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Supplemental Information

Adiponutrin Functions as a Nutritionally Regulated Lysophosphatidic Acid Acyltransferase

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INVENTORY OF SUPPLEMENTAL INFORMATION

1) **Supplemental Figures and legends**

Figure S1 related to Figure 1

Figure S2 related to Figure 2

Figure S3 related to Figure 3 and 4

Figure S4 related to Figure 5

Figure S5 related to Figure 6

Figure S6 and S7 related to Figure 7

2) **Supplemental Tables**

Table S1 related to Figure 7

Table S2 related to Experimental Procedures “Cloning, expression and purification of mAdpn and hADPN” and Supplemental Experimental Procedures “Cloning of m*Adpn* and h*ADPN”*

Table S3 related to Supplemental Experimental Procedures “RNA isolation and real-time PCR”

3) **Supplemental Experimental Procedures**

4) **Supplemental References**

SUPPLEMENTAL FIGURES AND LEGENDS

Figure S1.

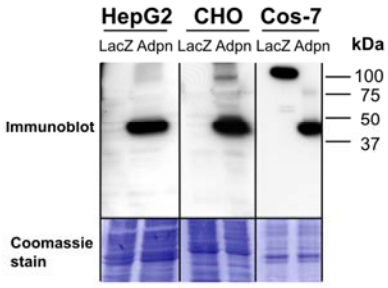

FIGURE S1. **Overexpression of Adpn in mammalian cells:** Murine Adpn was overexpressed in HepG2 and CHO cells using His-tagged Adpn expressing adenovirus, and in Cos-7 cells using His-tagged pcDNA4/HisMax expression vector encoding Adpn. Adenovirus or vector DNA encoding *β*-galactosidase (LacZ) was used as a control. LacZ expressing adenovirus produced untagged LacZ. Overexpression of His-tagged proteins was detected by Western blotting in the lysates of HepG2 and CHO cells (20 µg protein) with an anti-His (C-terminal) polyclonal antibody and in the lysates of Cos-7 cells (5 µg protein) with an anti-His (N-terminal) monoclonal antibody.

Figure S2.

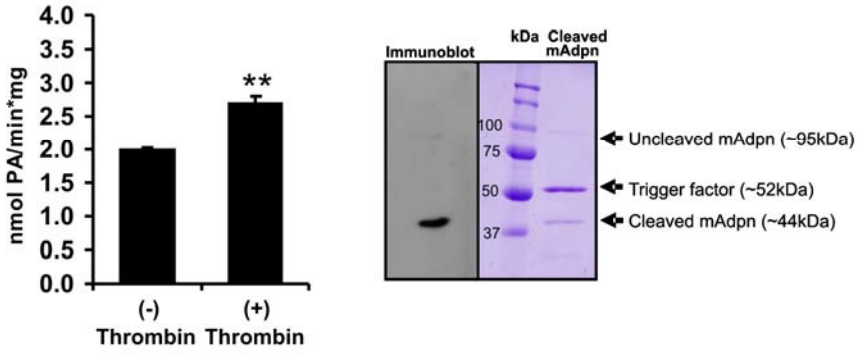

FIGURE S2. **LPAAT activity of purified mAdpn following protease cleavage:** LPAAT activity of purified recombinant mAdpn was measured after thrombin cleavage of Trigger Factor tag from the N-terminal region of mAdpn. Western blot analysis was performed using a polyclonal antibody against mAdpn. Data are shown as mean ± SD and are representative of three independent experiments with each sample in duplicates, $p<0.01$ (**).

Figure S3.

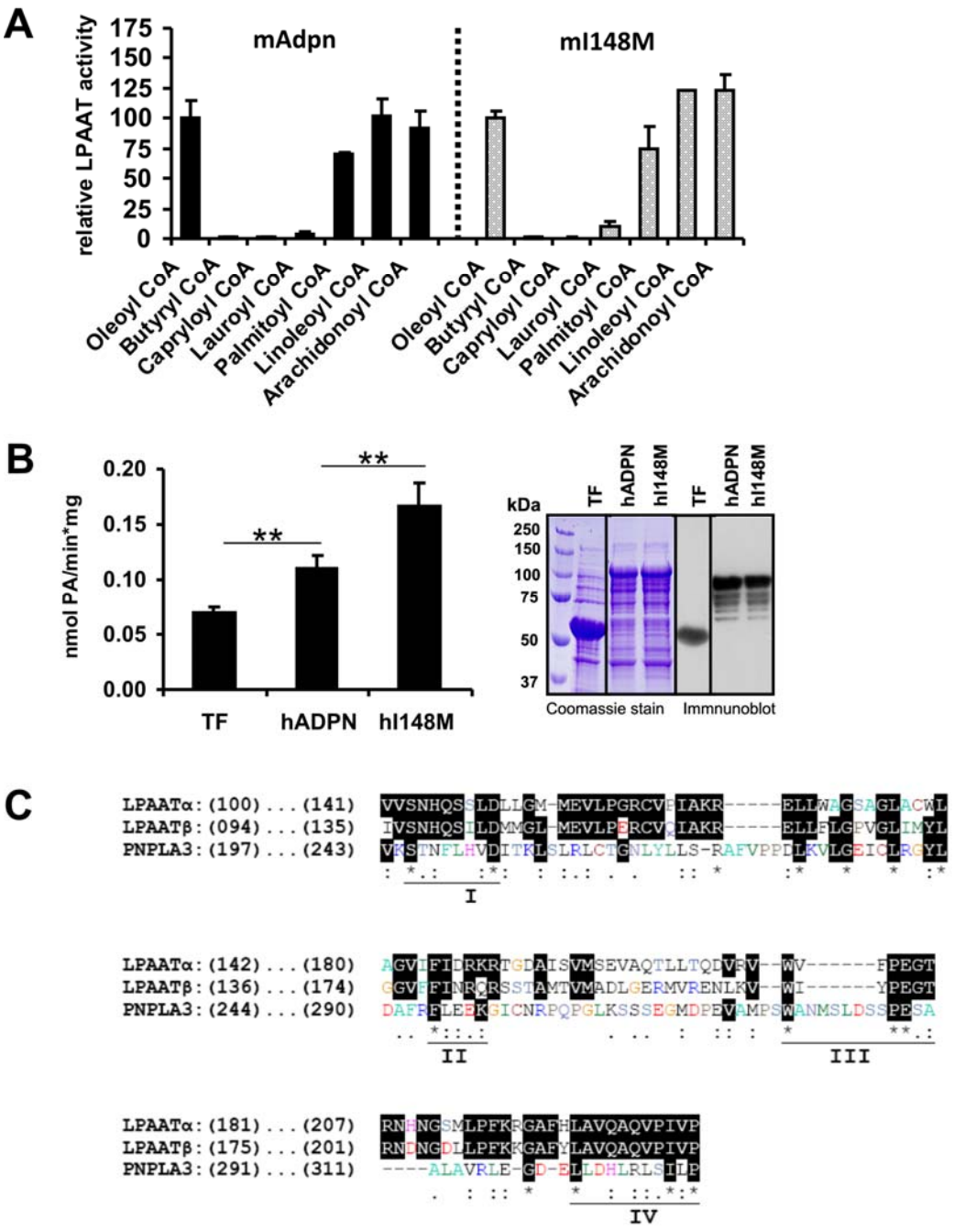

FIGURE S3. **Enzyme properties of wild-type and I148M mutant of ADPN:** (**A)** Acyl-CoA preference of purified mI148M. mI148M was overexpressed in *E. coli* and purified under identical conditions as mAdpn. LPAAT activity of mAdpn and mI148M was determined using 2 µg of purified proteins in the presence of [^{3}H] LPA as acceptor and acyl-CoAs of various chain lengths as donor. Purified Trigger Factor was used as negative control with negligible LPAAT activity (<0.1nmol PA/min*mg). Relative LPAAT activity was determined by normalising the specific activity of mAdpn and mI148M (2.4 and 5.0 nmol/min*mg, respectively) in the presence of [^{3}H] LPA and oleoyl-CoA to 100%. **(B**) LPAAT activity was determined in bacterial total lysates (10 µg of lysate protein) overexpressing TF, hADPN or hI148M. Overexpression of TF, hADPN and hI148M was confirmed by Coomassie blue-

stained SDS-PAGE gel and Western blotting of bacterial total lysate (40 µg lysate protein) using anti-His monoclonal antibody. Data are shown as mean ± SD and represent two independent experiments with each sample in duplicates. Statistical significance was determined by a two-tailed student's *t*-test, $p<0.01$ (**). **(C)** The amino acid sequence alignment of human PNPLA3/hADPN (GenBank No. NP_079501) with human LPAAT-*alpha* (GenBank No. U56417) and LPAAT-*beta* (GenBank No. U56418). Amino acid sequences were retrieved from NCBI database and aligned by Clustal X and viewed by Bioedit softwares, respectively. Identity is indicated by black shading and degree of conservation is indicated by '*', ':' and '.'. The amino acid numbers are given in parenthesis and I-IV indicates conserved LPAAT motifs.

Figure S4.

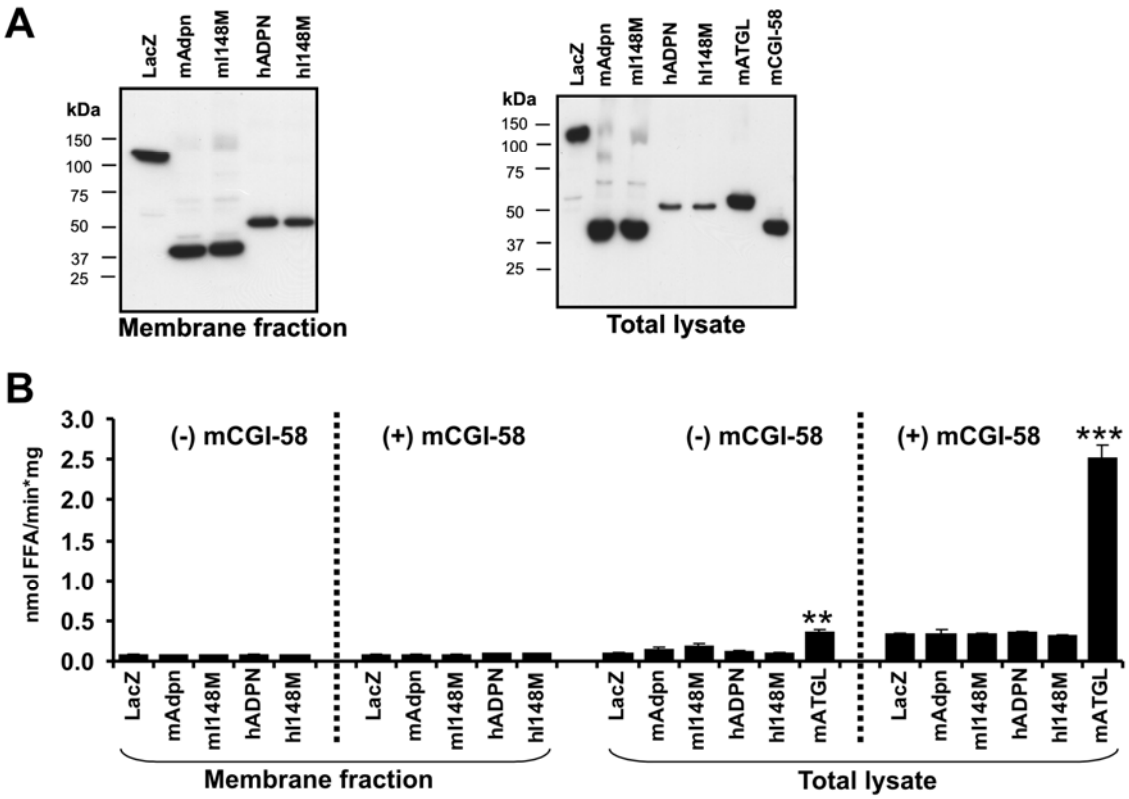

FIGURE S4. **TG hydrolase activity of ADPN: A)** Cos-7 cells were transfected with His-tagged pcDNA4/HisMax expression vector encoding mATGL, mAdpn, mI148M, hADPN and hI148M. LacZ was used as a negative control. Expression of His-tagged recombinant proteins was detected by immunoblotting 5 µg of protein using anti-His monoclonal antibody. **B)** TG hydrolase activity using radiolabeled triolein as substrate was measured in the membrane fraction (40 µg protein) and total cell lysates (40 µg protein) of Cos-7 cells expressing above mentioned recombinant proteins in the absence or presence of total cell lysate expressing mCGI-58 (40 µg protein). Data are shown as mean ± SD and are representative of three independent experiments, $p<0.01$ (**), $p<0.001$ (***). FFA, free fatty acid.

Figure S5.

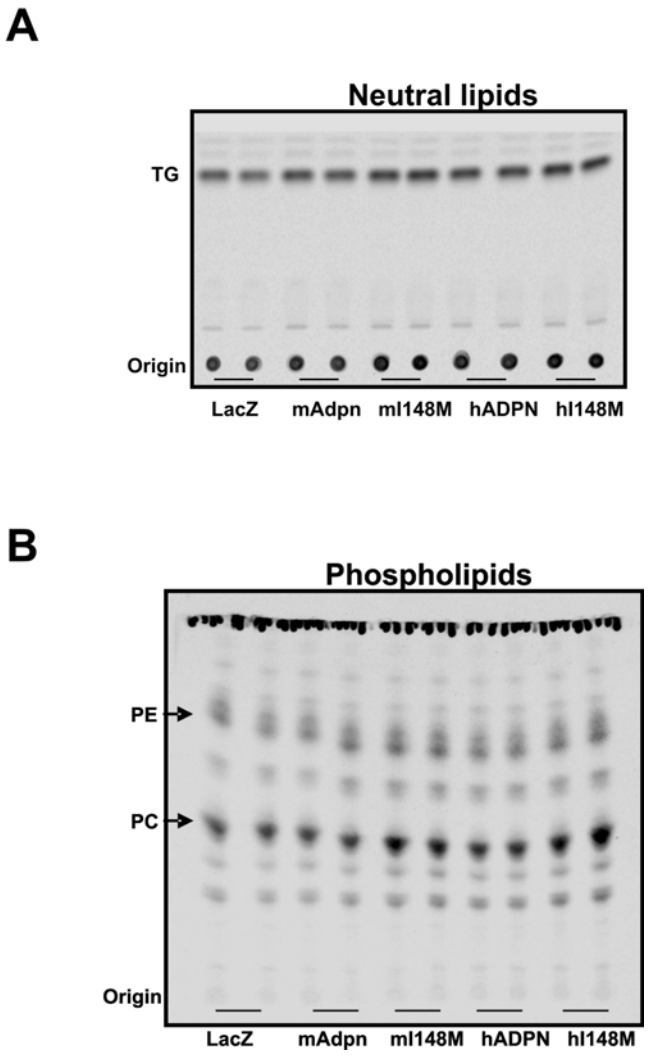

FIGURE S5. **TG and phospholipid synthesis with ADPN overexpression in mammalian cells:** Cos-7 cells were transfected with His-tagged pcDNA4/HisMax expression vector encoding various recombinant proteins. LacZ was used as a control. After 36 h, cells were incubated with 250 μM oleic acid (containing 9.5 μM [1-^{14}C] oleic acid) complexed with BSA (3:1) in the presence of high-glucose medium for 6 h. Cellular lipids were extracted and separated by TLC using solvent systems for neutral and phospholipids. Phosphorimager scan of silica gel TLC plates showing comparative [1-^{14}C] oleic acid incorporation into **A)** neutral lipids (TG indicates triacylglycerol) and **B)** PLs (arrows indicates the band for phosphatidylcholine (PC) and phosphatidylethanolamine (PE).

Figure S6.

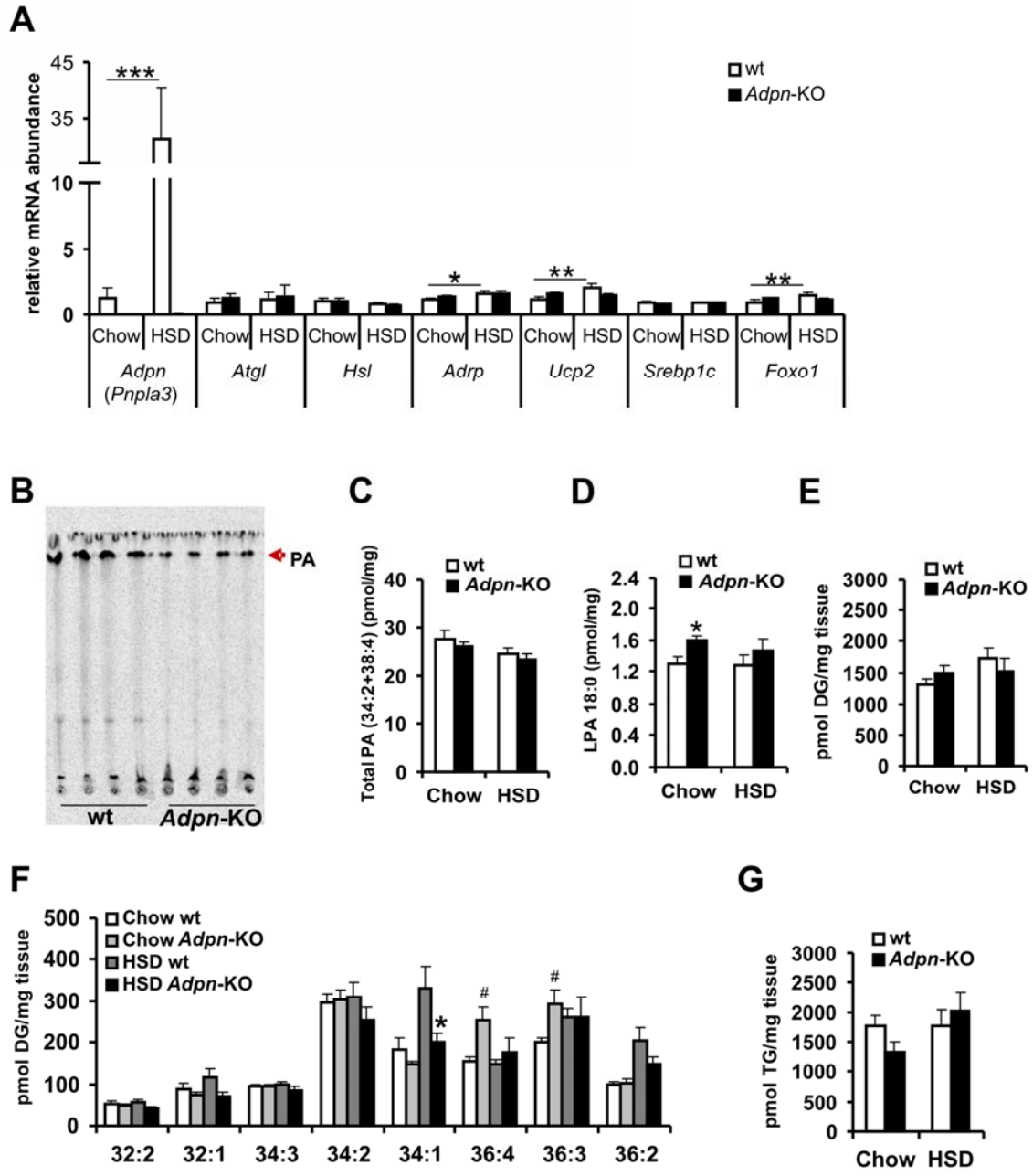

FIGURE S6. **Gene expression and lipidomic analysis of liver samples from wild-type (wt) and *Adpn*-KO mice:** 5 h post-feeding, male wt and *Adpn*-KO mice fed a chow diet or HSD were sacrificed and the liver was excised. **A)** Relative liver mRNA expression of the genes shown in graph as determined by RT-qPCR was normalized to *β-actin* as a reference gene and compared to wt mice fed chow diet, n=6. Data are presented as mean ± SD, n=6, $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***). **B)** Phosphorimager scan of a silica gel TLC plate showing LD-associated liver LPAAT activity of wt and *Adpn*-KO mice receiving a HSD, n=4. **C-G)** Liver samples were collected from chow diet and HSD fed wt and *Adpn*-KO mice after 2 min organ perfusion with Krebs-Henseleit Buffer 2. Lipidomic analysis of total liver lipid extracts showing PA **(C)**, LPA **(D)**, DG **(E, F)** and TG **(G)** levels. Data are shown as mean ± SEM. Statistical significance was determined by post hoc analysis, $p<0.05$ ($^{\#}$KO/wt for chow, n=6 for each genotype), $p<0.05$ (*KO/wt for HSD, n=6 for each genotype).

Figure S7.

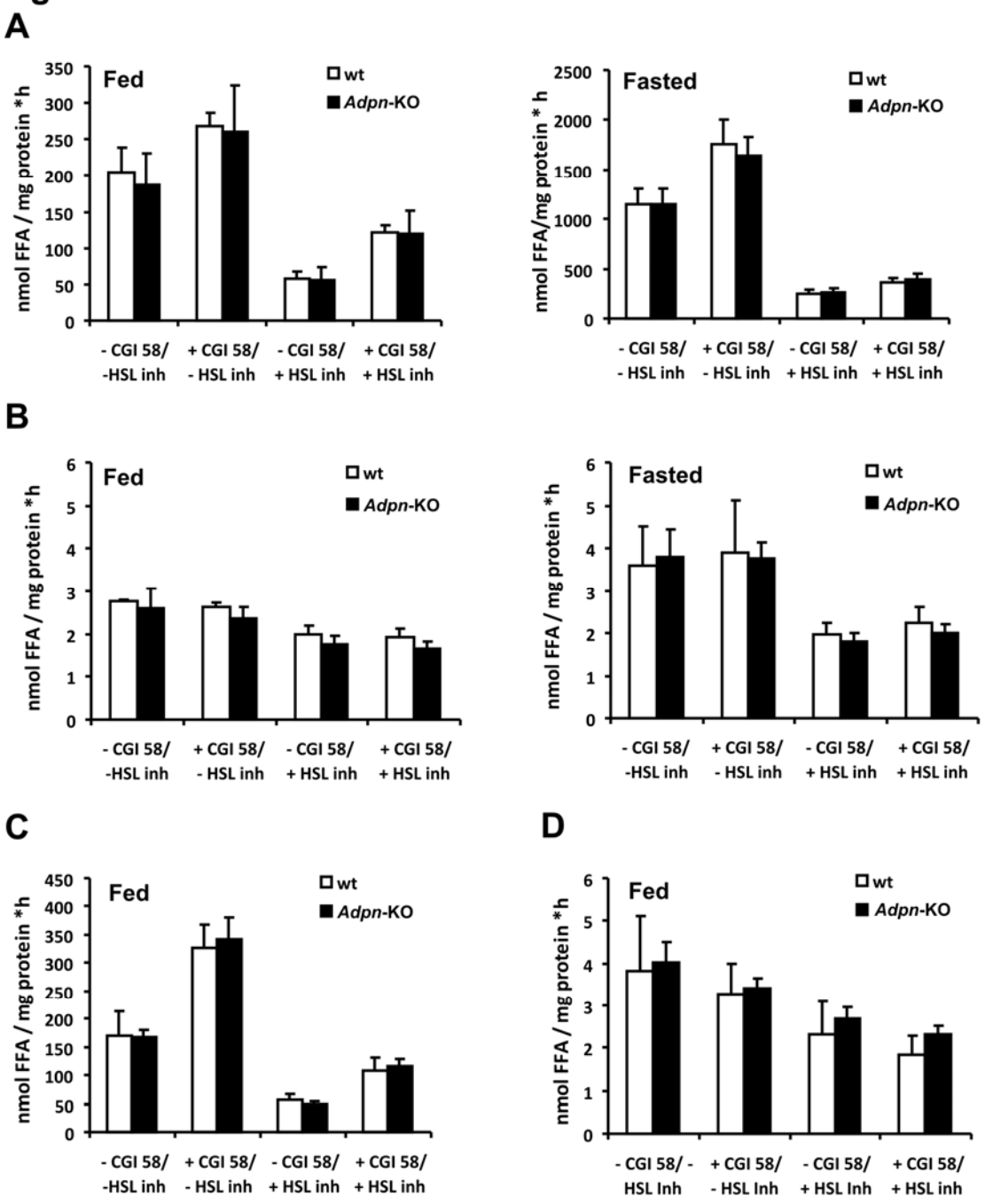

FIGURE S7. **TG hydrolase activities in white adipose tissue (WAT) and liver of *Adpn*-deficient mice:** TG hydrolase activity was performed in **A)** WAT and **B)** liver lysates (20 µg and 200 µg protein, respectively) of wild-type and *Adpn*-KO mice on a chow diet. Mice were given a HSD for three weeks and TG hydrolase activity was measured in **C)** WAT and **D)** liver lysates of 5 h post-fed mice. The activity was determined in the absence or presence of purified GST-tagged murine CGI-58 (100 ng protein) and / or 25 µM hormone-sensitive lipase inhibitor (indicated as HSL Inh) (NNC 0076-0000-0079, Novo Nordisk, Bagsvaerd, Denmark) using radiolabeled triolein.

Table S1: Plasma parameters of fed or fasted *Adpn*-KO mice in comparison to wt littermates. 12- to 14-week-old male mice were used for the analysis, n=6 per group.

Plasma parameter	Fed		Fasted	
	wt	***Adpn*-KO**	**wt**	***Adpn*-KO**
TG (mg/dl)	100.2±30.3	90.9±25.9	82.8±24.8	120.9±36.7
FFA (mM)	0.4±0.1	0.4±0.1	0.6±0.1	0.8±0.1
Glucose (mg/dl)	144.0±19.0	164.0±25.0	79.0±12.0	78.0±8.0
Cholesterol (mg/dl)	87.9±8.6	64.9±19.7	65.6±12.4	69.3±10.9

Table S2: Primers used for cloning and expression of wild-type (wt) and mutant mouse adiponutrin (mAdpn) and human adiponutrin (hADPN) proteins using pCold and pcDNA4/HisMax expression vectors (GenBank accession numbers for mouse and human adiponutrin are NM_054088 and NM_025225, respectively).

	Primer name	Sequence (5'-3')*	Restriction site	Purpose
Mouse Adiponutrin	mAdpn fw	AA**CTCGAG**ATGTATGACCCAGAGCGC	XhoI	Expression of wt mADPN[a]
	mAdpn rv	TA**TCTAGA**TTACCTGGTAGAGGGGAGCAG	XbaI	
	mAdpn I148M fw	GTTCCTGCTTCAT**G**CCCCTCT[#]	NA	Expression of mutant mADPN (I148M)
	mAdpn I148M rv	ATGAAGCAGGAACACACCAGGG	NA	
Human Adiponutrin	hADPN fw	ATT**CATATG**TACGACGCAGAGCGCG	NdeI	Expression of wt hADPN[b]
	hADPN rv	CGG**AAGCTT**TCACAGACTCTTCTCTAGTG	HindIII	
	hADPN_h fw	AA**GAATTC**ATGTACGACGCAGAGC	EcoRI	Expression of wt hADPN[c]
	hADPN_h rv	GGCC**TCTAGA**TCACAGACTCTTCTCTA	XbaI	
	hADPN I148M fw	TTCCTGCTTCAT**G**CCCTTCTAC[#]	NA	Expression of mutant hADPN (I148M)
	hADPN I148M rv	ATGAAGCAGGAACATACCAAGGC	NA	
	hADPN C15S fw	CTTCGCGGGCT**C**CGGCTT[#]	NA	Expression of mutant hADPN (C15S)
	hADPN C15S rv	AGCCCGCGAAGGACAAGCTC	NA	
	hADPN S47A fw	TTCGGCGCT**G**CGGCCG[#]	NA	Expression of mutant hADPN (S47A)
	hADPN S47A rv	AGCGCCGAACAACATGCGC	NA	
	hADPN D206A fw	ACTTTCTTCATGTGG**C**CATCACCAA[#]	NA	Expression of mutant hADPN (D206A)
	hADPN D206A rv	CCACATGAAGAAAGTTCGTGGACTTG	NA	
	hADPN P311G fw	CAGCATCCTG**GG**CTGGGATG[#]	NA	Expression of mutant hADPN (P311G)
	hADPN P311G rv	CAGGATGCTGAGACGCAGGTGG	NA	

* - nucleotides marked red and bold indicate the restriction cleavage sites. [#] - nucleotides marked bold and underlined are base replacements to generate point mutations. [a] - same primer pair was used for cloning mADPN in both pCold and pcDNA4/HisMax expression vectors. [b] - primers used for cloning hADPN in pCold expression vector. [c] - primers used for cloning hADPN in pcDNA4/HisMax expression vector. NA – not applicable.

Table S3: Primer sequences of genes used for the quantification of mRNAs by RT-qPCR with an amplicon size of less than150 bp. Primers were tested for specificity by melting curve analysis and agarose gel electrophoresis.

Gene analyzed (Symbol)	Primer sequence (5' to 3')	GenBank Acc. No.
Patatin-like phospholipase domain-containing protein 3 (PNPLA3)	**Fwd:** atg gca aac ttg tgg gag ac **Rev:** agc cct ccc tgt cct taa tc	NM_054088
Adipose triglyceride lipase (ATGL)	**Fwd:** gag acc aag tgg aac atc **Rev:** gta gat gtg agt ggc gtt	NM_001163689
Hormone sensitive lipase (HSL)	**Fwd:** tac acg tca ccc ata gtc a **Rev:** cgg caa ggt ctt cag cat	NM_010719
Adipose differentiation-related protein (ADRP)	**Fwd:** cag cca acg tcc gag att g **Rev:** cac atc ctt cgc ccc agt	NM_007408
Uncoupling protein 2 (UCP2)	**Fwd:** aga gca ctg tcg aag cct a **Rev:** tgg cat tac ggg caa cat t	NM_011671
Sterol regulatory element-binding protein-1c (SREBP1c)	**Fwd:** gtt act cga gcc tgc ctt cag g **Rev:** caa gct ttg gac ctg ggt gtg	NM_011480
Forkhead box protein O1 (Foxo1)	**Fwd:** aag gat aag ggc gac agc aa **Rev:** tcc acc aag aac tct ttc ca	NM_019739

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Materials

[1-^{14}C] oleoyl-CoA (59.3 mCi/mmol) and [1-oleoyl-9, 10-^{3}H] lysophosphatidic acid (53.4 Ci/mmol) was purchased from Perkin Elmer Lifesciences, Waltham, MA, USA. Glycerol-tri [9, 10(n)-^{3}H] oleate, [1-^{14}C] oleic acid and [9, 10 (n)-^{3}H] oleic acid were purchased from GE Healthcare. [1-^{14}C] diolein and [dioleoyl-1-^{14}C] phosphatidylcholine were purchased from American Radiochemicals, St. Louis, MO, USA. 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate (LPA, Na salt), L-α-lysophosphatidyl ethanolamine (LPE), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphoinositol (LPI), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phospho-L-serine (LPS) and 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC) were purchased from Avanti Polar Lipids, Alabaster, AL, USA. Oleic acid; 1,2-dioleoyl-*sn*-glycerol; 1-oleoyl-rac-glycerol; 1,2-dioleoyl-*sn*-glycerol-3-phosphocholine; 1,2-Di(cis-9-octadecenoyl)-*sn*-glycerol 3-phosphate (PA, Na salt); L-α-phosphatidylcholine; L-α-phosphatidylinositol; glycerol-3-phosphate (Mg salt); oleoyl-CoA; palmitoyl-CoA; lauroyl-CoA; capryloyl-CoA; butyryl-CoA and bovine serum albumin (essentially fatty acid free) were purchased from Sigma-Aldrich, Steinheim, Germany. Thrombin was purchased from GE Healthcare. Silicagel 60 TLC plates were purchased from Merck, Darmstadt, Germany.

Cloning of m*Adpn* and h*ADPN*

The coding sequences for m*Adpn* and h*ADPN* were amplified by PCR using Phusion® DNA polymerase (New England Biolabs, Frankfurt, Germany) and primers (Eurofins MWG Operon, Ebersberg, Germany) as given in Table S2. The purified amplicon was digested with restriction enzymes (as in Table S2) and ligated in expression vector pCold (TAKARA BIO INC., Saint-Germain-en-Laye, France). The ligation mixture was incubated at 16°C overnight with T4 DNA ligase (Roche Diagnostics, Mannheim, Germany) and used to transform *Escherichia coli* BL21 (DE3) chemical competent cells. Plasmid DNAs of selected clones

were sequenced by AGOWA GmbH (Berlin, Germany) to confirm the open reading frames. Similarly, for expression in eukaryotic cells, the PCR products were ligated to compatible restriction sites of the eukaryotic expression vector pcDNA4/HisMax© C (Invitrogen Life Technologies, Carlsbad, CA, USA). A control pcDNA4/HisMax© C vector expressing *β*-galactosidase was provided by the manufacturer (Invitrogen Life Technologies, Carlsbad, CA, USA). Site-directed mutagenesis was performed using the GeneTailor Site-Directed Mutagenesis System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions using primers listed in Table S2. All the mutants were confirmed by sequence analysis.

Expression of wild-type and mutant recombinant proteins

Overnight cultures (5 ml) of the positive transformants (*Escherichia coli* BL21(DE3) cells harboring mouse or human *ADPN* in pCold plasmid) in Luria-Bertani (LB) medium with 100 mg/L ampicillin were inoculated into 500 ml of LB medium with the same antibiotic. Cells were grown until an OD_{600} of 0.5-0.6 was reached, induced with 0.5 mM isopropyl *β*-D-thiogalactopyranoside (Sigma, Taufkirchen, Germany), and grown further at 15°C for 24 h. The pCold TF DNA Vector system utilizes cold shock technology to obtain high yield protein expression along with Trigger Factor (chaperone) that facilitates correct protein folding (Qing et al., 2004).

Purification of wild-type and mutant recombinant proteins

The induced cell pellet was resuspended in 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 10% glycerol (buffer A) containing complete, EDTA-free protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany), 500 µM benzamidine, 200 µM tris-(2-carboxyethyl)-phosphine (TCEP), 100 µg/ml lysozyme and 30 mM imidazole. Cell lysis was carried out by sonication and cell debris was removed by centrifugation at 13,000 x *g* at 4°C for 20 min. The supernatant was loaded onto a Ni^{2+}-charged Ni-NTA (Qiagen, Hilden, Germany) affinity column equilibrated with buffer A. The affinity bound protein was eluted in

buffer A with 250 mM imidazole. The purification process was monitored by 10% SDS-PAGE followed by Coomassie Brilliant Blue staining. Protein expression was also determined by immunoblotting using monoclonal anti-His antibody (Zimmermann et al., 2004). The concentrated protein was used for further assays and experiments. Thrombin cleavage was performed to remove the Trigger Factor tag from the N-terminal of the recombinant protein (Chang, 1985).

Cloning of recombinant mAdpn adenovirus

For cloning of the mouse Adpn adenovirus construct, *a Mlu*I–*Cla*I flanked cDNA fragment was amplified by PCR from the cDNA-containing plasmid pcDNA4mycHisMaxC-mAdpn, using the following primers: mouse Adpn-Ad forward 5´- ATA CGC GTA ACC ATG GGG TAT GAC CCA GA -3`, mouse Adpn-Ad reverse 5´- CCA TCG ATG GCA CAG TCG AGG CTG ATC -3´ (restriction enzyme sites are underlined). Subsequently, the PCR product was ligated to pAvCvSv plasmid and the resulting shuttle plasmid was cotransfected with pJM 17 into HEK-293 cells using the calcium phosphate co-precipitation method (Graham and van der Eb, 1973). Large-scale production of high-titer recombinant adenovirus was performed as described (Teng et al., 1994). An adenovirus expressing *beta*-galactosidase (LacZ-Ad) was used as a control.

Mice

Adpn-KO mice were generated as described earlier (Basantani et al., 2011). The targeted *Adpn* allele was then backcrossed onto the C57BL/6 background strain for >10 generations. Experimental mice were generated by breeding *Adpn* $^{+/-}$ males to *Adpn* $^{+/-}$ females. *Adpn*-KO and wild-type mice of the same genetic background (C57BL/6) were kept on a standard chow diet (4.5% w/w fat) and on a regular 12-h light/dark cycle. Blood samples were collected from fed (*ad libitum* access to food overnight) or fasted (food was removed for 14 h) animals as described previously (Haemmerle et al., 2006). For diet studies 10-week-old male

mice were housed using a 12-h light/dark “switch cycle” to facilitate tissue collection at 5 h post meal.

Analysis of plasma parameters

Plasma levels of TG, free FA, and total cholesterol were determined using commercial kits (Thermo Electron Corp., Victoria, Australia; Wako Chemicals, Neuss, Germany and Roche Diagnostics, Vienna, Austria, respectively). Plasma glucose concentration was determined using an Accu-Check glucometer (Roche Diagnostics, Vienna, Austria).

RNA isolation and real-time PCR

Total RNA was extracted from the livers of experimental mice with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and quantified with an ND-1000 spectrophotometer (NanoDrop Technologies Wilmington, DE). One microgram of total RNA treated with DNAse I (Amp Grade, Invitrogen; Carlsbad, CA, USA) was reverse transcribed with a High-Capacity cDNA Reverse Transcription Kit (ABI, Foster City, CA). Quantitative real-time PCR was performed using Maxima® SYBR Green/ROX qPCR Master Mix (2X) (Fermentas Life Sciences, St. Leon-Rot, Germany) on a StepOnePlus real-time PCR system (ABI, Vienna, Austria). List of primer sequences is given in Table S3. Primer sequences of *Adpn* used for real-time PCR were previously described by Chen et al. (Chen et al., 2010) and adopted the following PCR cycle program: pre-incubation at 50°C for 10 min, denaturation at 95°C for 10 min, 40 cycles of denaturation (95°C for 15 sec) and annealing and elongation (60°C for 1 min). Relative expression was calculated by the $\Delta\Delta C_t$ method (where C_t is threshold cycle) with the use of StepOne software v.2.1 and values were normalized to *β-actin*.

Isolation of LDs from mouse liver

Isolation of LDs was performed as described previously (Schweiger et al., 2008) with minor changes. Freshly dissected liver was washed with PBS, homogenized with an ULTRA TURRAX® T25 Basic (IKA® WERKE, Staufen, Germany) in ice cold buffer B (20 mM

potassium phosphate, 0.25 M sucrose, 1 mM DTT, 20 µg/ml leupeptin, 2 µg/ml antipain, 1 µg/ml pepstatin). Tissue lysate was transferred to SW41 tubes, overlaid with buffer C (50 mM potassium phosphate, pH 7.4, 100 mM KCl, 20 µg/ml leupeptin, 2 µg/ml antipain, 1 µg/ml pepstatin), and centrifuged in a SW41 rotor (Beckman, Fullerton, CA) for 2 h, 100,000 x g at 4ºC. LDs were collected as a white band from the top of the tubes and concentrated by two centrifugation steps (20,000 x g, 15 min, 4ºC). The underlying solution was removed and LDs were resuspended in buffer C by brief sonication in a water bath. TG and protein content of the LDs was determined using commercial reagents (Thermotrace, Thermo Electron Corporation, Victoria, Australia and Bradford, Bio-Rad Laboratories GmbH, Munich, Germany, respectively).

Acyltransferase activity

The reaction mixture for LPAAT activity measurements contained 1-2 µg purified His-tagged protein in 50 mM Tris-HCl buffer, pH 7.5, 100 µM LPA and 20 µM [1-^{14}C] oleoyl-CoA (110,000 dpm/nmol) in a total volume of 100 µl. For analysis of total liver lysates and LD fractions (10 and 5 µg protein, respectively), 200 µM LPA was used in the reaction mixture along with 20 µM [1-^{14}C] oleoyl-CoA and 60 µM oleoyl-CoA to maintain the linearity of the reaction. The reaction mix was incubated at 37°C for 10 min and terminated by extracting lipids with 1.2 ml of chloroform:methanol (2:1 v/v) and 400 µl of acidified water (2% ortho-phosphoric acid). Total lipid extracts were separated by TLC using Silicagel 60 TLC plates in chloroform:methanol:acetone:glacial acetic acid:water (50:10:20:15:5, v/v) as the solvent system (Ghosh et al., 2008). Spots co-migrating with phosphatidic acid standard were cut and quantified by scintillation counting.

TG hydrolase activity

The reaction mixture for the determination of triacylglycerol hydrolase activity contained 5 µg of purified Adpn His-tagged protein (or 40 µg of cell lysate protein or 20 µg of WAT homogenate protein or 200 µg of liver homogenate protein) incubated with 100 µl of

substrate in a total volume of 200 µl at 37°C for 60 min (Haemmerle et al., 2006). In some reactions, cell lysate overexpressing murine CGI-58 (Lass et al., 2006) or purified GST-tagged murine CGI-58 (Schweiger et al., 2008) or purified His-Sumo-tagged murine CGI-58 (Gruber et al., 2010) was used to determine the simulated TG hydrolase activity in cell lysates, tissue lysate or purified protein samples, respectively. The reaction was terminated by addition of 3.25 ml of methanol:chloroform:heptane (10:9:7, v/v) and 1 ml of potassium carbonate, 0.1 M boric acid, pH 10.5. After centrifugation at 800 x g for 15 min, the radioactivity in 1 ml of the upper aqueous phase was determined by liquid scintillation counting. The substrate for TG hydrolase was prepared by emulsifying 33 nmol triolein/assay (glycerol-tri[9,10(n)-^{3}H] oleate, 40,000 cpm/nmol) and 45 µM phosphatidylcholine:phosphatidylinositol (3:1) by sonication in 100 mM potassium phosphate buffer, pH 7.4 and 2% fatty acid free BSA. For TG hydrolase assays in bacterial total cell lysates, murine *Atgl* (NM_001163689.1) was cloned and expressed in pASK-IBA5plus expression plasmid (IBA GmbH, Gottingen, Germany) to generate strep-tagged murine Atgl. Primers used for cloning were: Forward-5'-TCGGTACCCATGTTCCCGAGGGAGACCAA-3', Reverse-5'-ACCTCGAGTCAGCAAGGCGGGAGGC-3' with KpnI and XhoI restriction sites.

[1-^{14}C] oleic acid incorporation in cellular lipids

Cos-7 cells (passage >20) were cultured in low glucose (1 g/L) Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, Invitrogen) containing 10% fetal bovine serum (FBS, Sigma, Taufkirchen, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C with 5% CO_2. One day before transfection, ~1.5 x 10^5 cells were seeded in 6-well plates. Cells grown to 70–80% confluence were transfected with 1 µg of expression plasmid DNA (see above) using Metafectene® (Biontex GmbH, Martinsried/Planegg, Germany). Vector encoding *beta*-galactosidase (LacZ) was used as a control. After 36 h, cells were treated with 9.5 µM [1-^{14}C] oleic acid (52.6 mCi/mmol stock; Perkin-Elmer) and 0.25 mM oleic acid in high glucose (4.5 g/L) DMEM containing 0.5% BSA. After 6 h, cells were washed twice and lipids were extracted two times with hexane:isopropanol (3:2; v/v). To measure the incorporation of

radiolabeled oleate into different lipid species, an aliquot of extracted lipids was vacuum dried and separated by TLC using the solvent system for neutral lipids [hexane:diethylether:glacial acetic acid (70:29:1, v/v)] or polar phospholipids [chloroform:methanol:acetone:glacial acetic acid:water (50:10:20:15:5, v/v)].

HepG2 and CHO cells were cultured in high-glucose DMEM and DMEM/F12 (1:1), respectively, using 10% FBS and antibiotics at 37°C with 5% CO_2. Subsequently, HepG2 and CHO cells were seeded at a density of 6 x 10^5 cells/well in 6-well plates and infected with Adpn or LacZ expressing adenovirus at a multiplicity of infection (MOI) of 300 pfu/cell and 600 pfu/cell (for HepG2 and CHO cells, respectively) in DMEM. After 2 h, DMEM or DMEM/F12 (1:1) containing 10% FBS and antibiotics was added onto the cells. To determine oleic acid incorporation into TGs, after 36 h, 1 µCi/ml [1-^{14}C] oleic acid and 400 µM oleic acid complexed to FA-free BSA at a molar ratio of 3:1 (FFA/BSA) was added to the incubation medium and cells were further incubated for 6 h. After 6 h, cells were washed; lipids were extracted and separated as described above. To determine protein concentration, cells were lysed in 0.3 N NaOH containing 0.1% SDS and protein was quantified with BCA reagent using BSA as standard.

Membrane- and LD-associated LPAAT assay in Cos-7 cells

Cos-7 cells (passage >20) were seeded at a density of ~3 x 10^5 cells/10 cm dish and cultured overnight in low glucose (1 g/L) DMEM containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C with 5% CO_2 to reach 30% confluency. Cells were transiently transfected with expression plasmid DNA encoding various His-tagged recombinant proteins (see above) using Metafectene®. After 24 h of transfection, cells were supplemented with DMEM containing 10% FBS and 400 µM oleic acid complexed to FA-free BSA at a molar ratio of 3:1 (FFA/BSA) for further 20 h. Cells were harvested by washing two times with PBS and lysed in ice-cold buffer D (0.25 M sucrose, 1 mM DTT, 1 mM EDTA, 20 µg/ml leupeptine, 2 µg/ml antipain, 1 µg/ml pepstatin, pH 7.0) using Misonix Sonicator 4000 (Misonix, Inc. NY, USA). Cell lysates were centrifuged for 5 min, 1000 x g at 4°C to remove nuclei and

unbroken cells. Isolation of LDs was performed as described (Schweiger et al., 2008) with minor changes. Supernatant obtained from 1000 x g centrifugation was overlaid with buffer C and centrifuged in SW41 rotor for 1 h, 100,000 x g at 4°C. LDs were collected as a white band from the top of the tubes. Cytosolic fraction was collected after removing the overlay buffer. Membrane fraction was washed by overlaying with buffer D and one more centrifugation step in SW41 rotor for 30 min, 100,000 x g at 4°C. Supernatant was discarded and the total membrane fraction was lysed in buffer D using a Misonix Sonicator 4000. Protein content was measured by the Bradford method. LPAAT activity was determined in LDs, cytosolic and total membrane fractions using 20, 20 and 2 µg protein, respectively in the presence of 100 µM LPA and 20 µM [1-^{14}C] oleoyl-CoA.

Lipid extraction and mass spectrometry analyses

Liver samples were collected from *Adpn*-KO mice on chow diet or HSD after 2 min perfusion with Krebs-Henseleit Buffer 2. Glycerophospholipids from liver tissue were extracted using a modified Bligh and Dyer procedure (Bligh and Dyer, 1959). Approximately 10 mg of frozen mouse liver was homogenized in 800 µl of ice-cold 0.1 N HCl:CH_3OH (1:1) using a tight-fit glass homogenizer (Kimble/Kontes Glass Co, Vineland, NJ) for about 1 min on ice. Suspension was then transferred to cold 15 ml borosilicate glass tubes with screw caps and vortexed with 400 µl of cold $CHCl_3$ for 1 min. The extraction proceeded with centrifugation (5 min, 4^{o}C, 18,000 x g) to separate the two phases. Lower organic layer was collected and solvent evaporated. The resulting lipid film was dissolved in 100 µl of isopropanol:hexane:100 mM NH_4CO_2H (aq.) 58:40:2 (mobile phase A). Quantification of glycerophospholipids was achieved by an LC-MS technique using synthetic (non-naturally occurring) diacyl and lysophospholipid standards. Typically, 200 ng of each odd-carbon standard was added per 10-20 mg tissue. Glycerophospholipids were analyzed on an Applied Biosystems/MDS SCIEX 4000 Q TRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) and a Shimadzu high pressure liquid chromatography system with a Phenomenex Luna Silica column (2 × 250 mm, 5-µm

particle size) using a gradient elution as previously described (Ivanova et al., 2007; Myers et al., 2011). The identification of the individual species, achieved by LC/MS/MS, was based on their chromatographic and mass spectral characteristic. This analysis allows identification of the two fatty acid moieties but does not determine their position on the glycerol backbone (*sn-1* versus *sn-2*). Neutral lipids from frozen mouse liver tissue (10-15 mg) were extracted by homogenizing tissue in the presence of internal standards (24:0 diacylglycerol (DG), and 42:0 triacylglycerol (TG)) in 2 ml 1 X PBS and extracting with 2 ml ethyl acetate:trimethylpentane (25:75). After drying the extracts, the lipid film was dissolved in 1 ml hexane:isopropanol (4:1) and passed through a bed of Silicagel 60 Å to remove remaining polar phospholipids. Solvent from the collected fractions was evaporated and lipid film was redissolved in 100 µl CH_3OH:$CHCl_3$ (9:1), containing 10 µl of 100 mM CH_3COONa for MS analysis essentially as described (Callender et al., 2007). Statistical analysis of lipidomics data was achieved by Two-way ANOVA across feeding regimens and Adpn genotype. Post-hoc *t*-tests for genotype effects were also assessed for each feeding regimen.

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