIP)

Human HuH7 hepatoma cells were seeded in 15 cm dishes and transfected with a plasmid encoding the mature human SREBP-2 (tagged with a Flag epitope) (plasmid 26807, Addgene), using lipofectamine LTX (Invitrogen). Forty-eight h after transfection, cells were processed for ChIP according to the protocol described by Bennet et al. [4]. Briefly, protein-DNA complexes were cross-linked and chromatin was precipitated using M2 anti-Flag or irrelevant mouse IgG antibodies (Sigma). Genomic regions surrounding the SRE in *PLIN5* human promoter, the SRE in human *LDLR* promoter (positive control), and a region within intron 1 in *PLIN5* (negative control) were amplified by qPCR using 2 mL of the immunoprecipitates. Primers are available upon request. Data is represented as amplification enrichment in anti-Flag vs. IgG immunoprecipitates, as described [5].

Fatty acid oxidation and de novo lipogenesis assays

Mouse primary hepatocytes were seeded in 24-well plates, incubated in media supplemented with DMSO or 5 μmol/L simvastatin for 48 h, and then supplemented with 125 μmol/L [^{3}H]-palmitate:BSA (1:20 ratio; 1 μCi/mL specific activity), as described [6]. After 2 h, supernatants were collected and applied to an ion-exchange resin (DOWEX 1X2, Sigma) to measure [^{3}H]-water release. To measure *de novo* lipogenesis, cells were incubated in 0.5 mL of media supplemented with 1 μCi/mL [^{14}C]-acetate for 6h. The cells were then scraped in methanol and lipids were extracted according to the Bligh Dyer method [7]. The lipid extracts were resolved by thin layer chromatography, together with standards for different classes of lipids. The TG band for each condition was scrapped and the radioactivity counted by scintillation. For both fatty acid oxidation and lipogenesis assays, radioactivity was normalized with cell protein content.

Cell viability assays

Mouse primary hepatocytes were seeded in collagen-coated 48-well plates and treated as described. To estimate viability, the cells were incubated for 3 h in the presence of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Cayman); healthy cells cleave MTT to produce dark blue formazan crystals that are then solubilized with isopropanol and spectrophotometrically quantified. Cell toxicity was measured by the release of intracellular lactate dehydrogenase (LDH) into the culture medium using the LDH-Cytotoxicity assay kit (BioVision). Apoptosis was estimated using the luminescent Caspase-Glo 3/7 assay (Promega). Intracellular ROS production was determined using the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma) and normalized to protein content; non-fluorescent DCFH-DA diffuses into the cell and is enzymatically hydrolyzed to DCFH, which can be rapidly oxidized to highly fluorescent DCF in the presence of ROS.

Statistical analysis

Data are shown as mean ± SEM. Differences in mRNA expression and lipid contents between groups were analyzed by Student's *t*-test, or one-way ANOVA and post hoc Tukey's test, or two-way ANOVA followed by Mann-Whitney *U*-test, as appropriate.

Supplemental figure legends

Fig. S1. Transcriptional profile of selected genes in mice treated with atorvastatin. Mice were dosed with saline (open bars) or 20 mg/Kg/day atorvastatin (closed bars). After the last dose, mice were allowed access to food, or fasted for 24 h. The relative expression of selected SREBP-2 targets (*A*), PPARα targets (*B*), and lipogenic genes (*C*) was determined by RT-qPCR. Data are shown as mean ± SEM, and were analyzed by two-way ANOVA followed by Mann-Whitney *U*-test. * $P \leq 0.05$, fasted vs. fed; ** $P \leq 0.01$, fasted vs. fed; ¶ $P \leq 0.05$, atorvastatin vs. saline; ¶¶ $P \leq 0.01$, atorvastatin vs. saline.

Fig. S2. Transcriptional profile of selected genes in mouse and human primary hepatocytes incubated with statins. Moue (*A*) and human (*B*) cells were cultured as described in Fig. 1D–F. The relative expression of selected SREBP-2 targets and LD-related genes was determined by RT-qPCR. Data are shown as mean ± SEM, and were analyzed by one-way ANOVA and post hoc Tukey's test. * $P \leq 0.05$, ** $P \leq 0.01$, statin vs. DMSO.

Fig. S3. Incubation with statins and/or oleate is not cytotoxic primary hepatocytes. Mouse primary hepatocytes were incubated with or without 5 μmol/L simvastatin (SMV) and/or 0.6 mmol/L oleate (OA), as described in Fig. 3A, B. Cellular toxicity and metabolic activity were evaluated by the release of LDH activity into the supernatant (*A*) and intracellular MTT activity (*B*), as described in Methods. Data show no significant adverse effects of SMV and/or OA. Data are shown as mean ± SD.

Fig. S4. Specificity of siRNA oligonucleotides against *Plin5*, and lack of cytotoxic effects. Mouse primary hepatocytes were transfected with non-targeting (siNT) or increasing amounts of anti-*Plin5* (si*Plin5*) siRNA oligonucleotides, as described in Fig. 3E–G. (*A*) The relative mRNA levels of hepatic perilipins and PPARα targets were determined by RT-qPCR, providing compelling evidence of the specificity of the *Plin5* knock-down. (*B*) The potential lipotoxic consequences of loss of *Plin5* were evaluated by measuring the release of LDH activity into the supernatants, intracellular MTT activity, caspase-3/7 activity, reactive oxygen species (ROS) production, and cytokine production, as described in Methods. Data show that only the highest siRNA dose (where both mRNA and protein levels for *Plin5* are decreased >

90%) leads to significant increase in cell toxicity and death. Data are shown as mean ± SEM, and were analyzed by one-way ANOVA and post hoc Tukey's test. * P < 0.05, **P < 0.01, si*Plin5* vs. siNT.

Fig. S5. *Plin5* overexpression does not change the expression of other hepatic perilipins. Mouse primary hepatocytes were transduced with an empty adenovirus or an adenovirus encoding Myc-tagged mouse *Plin5*, and incubated in the presence of BSA or 0.6 mmol/L oleate (OA), as described in Fig. 6. The relative mRNA levels of *Plin2*, *-3* and *-5* were determined by RT-qPCR. Data show that the expression of other hepatic perilipins does not change following overexpression of *Plin5*. Data are shown as mean ± SEM, and were analyzed by two-way ANOVA followed by Mann-Whitney *U*-test. *P < 0.05, OA (oleate) vs. BSA; §P < 0.05, adeno-Plin5 vs. adeno-empty.

Table S1

Transcript	Forward primer	Reverse primer
36b4	GGTGCCTCTGGAGATTTTCG	CACTGGTCTAGGACCCGAGAAG
Acc	TGACAGACTGATCGCAGAGAAAG	TGGAGAGCCCCACACACA
Atgl	GCCTCCTTGGACACCTCAATAA	CTTCCTCGGGGTCTACCACA
Cgi58	ATCTTTGGAGCCCGATCCT	CTTCTGGCTGATCTGCATACAC
Cpt1α	TGAGTGGCGTCCTCTTTGG	CAGCGAGTAGCGCATAGTCATG
Cpt2	CCAAAGAAGCAGCGATGG	TAGAGCTCAGGCAGGGTGA
Fasn	GCTGCGGAAACTTCAGGAAAT	AGAGACGTGTCACTCCTGGACTT
Gpat	GTCCAACACCATCCCCGACATC	AGTGACCTTCGATTATGCGATCA
Hmgcr	CTTGTGGAATGCCTTGTGATTG	AGCCGAAGCAGCACATGAT
Hsl	TTCTCCAAAGCACCTAGCCAA	TGTGGAAAACTAAGGGCTTGTTG
Ldlr	AGGCTGTGGGCTCCATAGG	TGCGGTCCAGGGTCATCT
Mcad	TTACCGAAGAGTTGGCGTATG	ATCTTCTGGCCGTTGATAACA
Pcsk9	GAAGACCGCTCCCCTGAT	GCACCCTGGATGCTGGTA
Pdk4	CGCTTAGTGAACACTCCTTCG	CTTCTGGGCTCTTCTCATGG
Plin2	CCTCAGCTCTCCTGTTAGGC	CACTACTGCTGCTGCCATTT
Plin3	CCACAGGATGCTGAAAAGG	TGATGTCCCTGAACATGCTG
Plin5	ACATGGTGCTGGGCAAGT	TCAGCTGCCAGGACTGCTA
Pparα	CTGAGACCCTCGGGGAAC	AAACGTCAGTTCACAGGGAAG
Scd1	CCGGAGACCCCTTAGATCGA	TAGCCTGTAAAAGATTTCTGCAAACC
Srebp1c	GGAGCCATGGATTGCACATT	GGCCCGGGAAGTCACTGT
Srebp2	GCGTTCTGGAGACCATGGA	ACAAAGTTGCTCTGAAAACAAATCA
Tnfα	CTGTAGCCCACGTCGTAGC	TTGAGATCCATGCCGTTG
36B4	CCCATCAGCACCACAGCC	CTCCAAGCAGATGCAGCA GA
ATGL	CTCCACCAACATCCACGAG	CCCTGCTTGCACATCTCTC
CGI58	GGACAAAATGATCTTGCTTGG	CCCAAGGCTCCACTAAAATG
HMGCR	CCATTTTGCCCGAGTTTT AG	TCAGCTATCCAGCGACTG TG
HSL	AACCAGTGCTCGGAATCAC	CAGGTCCATGTTGTGTGGATGA
LDLR	AGTTGGCTGCGTTAATGTGAC	TGATGGGTTCATCTGACCAGT
PCSK9	CGATGCCTGCCTCTACTCC	CTGGTCTTGGGCATTGGT
PLIN2	ACATTAAAGGGAAGAAGTTGAAGC	TTCTCCTGCTCAGGGAGGT
PLIN3	GATCACTTCCTGCCCATGAC	CACCGAACCCACTTCAGG
PLIN5	AGTTCCAAGCCAGGGACAC	CTGCTGGGCCTTTTCAATC

Supplemental References

[1] Allen RM, Marquart TJ, Albert CJ, Suchy FJ, Wang DQ, Ananthanarayanan M, et al. miR-33 controls the expression of biliary transporters, and mediates statin- and diet-induced hepatotoxicity. EMBO Mol Med 2012;4:882-895.

[2] Marquart TJ, Allen RM, Ory DS, Baldan A. miR-33 links SREBP-2 induction to repression of sterol transporters. Proc Natl Acad Sci U S A 2010;107:12228-12232.

[3] Carr TP, Andresen CJ, Rudel LL. Enzymatic determination of triglyceride, free cholesterol, and total cholesterol in tissue lipid extracts. Clin Biochem 1993;26:39-42.

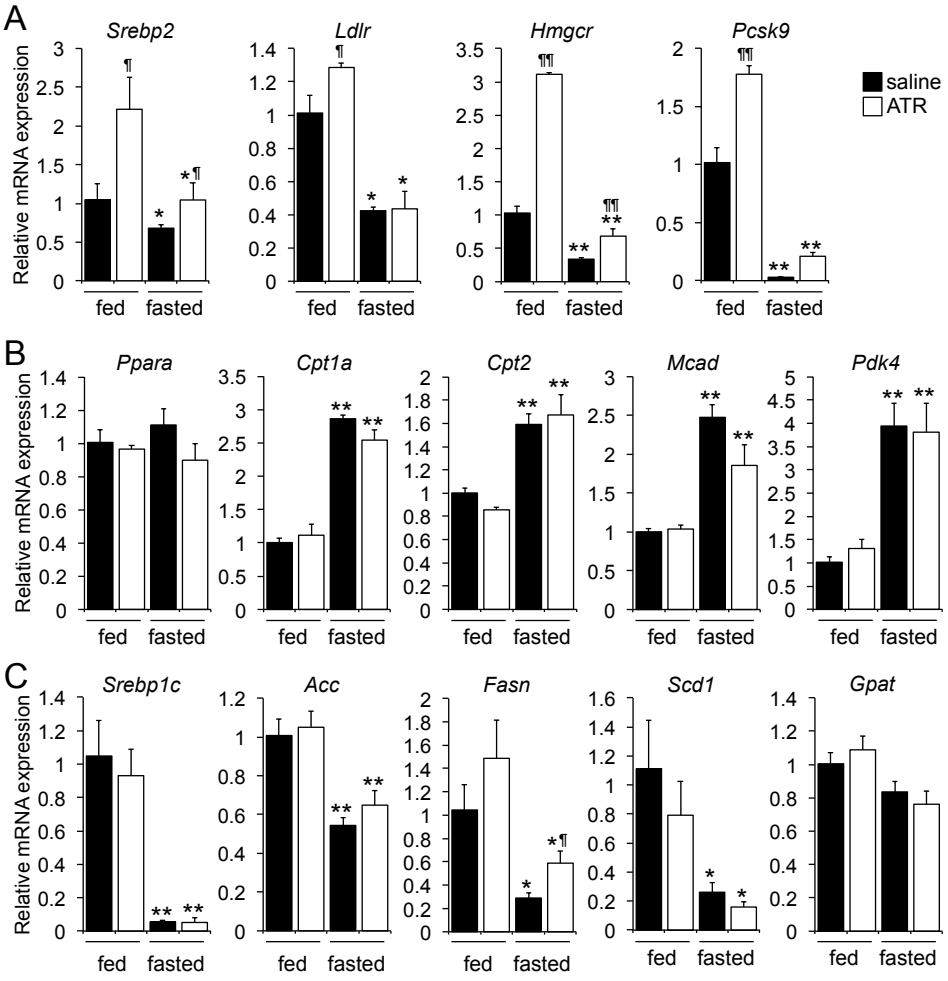
[4] Bennett MK, Toth JI, Osborne TF. Selective association of sterol regulatory element-binding protein isoforms with target promoters in vivo. J Biol Chem 2004;279:37360-37367.

[5] Mukhopadhyay A, Deplancke B, Walhout AJ, Tissenbaum HA. Chromatin immunoprecipitation (ChIP) coupled to detection by quantitative real-time PCR to study transcription factor binding to DNA in Caenorhabditis elegans. Nat Protoc 2008;3:698-709.

[6] Djouadi F, Bonnefont JP, Munnich A, Bastin J. Characterization of fatty acid oxidation in human muscle mitochondria and myoblasts. Mol Genet Metab 2003;78:112-118.

[7] Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959;37:911-917.

Fig. S1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Hmgcr
Pcsk9
Relative mRNA expression
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ATR
fed
fasted
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C
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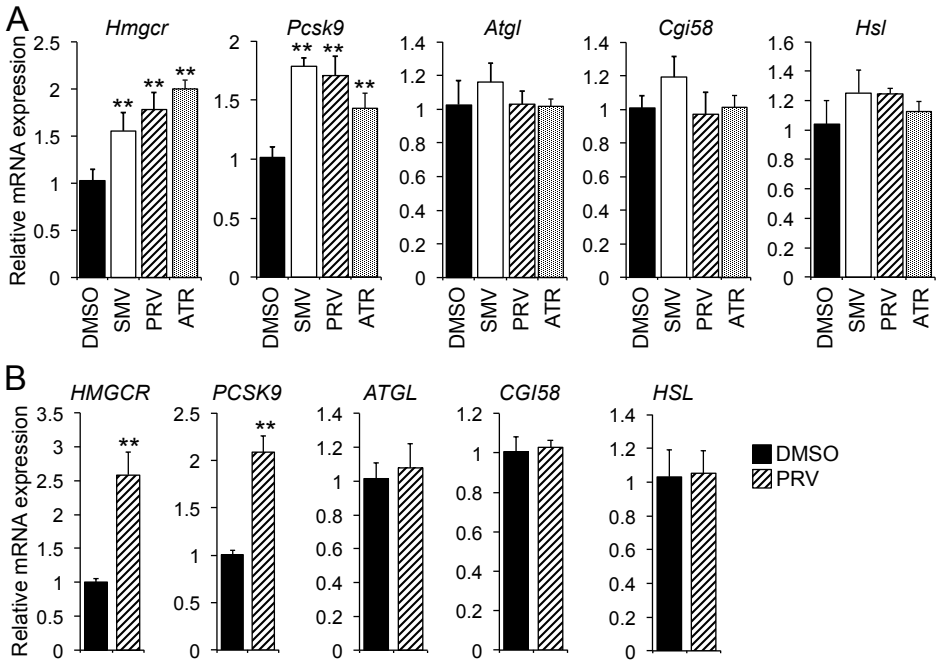
Acc
Fasn
Scd1
Gpat

Fig. S2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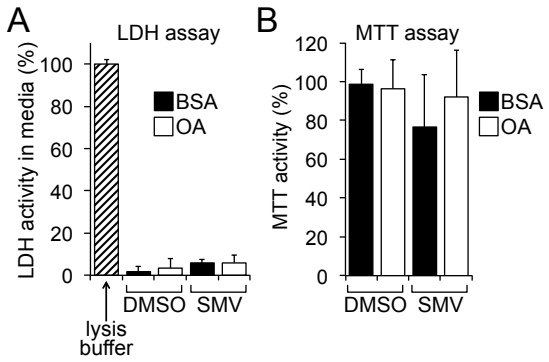
PRV

Fig. S3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LDH assay
LDH activity in media (%)
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60
40
20
0
BSA
OA
DMSO
SMV
lysis
buffer
B
MTT assay
MTT activity (%)
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100
80
60
40
20
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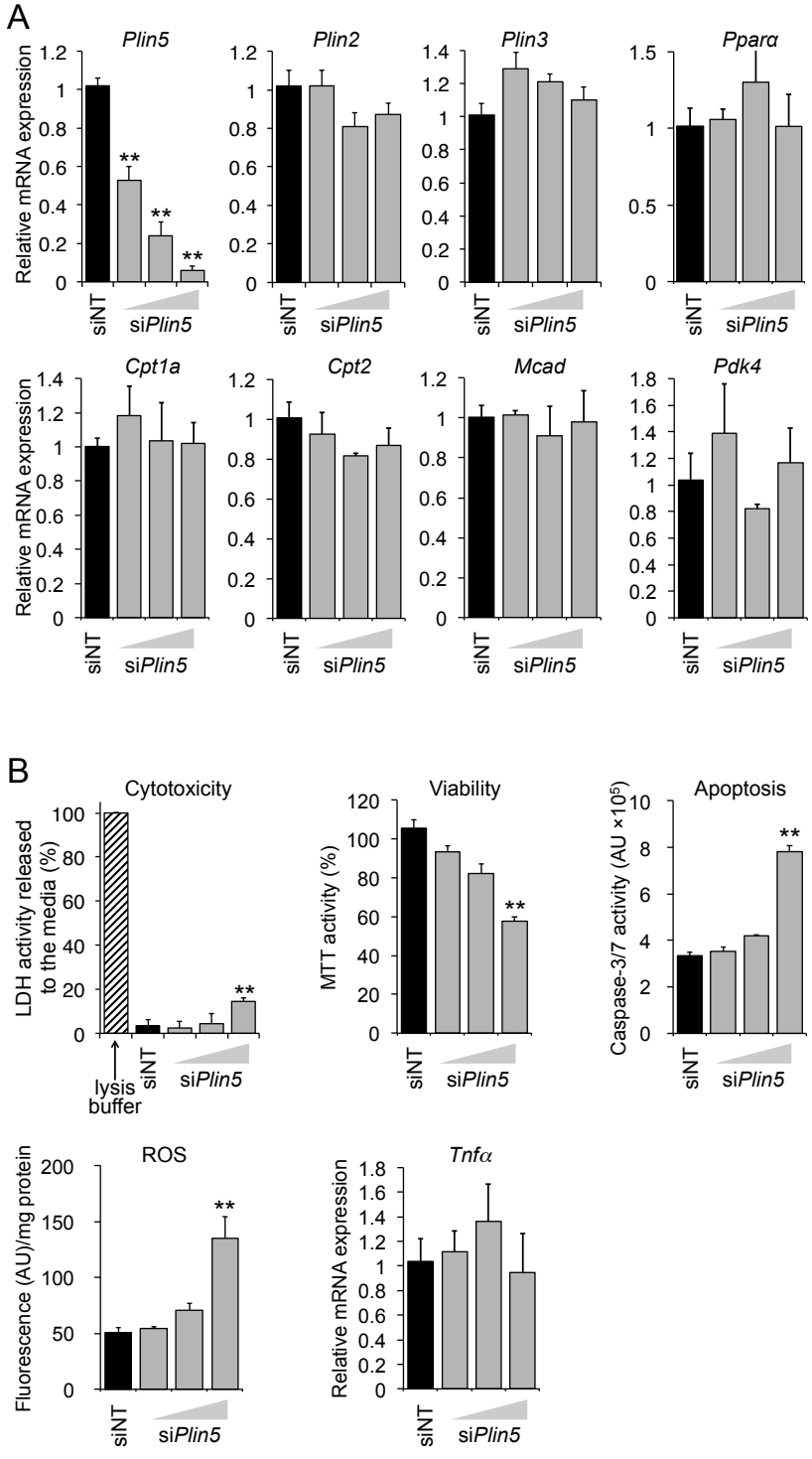
BSA
OA
DMSO
SMV

Fig. S4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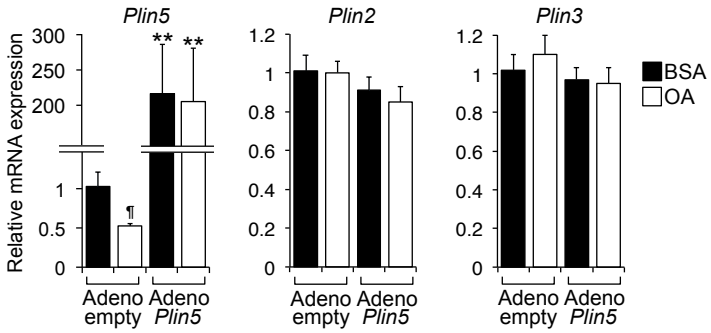
Plin2
Plin3
Pparα
Cpt1a
Cpt2
Mcad
Pdk4
Relative mRNA expression
siNT
siPlin5
B
Cytotoxicity
LDH activity released to the media (%)
lysis buffer
Viability
MTT activity (%)
Apoptosis
Caspase-3/7 activity (AU ×10^5)
ROS
Fluorescence (AU)/mg protein
Tnfα
Relative mRNA expression

Fig. S5


Plin5
Plin2
Plin3
Relative mRNA expression
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250
200
1
0.5
0
1.2
1
0.8
0.6
0.4
0.2
0
**
**
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BSA
OA
Adeno empty
Adeno Plin5