Supporting Information

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SI Materials and Methods

Study Populations. The Dallas Heart Study (DHS) is a population-based probability sample of Dallas County (52% African American, self-identified as "black"; 29% European-American, self-identified as "white"; 17% Hispanic, and 2% other ethnicities) in which ethnicity was self-assigned according to United States census categories (1). The study was approved by the Institutional Review Board of University of Texas Southwestern Medical Center, and all subjects provided written informed consent before participation. The Atherosclerosis Risk in Communities Study (ARIC) study is a prospective study of atherosclerosis initiated in 1987 (2) in four communities in the United States (Jackson, MS; Minneapolis, MN; Forsyth County, NC; and Washington County, MD). A randomly selected cohort of ~4,000 persons, ages 45–64 y, was selected from each community. The protocol for the study was approved by the Institutional Review Boards of all centers, and all participants provided written informed consent that included consent for genetic studies. The Biobank is a study approved by the University of Texas Southwestern Institutional Review Board with the goal of obtaining large numbers of samples from unrelated African Americans and Hispanics in Dallas and Tarrant County. After informed consent is obtained, each participant provides medical information and a fasting blood sample. Plasma lipid levels are measured and genomic DNA isolated as previously described (1).

Real-Time PCR Assay to Quantify mRNA Levels. Total RNA from mouse tissues was isolated by homogenization in STAT-60 reagent (TEL-TEST). Assays were performed using pooled total RNA from liver-specific sterol regulatory binding protein (SREBP) cleavage-activating protein knockout mice ($Scap^{-/-}$) (four mice per group), SREBP-1a, SREBP-1c, SREBP-2 Tg mice (five mice per group) and their littermate controls (3). Pooled RNA from livers of C57BL/6J mice (five mice per group) fed a chow diet with or without 0.015% of the LXR agonist (T0901317) for 4 d. cDNA was obtained from 2 μg of RNA using TaqMan (Applied Biosystems) with random hexamer primers. Oligonucleotides specific for each transcript (available on request) were used to amplify from total RNA by PCR in 2×SYBR Master Mix (Applied Biosystems) according to the manufacturer's instructions. The mRNA levels were normalized to the levels of 36B4 or GAPDH transcripts.

Lipoprotein Isolation. A total of 3 mL of plasma in EDTA (0.04%), sodium azide (0.05%) and aprotinin (1.7 μg/mL) was adjusted to a density of 1.215 g/mL with KBr. The plasma was added to a 11.5 mL Ultracrimp Polyallomer centrifugation tube (Thermo Scientific). Saline adjusted to a density of 1.215 g/mL with KBr was layered on top of the plasma to fill the ultracentrifugation tubes. Samples were spun at 269,600 × *g* at 11 °C for 24 h using a T-890 rotor (Thermo Scientific). The top 2 mL (supernatant) and the remaining bottom fraction (~8.8 mL) were harvested by tube-slicer. Ten volumes of ethanol:ether (1:1) was added to 10 μL of supernatant, 10 μL of infranatant and 3.43 μL plasma. After an overnight incubation at −20 °C, samples were centrifuged at 2,700 × *g* for 20 min at 4 °C. Organic solvent was removed and samples were air dried for 5 min before adding 40 μL of 2× sample buffer (Santa Cruz). A total of 9 μL of supernatant, 40 μL infranatant, and 40 μL plasma samples were heated to 95 °C for 5 min and loaded onto a 12% (vol/vol) SDS/PAGE gel.

Cell Culture. HepG2 cells were seeded in six-well plates and grown in low glucose DMEM (Sigma) with 10% FCS until 80% confluence. At day 0 cells were infected with 1×10^9 viral particles expressing no insert (control), angiopoietin-like protein (ANGPTL) 3, or ANGPTL6 with coinfection of either ANGPTL8-FLAG or control virus. After 48 h, cells were washed twice with DPBS (Cellgro) and then grown in serum-free DMEM. After 10 h, the medium was collected and subjected to centrifugation for 5 min (5,000 × *g* at 4 °C). The supernatant was concentrated 10× using Centrifugal Filters (Amicon Ultra, 0.5 mL–10 K; Millipore). HuH7 cells were cultured in High Glucose DMEM with 10% FCS and 100 units/mL penicillin G/streptomycin. At day 0, HuH7 cells were seeded in 60-mm dishes at a density of 15×10^5 per dish. On day 1, the medium was replaced with medium containing 0.34×10^{11} per dish recombinant adenoviral particles: vector alone or ANGPTL8-FLAG. On day 2, medium was replaced with 3 mL of serum-free medium. After 24 h, the medium was removed and saved. Mouse ANGPTL3 (1 μg/mL) was added to dishes containing the infected HuH7 cells or conditioned medium alone. All cells and medium were incubated for 16 h at 37 °C, 5% CO_2 before the medium was harvested for immunoblot analysis.

Adenoviral Infection of Mice. Recombinant adenoviruses expressing human ANGPTL8-FLAG, and wild-type ANGPTL3 were generated using AdEasy Vector System (Qbiogene), as described by the manufacturer. A total of 1.25×10^{11} viral particles were injected into the tail veins of each mouse. The mice were killed 3 d later after a 4-h fast and blood and liver tissue was collected.

Measurement of Plasma Lipids. Blood was collected in EDTA tubes and plasma was separated by centrifugation at 1,700 × *g* for 10 min. Plasma levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), triacylglycerol (TAG), and cholesterol, were measured using the Vitros 250 system (GMI). Phospholipids were measured by use of Phospholipid C kit (Waco). The plasma lipoprotein classes were separated by FPLC using a Superose 6 column (GE Healthcare). Pooled mouse plasma (200 μL) from each group was applied to the column and 40 fractions were collected. Cholesterol and TAG content of each fraction was measured using enzymatic assays (Infinity; Thermo Scientific). Human plasma (200 μL) was fractionated using the same procedure. Indicated pooled fractions were precipitated overnight at −20 °C by use of 10-fold volume of a mixture of ethanol: ether (1:1) and resuspended in 2× sample buffer (Santa Cruz).

ELISA Assays. Human and mouse ANGPTL3 levels were assayed using ELISA kits obtained from AdipoGen (AG-45A-0014 and AG-45A-0015, respectively) and the assays were performed according to instructions of the manufacturer. The ELISA to measure human ANGPTL3 used a mAb to the C-terminal domain of ANGPTL3 as the capture antibody and a polyclonal antibody to the N-terminal domain as the detection antibody in the assay. To measure mouse ANGPTL3, both the capture and detection antibodies were polyclonal antibodies directed against full length mouse ANGPTL3. Plasma was diluted 1:40 and 1:2,000 to measure human and mouse ANGPTL3, respectively.

Plasma Lipolytic Activity. TAG lipase activity was determined by incubating 10 μL of pre- and postheparin plasma pooled from mice (four to five per group) with a glycerol-stabilized emulsion composed of 9,10-[^{3}H](N) triolein (American Radiolebeled Chemicals), triolein, and phosphatidylcholine. The mixture was

incubated at 37 °C for 1 h and stopped by adding heptane: chloroform:methanol (1:1.25:1.41). After centrifugation at 1,800 × *g* for 15 min, 1 mL of the upper phase was used for counting. The amount of free fatty acids liberated was calculated as described (4).

VLDL Secretion. C57BL/6J male mice were infected with recombinant adenovirus expressing human ANGPTL8-FLAG and control virus (four mice per group). After 3 d, mice were fasted 4 h and injected with 500 mg/kg body weight of TRITON WR 1339 (Sigma), which blocks lipoprotein lipase (LPL) activity. Blood was obtained prior injection and then after 60, 90, and 120 min postinjection from tail vein.

Immunoblotting and Immunoprecipitation. Immunoblot analysis was performed exactly as described previously (5), unless otherwise indicated.

For detection of ANGPTL8 in human blood, 2.5 μL of plasma was size-fractionated on a 12% SDS-polyacrylamide gel and then transferred to a PVDF membrane (Amersham Biosciences). The membrane was incubated in 5% (wt/vol) nonfat milk, 5% newborn calf serum in TBST (Tris-buffered Saline with Tween 20) buffer for 1 h. The primary antibody (1:500) was added to the medium for an overnight incubation.

To measure ANGPTL8-FLAG in mouse livers, proteins were extracted from 100 mg of liver tissue. The tissue was homogenized in Triton buffer (80 mM NaCl, 2 mM $CaCl_2$, 1% Triton, 50 mM TrisCl, pH 8.0,) and 30 μg of protein was subjected to immunoblotting using a rabbit polyclonal anti-FLAG M2 antibody (Sigma; 1:1,000). The plasma of the mice was diluted 10-fold in PBS and incubated at 95 °C for 5 min in sample buffer (31 mM TrisCl, pH 6.8, 1% SDS, 12.5% glycerol, 0.0025% bromophenol). A total of 3 μL of plasma was subjected to immunoblot analysis with polyclonal anti-FLAG M2 antibody. The membranes were then exposed to F-BX810TM Blue X-Ray films (Phoenix Research Products).

To immunoprecipitate ANGPTL8-FLAG, 200 μL of mouse plasma was diluted to 15 mL in PBS buffer containing mixture protease inhibitor (Roche). A total of 100 μL of anti-Flag M2 beads (Sigma) was added to the samples. Samples were rotated overnight at 4 °C and then the beads were washed 6× with 1 mL PBS. Proteins were eluted from the beads in 100 μL of sample buffer by boiling the beads at 95 °C for 5 min. ANGPTL8 and ANGPTL3 were visualized by immunoblotting.

Statistical Methods. Linear regression models with adjustment for age, sex, body mass index (BMI), and ethnicity as appropriate. We applied a square-root transformation to LDL-cholesterol (C) values before analysis to achieve approximate normality in the distribution. BMI, HDL-C, TAG, and homeostatic model assessment-insulin resistance values were log-transformed to remove skewness. To control for possible population substructure and eliminate possible mismatches between true and self-reported ethnicity, we repeated the analysis with adjustment for percentage of African and European ancestry, as estimated using STRUCTURE (6) under a linkage model with 2,270 ancestry-informative markers (7).

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4. Nilsson-Ehle P, Schotz MC (1976) A stable, radioactive substrate emulsion for assay of lipoprotein lipase. *J Lipid Res* 17(5):536–541.
5. Romeo S, et al. (2009) Rare loss-of-function mutations in ANGPTL family members contribute to plasma triglyceride levels in humans. *J Clin Invest* 119(1):70–79.
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7. Smith MW, et al. (2004) A high-density admixture map for disease gene discovery in African Americans. *Am J Hum Genet* 74(5):1001–1013.

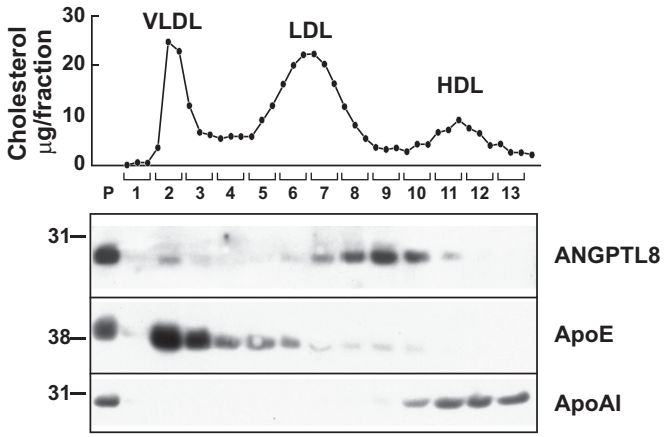

Fig. S1. ANGPTL8 distribution in human plasma. Two hundred microliters of human plasma were separated on a Superose 6 column in 40 fractions and assayed for cholesterol content. Thirteen pooled fractions were delipidated by use of a mixture of ethanol:ether and then the proteins were resuspended in 2× sample buffer. One-fifth of precipitated proteins was subjected to SDS/PAGE separation and ANGPTL8 distribution among fractions detected by immunoblot. ApoE, ApoAI, were used as lipoprotein markers, (P, plasma).

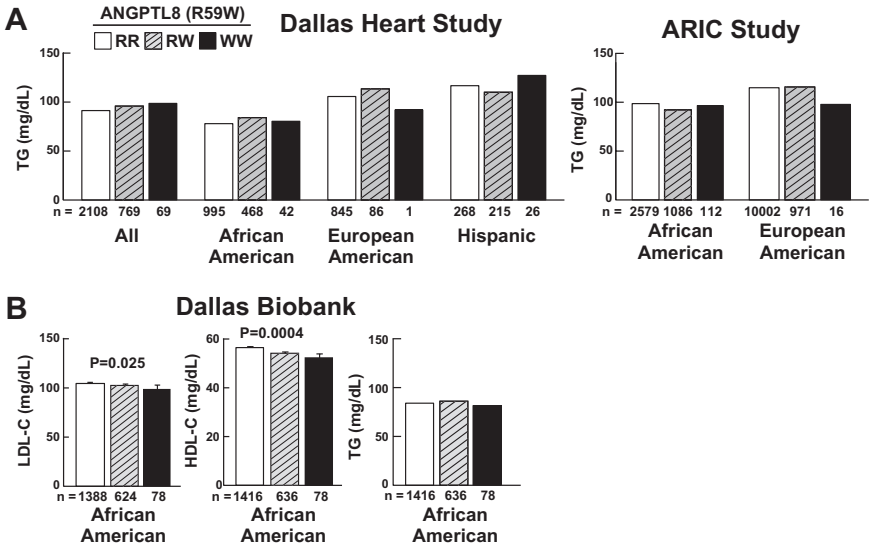

Fig. S2. Plasma TAG and lipid levels in three populations. (*A*) Median TAG levels in DHS and ARIC Study participants stratified by ethnicity and R59W genotype. (*B*) Mean LDL-C, mean HDL-C and median TAG levels in the African American Dallas Biobank participants by R59W genotype. All *P* values were calculated using linear regression with adjustment for age, sex, ethnicity, and BMI.

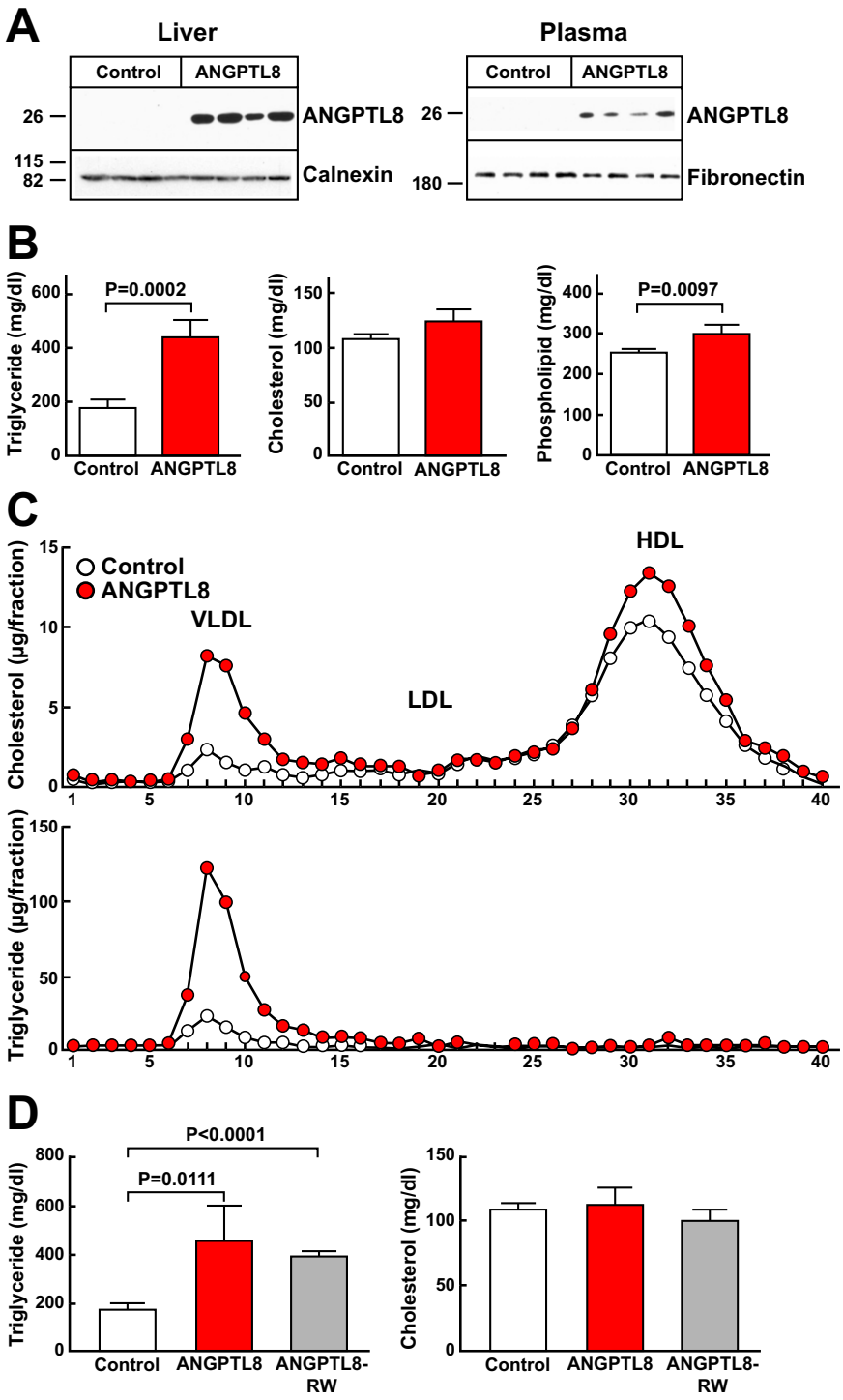

Fig. S3. Adenoviral-mediated expression of human ANGPTL8 in mouse liver results in an increase in plasma TAG levels. (*A*) Immunoblot analysis of ANGPTL8 from liver (30 μg protein) and plasma (3.0 μL) of C57BL/6J mice injected with recombinant adenoviruses particles expressing no insert (control) and ANGPTL8-FLAG. Three days after the mice were infected with the adenoviruses, they were killed after a 4-h fast. Immunoblot analysis was performed using an anti-FLAG M2 antibody to detect ANGPTL8. Calnexin and fibronectin were used as loading controls. (*B*) Plasma TAG, cholesterol and phospholipid levels. (*C*) Plasma lipoproteins were fractionated using FPLC as described in the *SI Materials and Methods*. Cholesterol and TAG were measured enzymatically in 50 μL of each fraction (325 μL each). (*D*) Plasma triglyceride and cholesterol levels in mice overexpressing wild-type ANGPTL8 and ANGPTL8-59W.

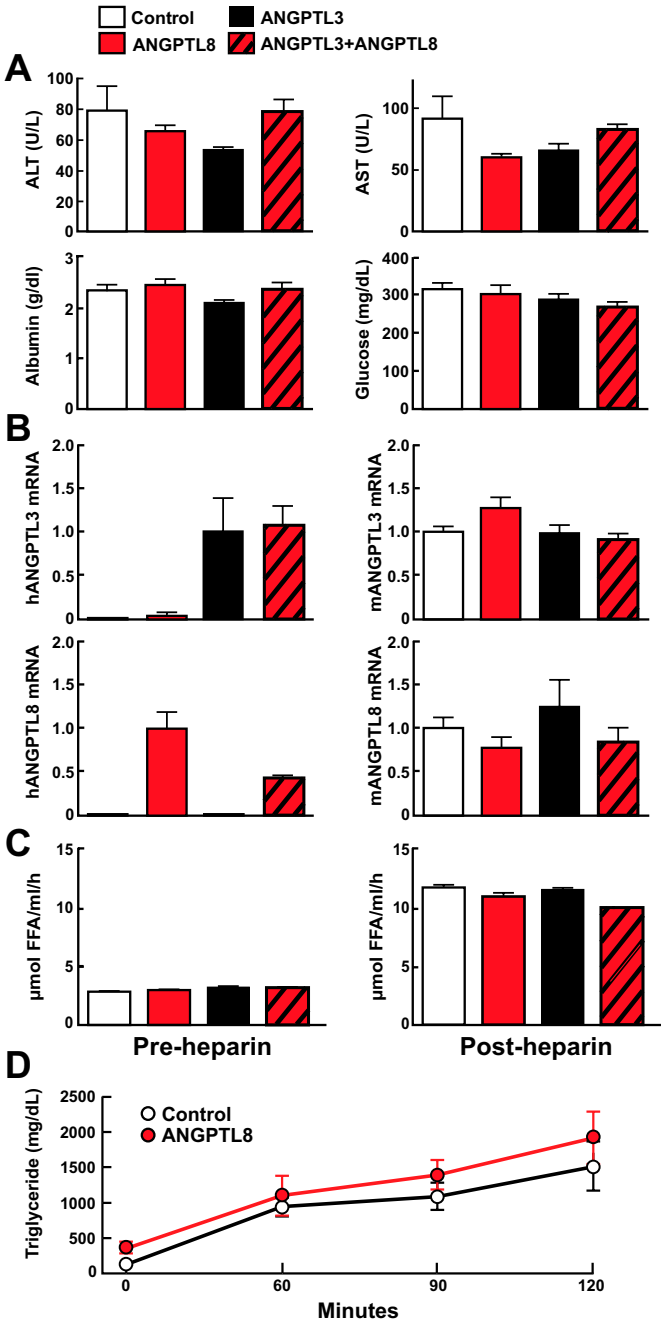

Fig. S4. Plasma chemistries, hepatic mRNA levels, lipolytic activity, and VLDL secretion in mice infected with recombinant ANGPTL8 and ANGPTL3 adenoviruses. (*A*) Mice described in the legends to Fig. 3 *A–C* were killed after a 4-h fast and levels of ALT, AST, albumin, and glucose were measured in plasma. (*B*) Hepatic mRNA levels were measured using quantitative real-time PCR as described in *Materials and Methods*. All mRNA levels were normalized to GAPDH. The value for the control mice was arbitrarily set to 1. (*C*) Total lipolytic activity was measured in pre- and postheparin plasma as described in *Materials and Methods*. (*D*) TAG secretion from mice injected with recombinant adenoviruses particles expressing no insert (Control) and ANGPTL8-FLAG.

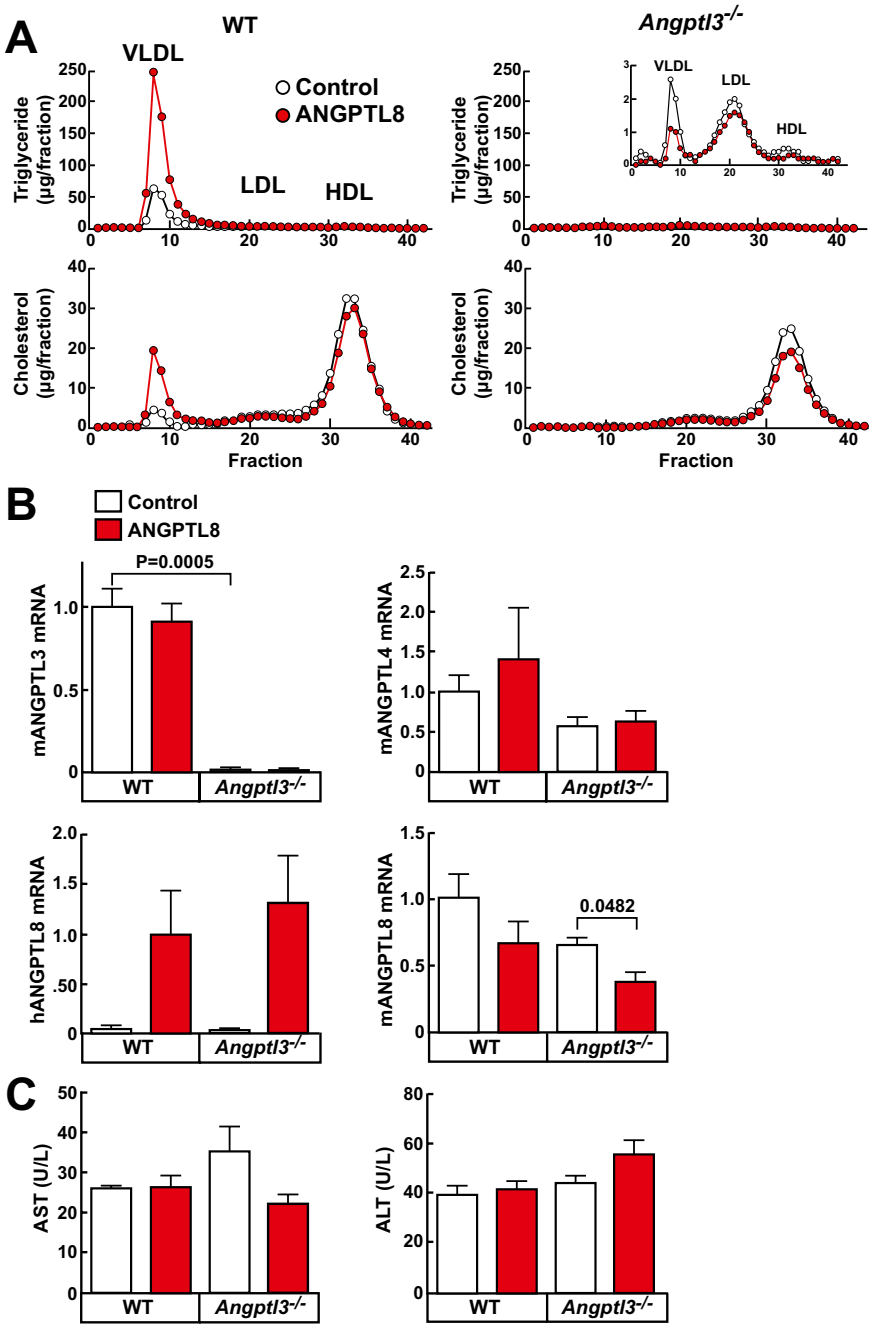

Fig. S5. FPLC analysis of plasma lipoproteins, hepatic mRNA levels and plasma liver enzyme levels in wild-type and *Angptl3*$^{-/-}$ mice infected with adenoviruses expressing ANGPTL8. (*A*) Lipoprotein profile of plasma from mice used in Fig. 3*F*. A total of 200 µL of pooled plasma from each group of mice was analyzed using Superose 6 column (GE Healthcare) as described in *SI Materials and Methods*. (*B*) Hepatic mRNA levels were measured using quantitative real-time PCR as described in *Materials and Methods*. All mRNA levels were normalized to GAPDH. (*C*) Plasma liver enzymes were measured as described in *Materials and Methods*.

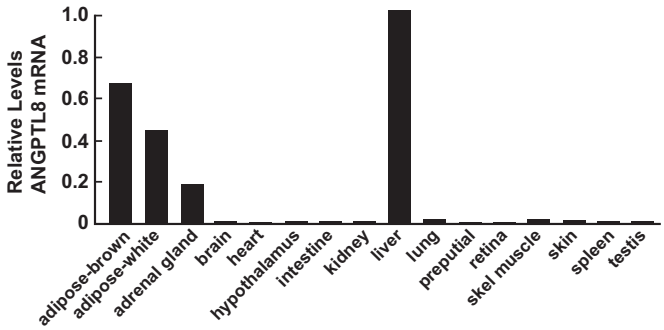

Fig. S6. Tissue distribution of mouse ANGPTL8 mRNA in 16 tissues. C57BL/6J mice 10-wk-old were fed ad libitum on standard chow diet. Equal aliquots of total RNA were pooled from five mice and assayed by real-time PCR. All measurements were standardized using 36B4 and showed as a ratio of liver expression that was set to 1.

Table S1. Clinical characteristics of the DHS participants stratified by ethnicity and rs2278426 genotype

	African American (n = 1,744)				European-American (n = 1,001)				Hispanic (n = 579)			
Characteristic	C/C	C/T	T/T	P	C/C	C/T	T/T	P	C/C	C/T	T/T	P
n	1161	534	49	0.197*	909	91	1	0.718*	308	239	32	0.108*
Male (%)	42.1	40.4	51.0	0.332	47.5	57.1	0.0	0.098	39.6	44.8	46.9	0.405
Age (y)	45 ± 10	44 ± 10	45 ± 10	0.398	45 ± 10	43 ± 10	49 ± 0	0.180	41 ± 9	39 ± 9	42 ± 9	0.179
BMI (kg/m^2)	31.9 ± 8.3	32.1 ± 8.3	29.1 ± 5.8	0.535	29.0 ± 6.6	29.8 ± 6.9	25.7 ± 0	0.291	30.2 ± 6.9	30.9 ± 6.8	30.8 ± 4.8	0.091
Glucose (mg/dL)	91 ± 12	92 ± 13	89 ± 13	0.287	92 ± 12	91 ± 13	112 ± 0	0.879	93 ± 11	94 ± 10	100 ± 9	0.005
Diabetes (%)	14.3	12.4	14.3	0.538	6.9	5.5	0.0	0.838	13.0	10.0	18.8	0.269
HOMA-IR (U)	3.3 (2 – 6)	3.3 (2 – 6)	3.3 (2 – 5)	0.993	2.3 (1 – 4)	2.7 (1 – 4)	3.1 (–)	0.657	3.4 (2 – 6)	3.3 (2 – 5)	4.2 (2 – 7)	0.203
HTGC (%)	3.3 (2 – 5)	3.2 (2 – 5)	3.5 (2 – 5)	0.338	3.6 (2 – 7)	4.7 (3 – 9)	1.5 (–)	0.131	4.6 (3 – 10)	4.6 (3 – 12)	8.1 (3 – 17)	0.127
TAG[†] (mg/dL)	78 (59 – 113)	85 (62 – 116)	81 (59 – 113)	0.141	106 (73 – 160)	114 (81 – 170)	92	0.266	118 (79 – 179)	111 (79 – 153)	129 (105 – 182)	0.376
HDL (mg/dL)	53 ± 15	51 ± 15	54 ± 20	0.028	49 ± 15	46 ± 13	48 ± 0	0.298	47 ± 11	45 ± 12	43 ± 11	0.025
LDL (mg/dL)	106 ± 37	103 ± 36	91 ± 35	0.005	108 ± 35	106 ± 33	86 ± 0	0.531	110 ± 34	103 ± 31	105 ± 33	0.033

Continuous characteristics are shown as mean ± SD or median (first – third quartile). P values were calculated using linear regression with adjustment for age, sex, and BMI. HOMA-IR, Homeostasis Model of Assessment–Insulin Resistance; HTGC, hepatic triglyceride content.

*P value for deviation from Hardy–Weinberg proportions.

[†]Individuals with diabetes were excluded from this calculation.

Table S2. Clinical characteristics of ARIC participants stratified by ethnicity and rs2278426 genotype

	African Americans				European-Americans			
Characteristic	C/C	C/T	T/T	*P*	C/C	C/T	T/T	*P*
n	2,665	1,134	118	0.868*	10,017	971	16	0.153*
BMI (kg/m^2)	29.7 ± 6.1	29.4 ± 6.1	30.2 ± 6.6	0.466	27.0 ± 4.9	27.0 ± 4.8	26.7 ± 4.5	0.955
LDL (mg/dL)	139 ± 43	135 ± 43	129 ± 37	2.1×10^{-4}	137 ± 38	137 ± 39	147 ± 32	0.889
HDL (mg/dL)	55 ± 17	54 ± 18	51 ± 15	0.001	51 ± 17	49 ± 16	50 ± 18	0.006
Triglyceride (mg/dL)	98 (72 – 135)	92 (71 – 133)	97 (72 – 132)	0.637	115 (82 – 164)	116 (82 – 165)	99 (78 – 125)	0.702
HOMA-IR (U)	2.7 (1.7 – 4.3)	2.6 (1.7 – 4.1)	2.9 (1.9 – 4.8)	0.861	2.1 (1.4 – 3.2)	2.1 (1.3 – 3.1)	2.4 (2.0 – 2.7)	0.180

Data are shown as mean± SD or median (first – third quartile). *P* values were calculated using linear regression model with adjustment for age, sex, and BMI where necessary. **P* value for deviation from Hardy–Weinberg equilibrium.

Table S3. Clinical characteristics of the Dallas Biobank African American participants (*n* = 2,148) stratified by rs2278426 genotype

Characteristic	C/C	C/T	T/T	*P*
n	1,432	638	78	0.52*
Male (%)	34.7	37.9	31.2	0.27
Age (y)	44.7 ± 14.3	45.2 ± 14.2	43.9 ± 13.1	0.70
BMI (kg/m^2)	31.8 ± 7.9	31.7 ± 7.9	31.8 ± 8.9	0.97
Glucose (mg/dL)	82 (75 – 93)	82 (75 – 92)	84 (75 – 98)	0.23
Triglyceride (mg/dL)	83 (61 – 119)	86 (62 – 124)	81 (62 – 107)	0.705
HDL-C (mg/dL)	56.5 ± 17.5	54.1 ± 16.3	52.2 ± 15.5	3.5×10^{-4}
LDL-C (mg/dL)	105 ± 34	103 ± 35	98 ± 37	0.0252

Continuous characteristics are shown as mean± SD or median (first – third quartile). *P* values were calculated using linear regression with adjustment for age, sex, and BMI where necessary.
**P* value for deviation from Hardy–Weinberg equilibrium.