Supplemental Materials

Adipocyte lipid chaperone aP2 is a secreted adipokine regulating hepatic glucose production

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SUPPLEMENTAL FIGURES.

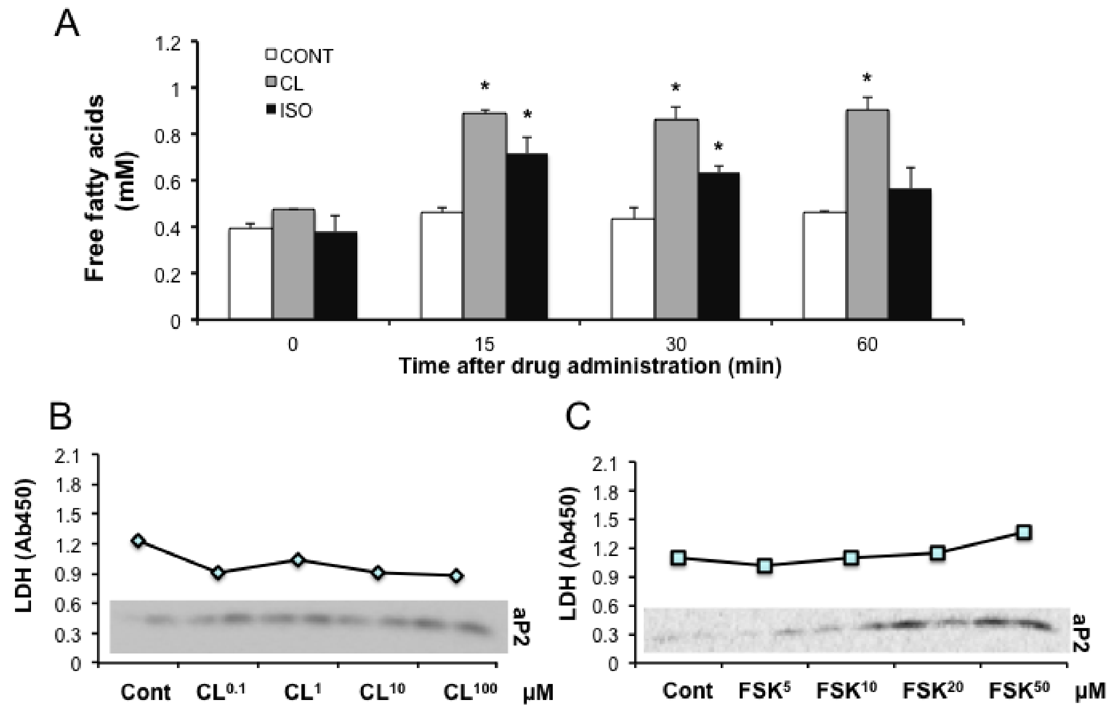

Figure S1, related to Figure 1. Serum fatty acid levels in mice and LDH release from adipocytes after stimulation of lipolysis.

a, Plasma nonesterified fatty acid (NEFA) levels in mice injected with saline (Control), CL *316243* (CL) or isoproterenol (ISO) to induce lipolysis. These are the same mice (n≥3) shown in figure 2b. Fatty acid measurements were done using a commercial assay system (Wako Chemicals USA, Inc.). Data are presented as means ± SEM. * $p<0.05$ in student's *t* test. **b,** Lactate dehydrogenase (LDH) activity in conditioned medium of WT adipocytes treated with CL*316243* (CL) or **c,** forskolin (FSK) for 1 hour at indicated concentrations compared to the activity in conditioned medium of untreated controls (Cont). The insets show aP2 western blots in the conditioned medium in duplicates. LDH activity (absorbance at 450nM) was measured using LDH-Cytotoxicity Assay KitII (Abcam, Cambridge, MA).

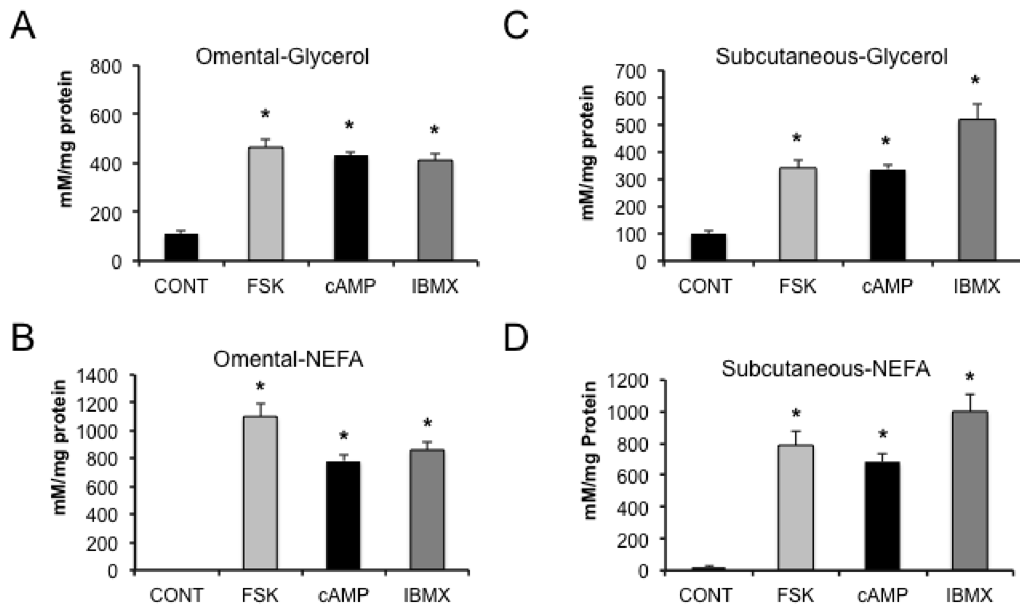

Figure S2, related to Figure 2. Fatty acid and glycerol release from primary cultured human adipocytes following stimulation of lipolysis.

Glycerol and nonesterified fatty acid (NEFA) levels in the conditioned medium collected from primary human omental (**a**-**b**) or subcutaneous (**c**-**d**) adipocytes treated with forskolin (FSK, 20μM), cyclic adenosine monophosphate (cAMP, 1mM), or isobutylmethylxanthine (IBMX, 1mM). These data correspond to figures 2E and 2F. Data are presented as means ± SEM. * $p < 0.05$ in student's t test.

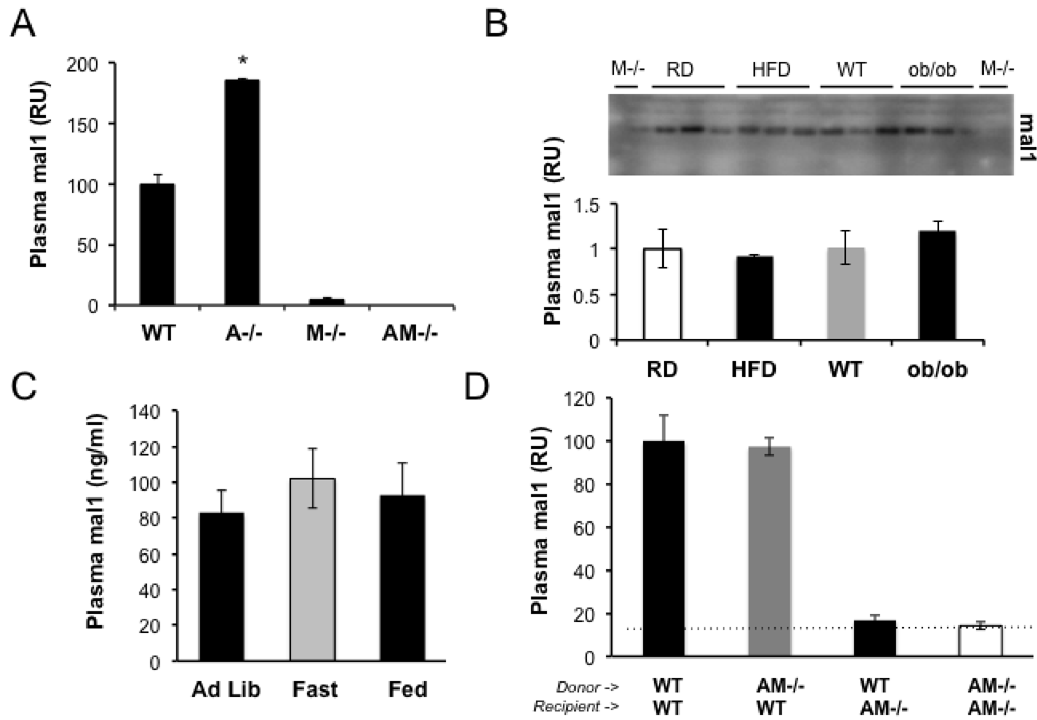

Figure S3, related to Figure 3. Plasma mal1 levels in different genetic backgrounds of mice.

a, Plasma mal1 levels from lean WT, mal1$^{-/-}$ (M-/-), aP2$^{-/-}$ (A-/-), and aP2-mal1$^{-/-}$ (AM-/-) mice following a daytime 6 hour food withdrawal. **b,** Western blot analysis of plasma mal1 levels in regular diet lean (RD), high fat-fed obese (HFD), wild type (WT) and genetically obese (ob/ob) mice. M-/- (mal1-deficient) sample was used as negative control. Quantitation of the data is shown in the graph below the blot. **c,** Plasma mal1 measurement in mice fed *ad libitum* (Ad Lib), following a 24 hour fast (Fast), and then refed for 4 hours (Fed), n=3. The same mouse were used for all three time points. **d,** Plasma mal1 levels in mice that have undergone bone marrow transplantation. Bone marrow transplantation was performed between WT and AM$^{-/-}$ mice (as donors and recipients) and mal1 levels were determined in the resulting 4 groups of chimeras. Values

in panels a, b, and d are given in relative values due to differences in assay systems. Data are presented as means ± SEM.

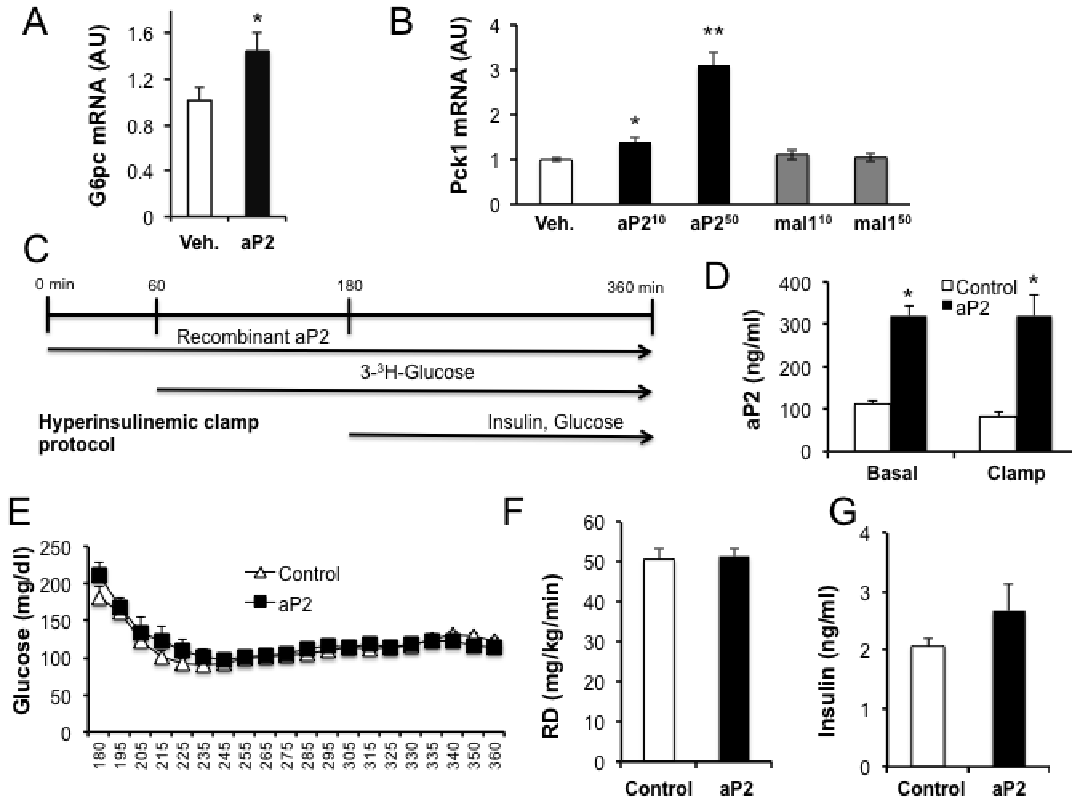

Figure S4, related to Figure 4. Effects of recombinant aP2 in cells and whole animals. **a,** Expression of glucose-6-phosphatase (G6pc) in primary mouse hepatocytes treated with aP2 (10 µg/ml). **b,** Phosphoenolpyruvate carboxykinase 1 (Pck1) mRNA expression in primary hepatocytes treated with 10 or 50 µg/ml recombinant aP2 ($aP2^{10}$ and $aP2^{50}$) or mal1 ($mal1^{10}$ or $mal1^{50}$). **c,** Hyperinsulinemic euglycemic clamp protocol used in the study of whole body glucose fluxes in WT lean mice that were either infused with control or recombinant aP2. **d,** Plasma aP2 levels measured at basal and clamp periods of WT lean mice infused with recombinant aP2 protein. **e,** Blood glucose levels during the hyperinsulinemic euglycemic clamp period (between 180- and 360 minutes). **f,** Whole

body rate of glucose disposal (RD) during the clamp study in WT mice following recombinant aP2 protein infusion. At least 10 male mice were used in each experiment. **g,** Serum insulin measurements during the clamp period in the same animals. Statistical analysis was done by student's *t* test. Data are presented as means ± SEM. * $p < 0.05$, ** $p < 0.01$.

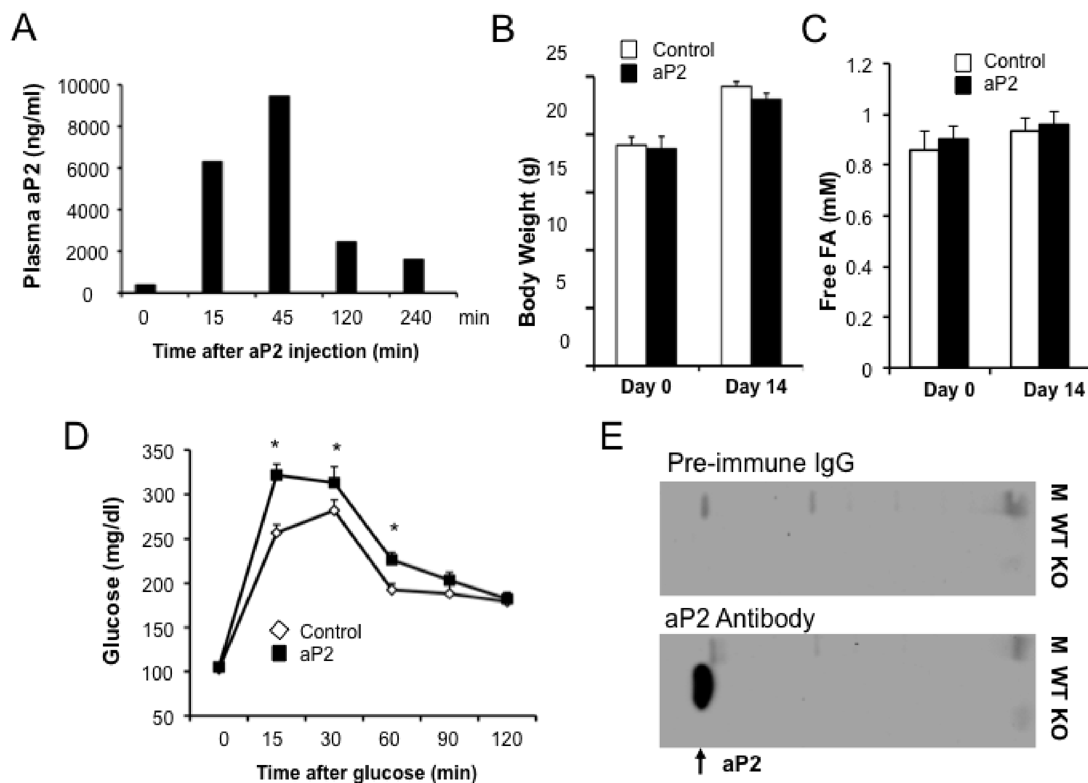

Figure S5, related to Figure 5. Serum aP2, body weight, serum free fatty acids and glucose tolerance in mice treated with recombinant aP2.

a, Plasma aP2 levels followed over time in WT mice on regular diet after a single dose intraperitoneal (i.p.) injection of 100μg of recombinant aP2. **b,** Body weight and **c,** Serum free fatty acid measurements at the onset (day 0) and two weeks after (day 14)

administration of recombinant control (Gus) or aP2 protein, twice daily at 100 μg dose intraperitoneally. **d,** Glucose tolerance test performed in WT mice on regular diet that received control or aP2 protein for two weeks. **e**, Validation of the anti-aP2 antibody and the control preimmune IgG using protein extracts from wild type (WT) and aP2$^{-/-}$ (KO) adipocyte extracts for western blot analysis. M=protein marker. Statistical analysis was performed by student's *t* test. Data are presented as means ± SEM. * $p < 0.05$.

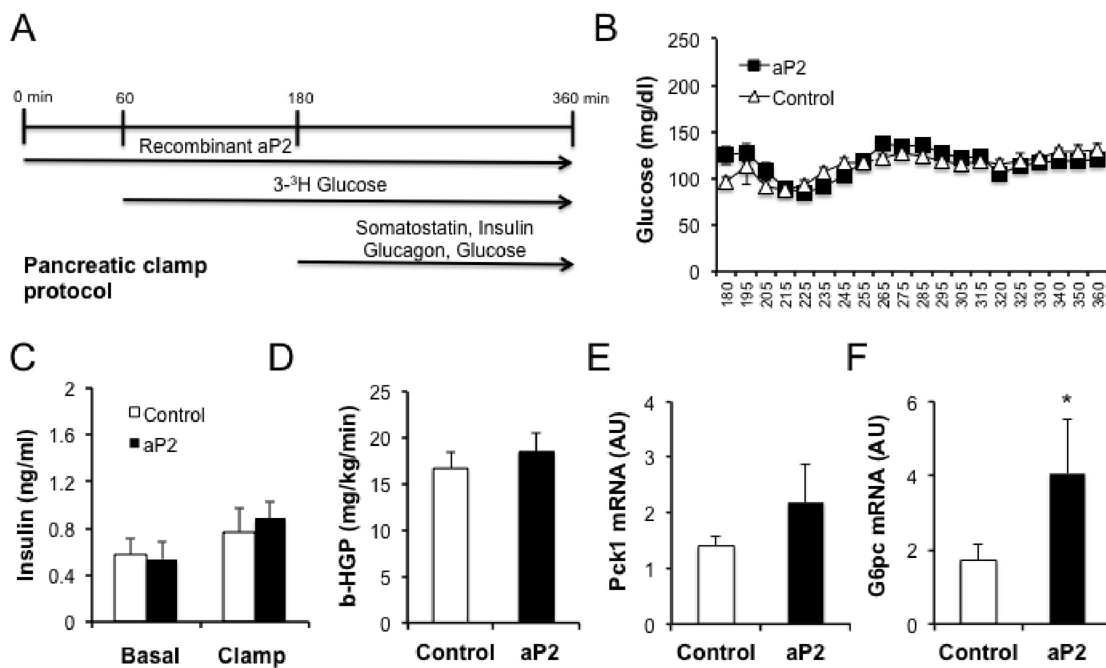

Figure S6, related to Figure 6. Pancreatic clamp studies in aP2$^{-/-}$ mice treated with recombinant aP2.

a, Pancreatic clamp protocol performed in aP2$^{-/-}$ mice after infusion with control (n=6) or recombinant aP2 protein (n=8). **b**, Blood glucose measurements during the pancreatic clamp period. **c,** Serum insulin levels during the basal and clamp periods in the same animals. **d,** Basal hepatic glucose production (b-HGP) in 5h fasted aP2$^{-/-}$ mice after 3

hour infusion with recombinant aP2 compared to controls. **e,** Hepatic phosphoenolpyruvate carboxykinase 1 (Pck1) and **f,** glucose-6-phosphatase (G6pc) mRNA expression in the same animals. Data are presented as means ± SEM. * $p < 0.05$.

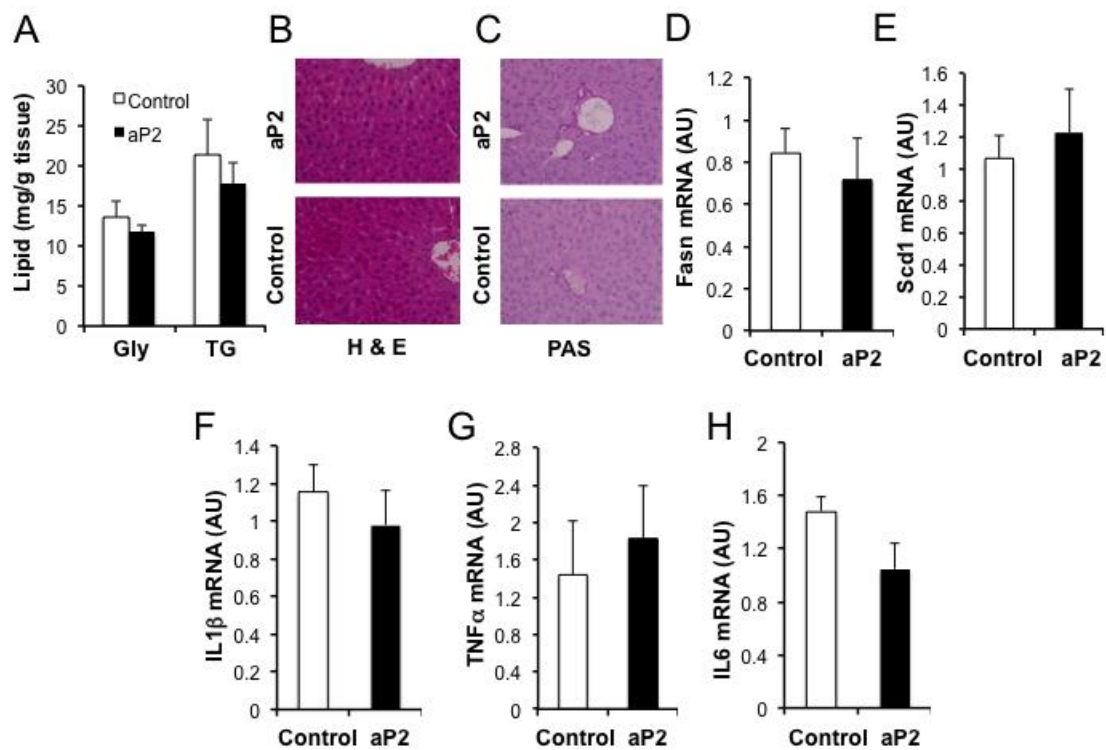

Figure S7, related to Figure 7. Effect of recombinant aP2 on liver lipid and glycogen content and lipogenic and inflammatory gene expression profiles.

a, Liver tissue glycerol and triglyceride content in $aP2^{-/-}$ mice infused with recombinant aP2 compared to controls. **b**, Liver sections of $aP2^{-/-}$ mice after recombinant aP2 administration stained with Hematoxylin-Eosin and **c,** Periodic Acid-Schiff to detect glycogen content. **d,** Fatty acid synthase (Fasn), **e,** Stearoyl-CoA desaturase-1 (Scd1), **f,**

Interleukin-1 beta, **g,** Tumor necrosis factor-alpha, and **h,** Interleukin-6 mRNA expression levels relative to ribosomal protein 36B4 mRNA in the same animals. Data are presented as means ± SEM.

Supplemental Experimental Procedures

Plasma aP2, mal1, glucagon, adiponectin, and insulin ELISAs

Blood was collected from the antecubital vein into EDTA tubes from humans following an overnight fast, and from mice by tail bleeding after 6 hours or overnight food withdrawal. The samples were spun in a microcentrifuge at 13,000 rpm for 15 minutes at 4°C. Plasma aP2 was determined by an ELISA system according to the manufacturer's instructions (Biovendor Inc.). To monitor nutritional regulation of aP2, blood samples were collected from mice immediately prior to the start of the dark cycle (*ad libitum* samples), after which the animals were placed in cages without food. After 24 hours of fasting, a second set of blood samples were collected (24 hour fasting samples) and food was provided. Final blood sampling was performed 4 hours after re-feeding (fed samples). To determine aP2 levels during lipolysis, blood was collected from 12 mice at baseline levels. After this, 6 mice were injected with isoproterenol (1 mg/kg) and the other 6 received vehicle control. Blood samples for aP2 measurement were collected at the indicated time points following injection. A similar protocol was used for CL*316243* compound (0.1 mg/kg) administration.

Insulin (insulin-mouse ultrasensitive ELISA, Alpco Diagnostics, Salem, NH), mal1 (Circulex Mouse mal1 ELISA, CycLex Co., Ltd., Japan), glucagon, and adiponectin (Quantikine ELISA, R&D Systems, Minneapolis, MN) measurements were performed according to the manufacturer's instructions. Western blot analysis was also used for serum mal1 measurements. For glucagon measurements, 500KIU of aprotonin was added per ml of blood collected. For assessing nutritional regulation of plasma mal1, blood collection scheme was the same as that for aP2.

Gene expression

RNA isolation was performed using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. For first strand cDNA synthesis 5x iScript RT Supermix was used (BioRad, Herculus, CA). Quantitative real time PCR (Q-PCR) was performed using Power SYBR Green PCR master mix (Applied Biosystems, Life Technologies, Grand Island, NY) and samples were analyzed using a ViiA7 PCR machine (Applied Biosystems, Life Technologies, Grand Island, NY). Primers used for Q-PCR were as follows: 18S: 5'-agtccctgccctttgtac-3', 5'-cgatccgagggcctcacta-3', 36B4: 5'-cactggtctaggacccgagaa-3', 5'-agggggagatgttcagcatgt-3', TNFα: 5'-ccctcacactcagatcatcttct-3', 5'-gctacgacgtgggcta cag-3', IL-1β: 5'-gcaactgttcctgaactcaact-3', 5'-atcttttggggtccgtcaact-3', IL-6: 5'-acaacc acggccttccctactt-3', 5'-cacgatttcccagagaacatgtg-3', FAS: 5'-ggaggtggtgatag ccggtat-3', 5'-tgggtaatccatagagcccag-3', SCD1: 5'-ttcttgcgatacactctggtgc-3', 5'-cgggatt gaatgttctt gtcgt-3', Pck1: 5'-ctgcataacggtctggacttc-3', 5'-cagcaactgcccgtactcc-3', G6pc: 5'-cgactcgctatctccaagtga-3', 5'-gttgaaccagtctccgacca-3'.

Vector construction, transfection, and cell culture

Flag-tagged GFP (plasmid ID 10825) and AKT (plasmid ID 9021) were obtained from Addgene (Cambridge, MA). Flag-tagged GFP-aP2 was produced by cloning aP2 cDNA in a lentiviral vector pRR.LCMV.GFP (a kind gift of Rob C. Hoeben, Leiden University Medical Center, the Netherlands). HEK 293 cells were transfected with the indicated constructs using Lipofectamine 2000 (Invitrogen) and maintained in DMEM with 10% fetal bovine serum. FABP-deficient cell lines were established as previously described

(Cao et al., 2008; Makowski et al., 2001). 3T3-L1 or in house pre-adipocytes were maintained in DMEM with 10% bovine calf serum and differentiated into adipocytes in DMEM with 10% cosmic calf serum (CCS) using a standard differentiation protocol. To induce lipolysis, differentiated adipocytes were treated with forskolin (20 μM), 3-isobutyl-1-methylxanthine (IBMX, 1mM) or N^6,2'-O-Dibutyryladenosine 3',5'-cyclic monophosphate sodium salt (db-cAMP, 1mM) for one hour. At the end of one hour, the medium was replaced with fresh medium, and conditioned medium was collected an additional hour later. To collect fat explants, epididymal adipose tissue depots were dissected from mice and rinsed twice in PBS. Adipose tissue samples were then transferred into DMEM with 10% CCS and minced into an average size of 1 to 2 mm. The tissue explants were washed extensively with DMEM and cultured in DMEM containing 10% CCS. Human primary adipocyte cultures (ZenBio, Research Triangle Park, NC) were treated in DMEM + 0.5% BSA for 1h with FSK (20 μM) db-cAMP (1 mM) or IBMX (1mM). Medium and lysates were collected after 2.5 hours of incubation from omental, and 3 hours from subcutaneous adipocytes for aP2 measurements. NEFA and glycerol levels were normalized to mg protein of cell lysate. For CMV-driven aP2 expression in adipocytes, a mouse aP2 construct was prepared by addition of a Flag tag at the N-terminus of aP2 cDNA by high fidelity PCR, and ligation into a pcDNA3 vector (Invitrogen). The $aP2^{-/-}$ preadipocytes were cultured and differentiated in 10 cm tissue culture plates. On day 5 of differentiation, adipocytes were trypsinized and electroporated using Amaxa Nucleofector™ system V (VCA-1003) and an electroporator (Lonza, program A-33). On day 8 of differentiation, cells were treated with 20 μM forskolin or vehicle (DMSO) in culture medium for 1 hour, medium was refreshed with DMSO or

forskolin, and conditioned media (CM) was collected after 1 hour and centifuged at 5000 rpm for 10 min. Then, media was incubated with anti-Flag® M2 affinity gel (Sigma A2220) overnight at 4°C for immunoprecipitation of Flag-aP2, and beads were boiled with 2x SDS loading dye and subjected to electrophoresis and western blotting with a polyclonal in-house anti-aP2 antibody. Cell lysates were prepared using standard RIPA buffer and 30 μg of cell lysate was subjected to western blotting with antibodies against aP2 or tubulin (Santa Cruz Biotechnology, Inc. sc-9104).

Immunoprecipitation and immunoblotting

Tissue protein lysates and conditioned medium from adipocytes were separated on SDS-PAGE gels and immunoblotted using the following antibodies: adiponectin (for westerns, Santa Cruz Biotechnology), AKT (Cell Signaling Technology), Flag (Sigma), and beta-tubulin (Santa Cruz Biotechnology). Flag-tagged aP2 was immunoprecipated using 4 ml of conditioned medium from transfected HEK 293 cells after incubation with 30 μl Flag agarose beads (Sigma) overnight at 4°C. Proteins bound to agarose beads were eluted with SDS loading buffer and resolved with SDS-PAGE.

Details of the hyperinsulinemic-euglycemic and pancreatic clamp studies with aP2 infusion

After a 5 hour fast, (preceding the clamp), vehicle control and recombinant aP2 protein were infused at 8 μg/kg/min for 6 hours (Suppl. Fig. 4c, 6a). HPLC purified [3-^{3}H]-glucose (Perkin Elmer Life and Analytical Sciences, Boston, MA) was infused at 0.05 μCi/min during the 2-h basal period, and blood samples were collected at the end to estimate the rate of basal hepatic glucose production (HGP). After the basal period, a

180-min hyperinsulinemic-euglycemic clamp was conducted with a primed-continuous infusion of human insulin (Humulin R; Eli Lilly, Indianapolis, IN) at a rate of 2.5 mU/kg/min (Furuhashi et al., 2007). Insulin-stimulated whole-body glucose turnover was estimated with a continuous infusion of [3-^{3}H]-glucose throughout the clamps (0.1 μCi/min). Pancreatic clamps were performed by a modification of a reported procedure (Rossetti et al., 1997). Pancreatic clamps were performed similarly to hyperinsulinemic clamps, except mice were infused with somatostatin (3 μg/kg/min, Sigma), glucagon (0.5 ng/kg/min, Sigma), and human insulin (1 mU/kg/min, Humulin R, Eli Lilly, Indianapolis, IN) instead of insulin alone during the clamp period. For hyperinsulinemic and pancreatic clamps, blood was collected at 10 min intervals for the immediate measurement of blood glucose concentrations, and 25% glucose solution was infused at variable rates to maintain blood glucose at euglycemic levels. All infusions were performed using flow-controlled microdialysis pumps (CMA/Microdialysis, North Chelmsford, MA). Blood samples were taken at 135, 140, 145, 150, 160, 170, and 180 (last 7 time points in the clamp diagram) min after the start of clamps for the determination of plasma 3-[^{3}H]-glucose, and $^{3}H_2O$ concentrations. At the end of clamps, animals were sacrificed and tissues were harvested, and immediately frozen and stored at –80°C. Rates of basal hepatic glucose production and whole-body glucose disposal were determined as the ratio of the ^{3}H-glucose infusion rate to the specific activity of plasma glucose at the end of the basal period and during the final 45 minutes of the clamp period, respectively. Hepatic glucose production during the clamp period was determined by subtracting the glucose infusion rate from the whole body glucose disposal.

Human Samples

The samples used for serum FABP measurements were from selected female participants (n=910) in the Nurses' Health Study (NHS) and male participants (n=904) in the Health Professionals Follow-up Study (HPFS). NHS and HPFS cohort members were age 43-69 and 48-83, respectively, at blood draw. We had control groups available to us for serum aP2 measurements from several previously conducted nested case-control studies (Cassidy et al., 2009; Giovannucci et al., 2008) from within these two populations. The institutional review board of the Brigham and Women's Hospital and the Harvard School of Public Health Human Subjects Committees approved the study protocol.

Supplemental References

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