Supporting Online Material for

miR-33 Contributes to the Regulation of Cholesterol Homeostasis

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Supporting Online Material

miR-33 coordinates genes regulating cholesterol homeostasis

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Material and Methods

Materials

Chemicals were obtained from Sigma unless otherwise noted. Human lipoproteins (acetylated LDL, HDL) were obtained from Biomedical Technologies Inc (Stoughton, MA). The synthetic LXR ligand TO901317 is from Cayman Chemical. Human apoAI was obtained from Meridian Life Sciences. Mouse monoclonal antibody against ABCA1 (1:1000) was purchased from Abcam. Rabbit polyclonal antibodies against ABCG1 (1:1000), SR-B1 (1:250) and NPC1 (1:1000) were obtained from Novus and mouse monoclonal HSP-90 antibody was from BD Bioscience. Polyclonal antibodies against HMGCR (1:200) and SCAP (1:200) were obtained from Santa Cruz. Secondary fluorescently-labeled antibodies were from Molecular Probes (Invitrogen).

Cell Culture

THP-1, HepG2, J774, HEPA, Fu5AH, EAhy296, COS-7 and 293T cells were obtained from American Type Tissue Collection. THP-1 and J774 cells were maintained in RPMI 1640 media (Sigma) supplemented with 10% fetal bovine serum (FBS) and 2% penicillin-streptomycin in 10 cm^2 dishes at 37°C and 5% CO2. THP1 differentiation into macrophages was induced using 100nM phorbol-12-myristate acetate (PMA) for 72h. HepG2, HEPA, Fu5AH, COS-7 and 293T were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS and 2% penicillin-streptomycin. EAhy296 cells were grown in DMEM containing 10% FBS and penicillin-streptomycin, L-glutamine and HAT (Sigma). Peritoneal macrophages from adult female C57BL/6J mice were harvested by peritoneal lavage four days after intraperitoneal injection of thioglycollate. The cells were maintained in culture as adherent monolayer in medium containing DMEM, 10% FBS, and 20% L-cell-conditioned medium. Cells were stimulated with 37.5 µg/ml acLDL, 10µM TO901317, and 5µM simvistatin at the time points as indicated in figure legends.

miRNA Microarray Analysis

Differentiated THP-1 macrophages in 6-well plates (3 x 10^6 cells per well) were pre-treated overnight with 0.5% FBS in the presence or absence of 5µM simvastatin. Cells were then stimulated with 0.5% FBS media alone with either 37.5µg/ml AcLDL or 5µM simvastatin for 24 hours. Total RNA was extracted using Trizol (Invitrogen) and microRNA was purified from 40µg of total RNA using the RT^2 qPCR-grade miRNA Isolation Kit (SABiosciences). The purity and integrity of both the total RNA sample and the enriched miRNA was verified using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). A total of 400 ng of miRNA was reverse transcribed with the RT^2 miRNA First Strand kit (SABiosciences) and used for each set of Human Whole Genome miRNA Array (SABiosciences). 96-well plates were analyzed on a BioRad iCycler (BioRad Laboratories) and analysis was done using SABiosciences software. Each array was performed in triplicate from three independent experiments.

Mice

All animal experiments were approved by the Institutional Animal Care Use Committee of New York University Medical Center. Six-week old C57BL6 and Ldlr$^{-/-}$ mice were obtained from Jackson Laboratory. Ldlr$^{-/-}$ mice were placed on a either a chow diet or a high-fat diet (HFD) containing 0.3% cholesterol and 21% (wt/wt) fat (from Dyets Inc) for 12 weeks. C57BL6 mice were placed on either a chow diet, HFD, or a chow diet containing 0.005% (wt/wt) rosuvastatin (AstraZeneca UK Ltd), equaling 5 mg/kg body weight per day for 3 weeks. At sacrifice, mice were fasted for 12-14 h before blood samples were collected by retro-orbital venous plexus puncture. Liver samples were collected and stored at -80ºC and total RNA was harvested for miRNA and gene expression analysis.

RNA isolation and quantitative real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. For mRNA quantification, cDNA was synthesized using Taqman RT reagents (Applied Biosystems), following the manufacturer's protocol. Quantitative real-time PCR was performed in triplicate using iQ SYBR green Supermix (BioRad) on iCycler Real-Time Detection System (BioRad). The mRNA level was normalized to GAPDH as a house keeping gene. The primers sequences used were: ABCA1, 5'-GGTTTGGAGATGGTTATACAATAGTTGT-3' and 5'-CCCGGAAACGCAAGTCC-3; ABCG1, 5'-TCACCCAGTTCTGCATCCTCTT-3' and 5'-GCAGATGTGTCAGGACCGAGT-3'; NPC1, 5'-GGTCCGCCTGTGTACTTTGT-3' and 5'-GGCTTCACCCAGTCGAAATG-3'; 5'- GAPDH, 5'-AACTTTGGCATTGTGGAAGG-3' and 5'-ACACATTGGGGGTAGGAACA-3'; SREBP2, 5'-GCGTTCTGGAGACCATGGA-3' and 5'-ACAAAGTTGCTCTGAAAACAAATCA-3';SREBP1, 5'-ACTTCCCTGGCCTATTTGACC-3' and 5'-GGCATGGACGGGTACATCTT-3'; HMGCR 5'-CTTGTGGAATGCCTTGTGATTG -3' and 5'-AGCCGAAGCAGCACATGAT-3'.

For miRNA quantification, total RNA was reverse transcribed using the RT^2 miRNA First Strand kit (SABiosciences). Primers specific for human miR-33a, miR-611, miR-515-3p (SABiosciences) were used and values normalized to human SNORD44 and SNORD47 as housekeeping genes.
For mouse tissues, total RNA from liver, spleen, lung, kidney, brain, heart, aorta and peritoneal macrophages from C57BL6 mice was isolated using the Bullet Blender Homogenizer (Next Advance) in TRIzol. 1µg of total RNA was reverse transcribed using the QuantiMir kit from System Biosciences Inc (SBI) for miR-33 quantification and normalized to U6 using quantitative PCR as described above.

miR-33 and anti-miR-33 transfection

Mouse peritoneal macrophages, J774, HepG2, Hepa, and EAhy926 were transfected 40 nM mi*RIDIAN* miRNA mimics (miR-33) or with 60 nM mi*RIDIAN* miRNA inhibitors (anti-miR-33) (Dharmacon) utilizing Oligofectamine (Invitrogen). All experiment control samples were treated with an equal concentration of a non-targeting control mimics sequence (Con miR) or inhibitor negative control sequence (Con Inh), for use as controls for non-sequence-specific effects in miRNA experiments. Verification of miR-33 overexpression and knockdown was determined using qPCR, as described above. Additionally, lentiviral expression clones containing either a miR-33a precursor (miR-33) or an anti-sense to miR-33a (anti-miR-33) and scrambled controls (scr-miR) were obtained from System Biosciences and packaged into lentiviral particles in 293T cells using the pPACKH1 packaging system, with co-expression of copGFP for expression monitoring. Human THP-1 cells were transduced with lentivirus at an MOI of 1:10 and GFP-positive cells were sorted by FACS.

Western blot Analysis

Cell were lysed in ice-cold buffer containing 50 mM Tris-HCl, pH 7.5, 125 mM NaCl, 1% NP-40, 5.3 mM NaF, 1.5 mM NaP and 1mM orthovanadate, 175 mg/ml octylglucopyranoside and 1 mg/ml of protease inhibitor coctail (Roche) and 0.25 mg/ml AEBSF (Roche). Cell lysates were rotated at 4ºC for 1 h before

the insoluble material were removed by centrifugation at 12000 x g for 10 min. After normalizing for equal protein concentration, cell lysates were resuspended in SDS sample buffer before separation by SDS-PAGE. Following overnight transfer of the proteins onto nitrocellulose membranes. The membranes were probed with the indicated antibodies, and protein bands were visualized using the Odyssey Infrared Imaging System (LI-COR Biotechnology). Densitometry analysis of the gels was carried out using ImageJ software from the NIH (http://rsbweb.nih.gov/ij/).

3'UTR Luciferase Reporter Assays

cDNA fragments corresponding to the entire 3'UTR of hABCA1, hNPC1, mNPC1 and hABCG1 were amplified by RT-PCR from total RNA extracted from HepG2 cells with XhoI and NotI linkers. Additionally, 3'UTR of mABCG1 was cloned using cDNA from mouse liver with the same strategy. The PCR products were directionally cloned downstream *Renilla* luciferase open reading frame of the psiCHECK2^{TM} vector (Promega) that also contains a constitutively expressed firefly luciferase gene, which is used to normalize transfections. Point mutations in the seed region of predicted miR-33 sites within the 3'UTR of hABCA1, hNPC1 and mABCG1 were generated using Multisite-Quickchange (Stratagene) according to the manufacturer's protocol. All constructs were confirmed by sequencing. COS-7 cells were plated into 12-well plates (Costar) and co-transfected with 1μg of the indicated 3'UTR luciferase reporter vectors and the miR-33 mimic or negative control mimic (Dharmacon) utilizing Lipofectamine 2000 (Invitrogen). Luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega). *Renilla* luciferase activity was normalized to the corresponding firefly luciferase activity and plotted as a percentage of the control (cells co-transfected with the corresponding concentration of control mimic). Experiments were performed in triplicate wells of a 12-well plate and repeated at least three times.

Cholesterol Efflux Assays

THP-1 cells expressing either a control lentiviral construct, a pre-miR33a lentiviral construct or an anti-miR33 lentiviral construct (System Biosciences Inc.) were seeded with PMA in 24-well plates at a density of 1 x 10^6 cells per well three days prior to stimulation. On day 3, cells were labelled with 0.5µCi/ml of ^{3}H-cholesterol (PerkinElmer, Waltham, MA) for 24 hours. J774 macrophages, HepG2 or Fu5aH hepatocytes were transfected with either a control mimic, a miR-33 mimic, a control inhibitor or a anti-miR-33 inhibitor (Dharmacon) and seeded at a density of 1 x 10^6 cells per well one day prior to loading with 0.5µCi/ml ^{3}H-cholesterol for 24 hours. Then, cells were washed twice with PBS and incubated with 2mg/ml fatty-acid free BSA (FAFA, Sigma) in media for 1 hour prior to addition of 50µg/ml human apoAI or 50µg/ml HDL in FAFA-media with or without the indicated treatments. For cholesterol depleted experiments, cells were treated with 5µM simvistatin overnight prior to addition of apoAI. Supernatants were collected after 6 hours and expressed as a percentage of total cell ^{3}H-cholesterol content (total effluxed ^{3}H-cholesterol+cell-associated ^{3}H-cholesterol).

PCR Array Gene Expression Profiling

Total RNA was extracted from HepG2 or peritoneal macrophages over-expressing either a control mimic or a miR-33 mimic, as described above. Reverse transcription was performed on 1µg total RNA using the RT^2 First Strand kit and quantitative RT-PCR (QRT-PCR) analysis of 84 lipid-metabolism related genes was performed using Lipoprotein Signalling & Cholesterol Metabolism RT^2 Profiler PCR Arrays (SABiosciences) as per the manufacturer's protocol. The complete list of the genes analyzed is available online at http://www.sabiosciences.com. Data analysis was performed using the manufacturer's integrated web-based software package for the PCR Array System using $\Delta\Delta C_t$ based fold-change calculations. Data is the mean of three independent experiments and is represented by fold change compared to control mimic ± s.e.m.

Lentivirus and gene transfer

A lentivirus encoding the miR-33 precursor (miR-33) or an anti-sense to miR-33 (anti-miR-33) and scrambled controls (scr-miR) were obtained from System Biosciences Inc (SBI). For *in vivo* gene delivery, mice were injected with $2x10^9$ pfu/mouse of each lentiviral construct in 100 μl PBS via retro-orbital injection. At sacrifice, liver protein and RNA was harvested and stored at -80ºC. A total of 1ug of total RNA was reverse transcribed and used for PCR Array gene expression profiling as described above from three mice per group (control-miR, miR-33 and anti-miR33). Protein was analyzed by Western blots detecting ABCA1, ABCG1, NPC1, HMGCR, SR-B1, CD36, and HSP90.

Lipid analysis and Lipoprotein profile measurement

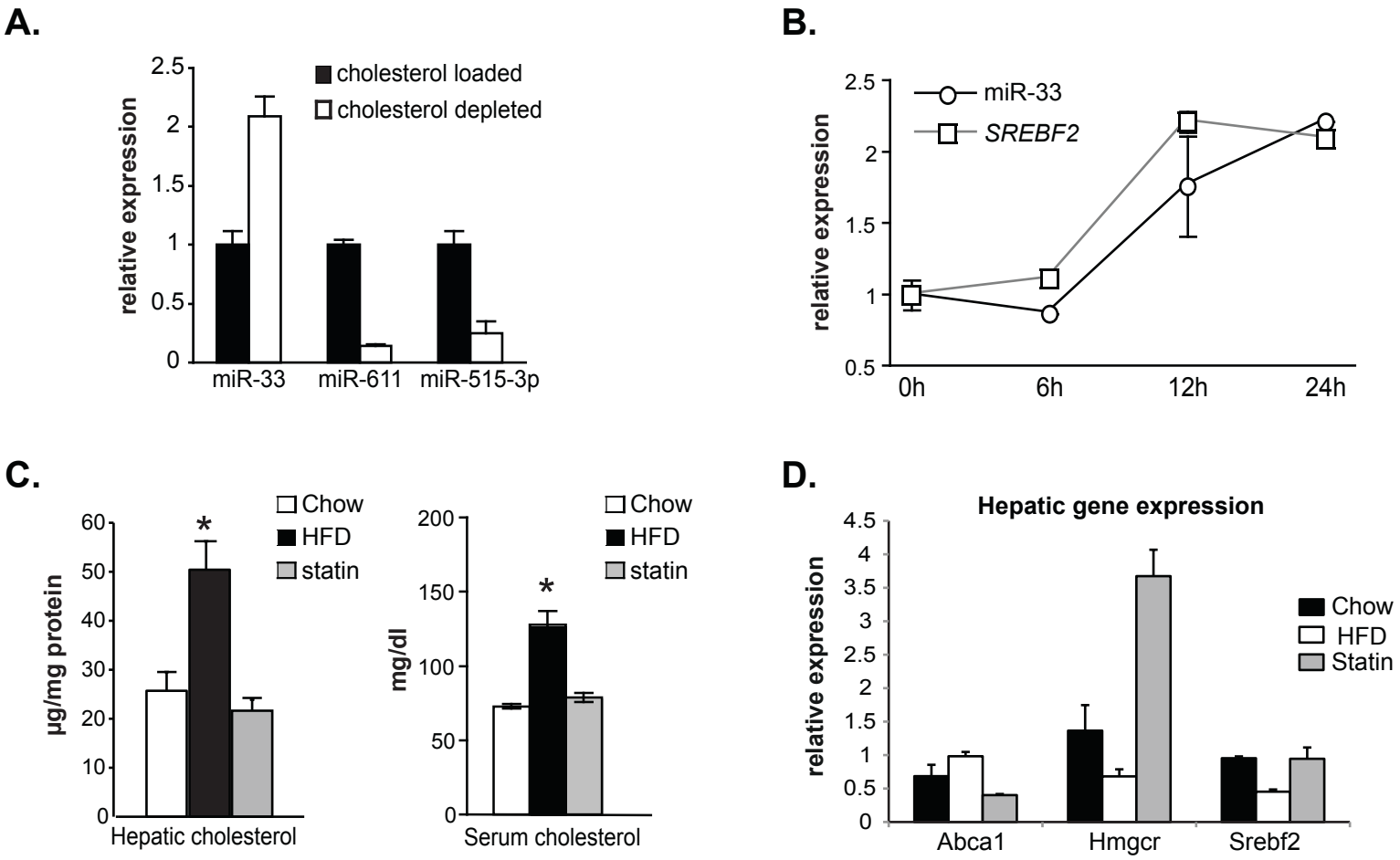
Mice were fasted for 12-14 h before blood samples were collected by retro-orbital venous plexus puncture. Plasma was separated by centrifugation and stored at -80ºC. Total plasma cholesterol and HDL-cholesterol were enzymatically measured with the Amplex red cholesterol assay kit (Molecular Probes), according to the manufacture's instructions. The lipid distribution in plasma lipoproteins fractions were assessed by fast-performance liquid chromatography (FPLC) gel filtration with 2 Superose 6 HR 10/30 columns (Pharmacia).

Immunohistochemistry

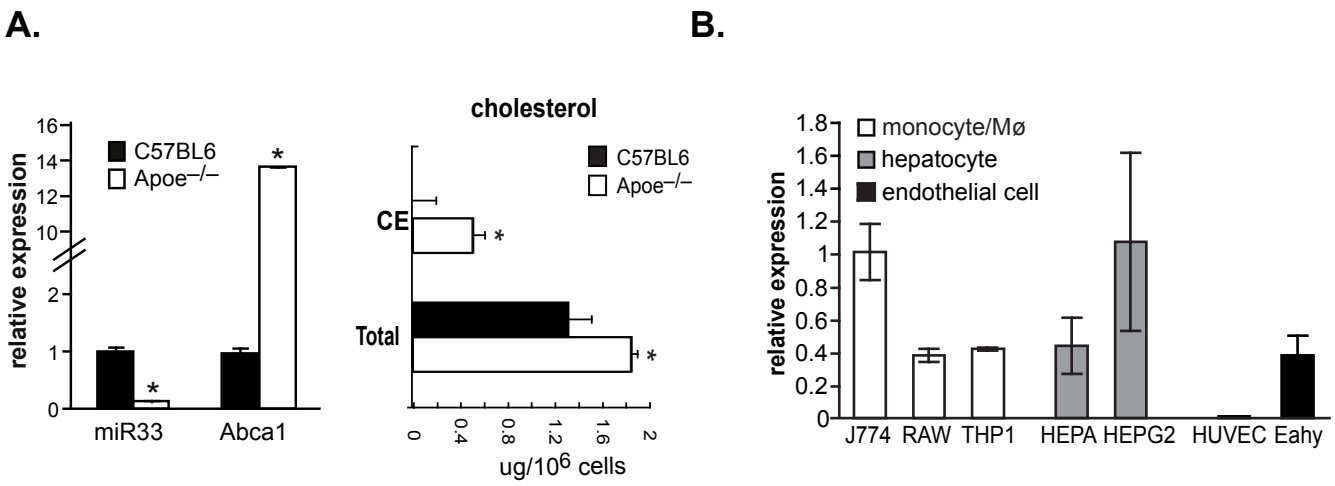
Snap-frozen fixed liver embedded in optimal cutting temperature (OCT) were sectioned, fixed in 4% PFA, and processed for Dapi staining according to standard protocols.

Statistics

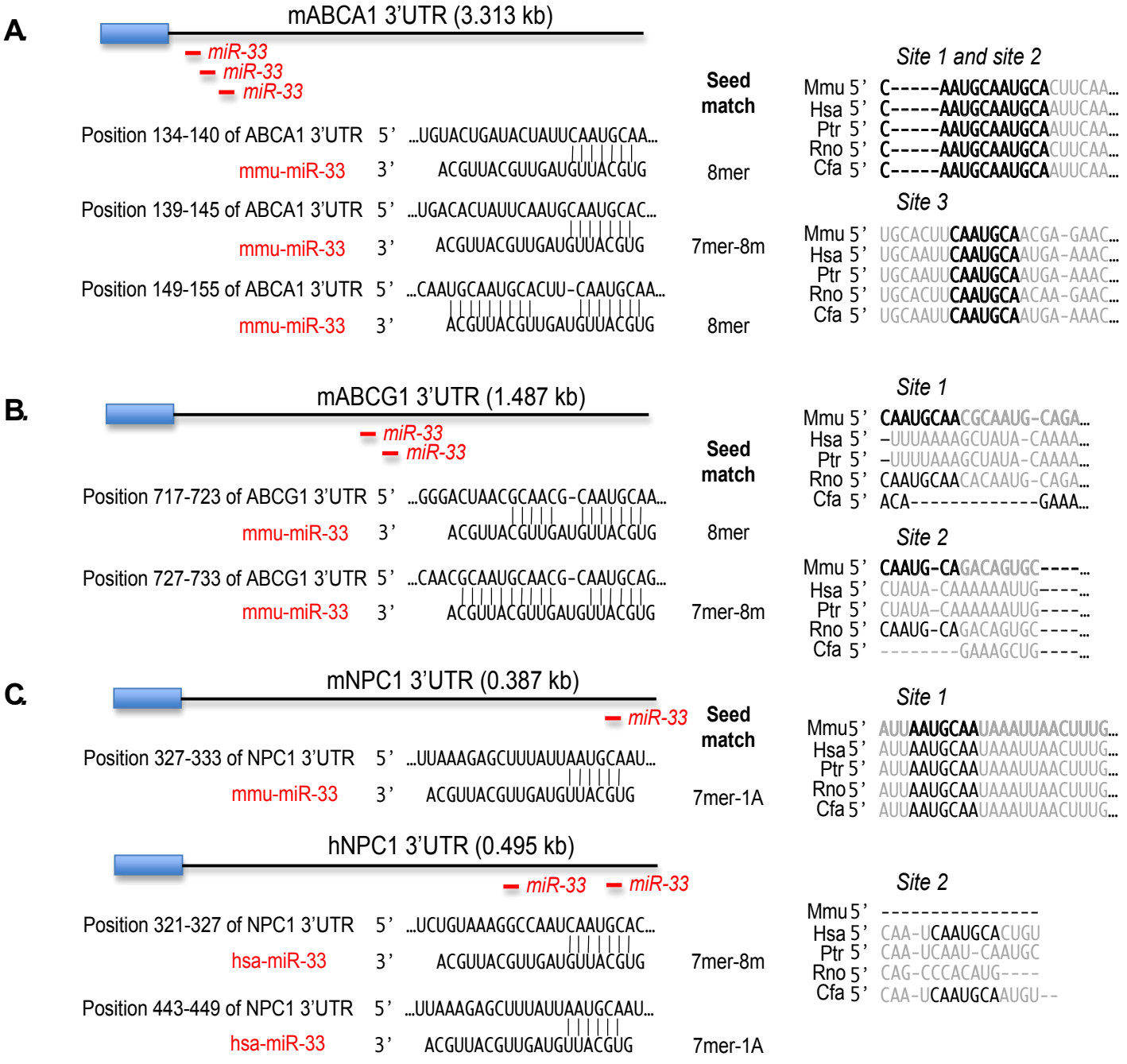
Data are presented as mean ± the standard error of the mean (SEM) (n is noted in the Fig legends), and the statistical significance of differences was evaluated with the Student's t test. Significance was accepted at the level of $p<0.05$.



Supplementary Figure 1. Characterization of miR-33 expression.
(A). Confirmation of miRNAs differentially expressed in cholesterol loaded and cholesterol depleted macrophages by QRT-PCR.
(B) QRT-PCR analysis of miR-33 and SREBF2 expression in human THP-1 macrophages treated with simvastatin for the indicated time.
(C) Analysis of total hepatic (left panel) and serum (right panel) cholesterol levels of C57BL6 mice (n=5 per group) fed a chow, high-fat (HFD) or rosuvastatin-supplemented diet (statin).
(D) Relative gene expression in liver from mice fed a chow, high-fat (HFD) or rosuvastatin-supplemented (statin) diet. Hmgcr mRNA expression was monitored as a cholesterol-responsive gene.
Data are the mean ± s.e.m and are representative of 3 or more experiments.

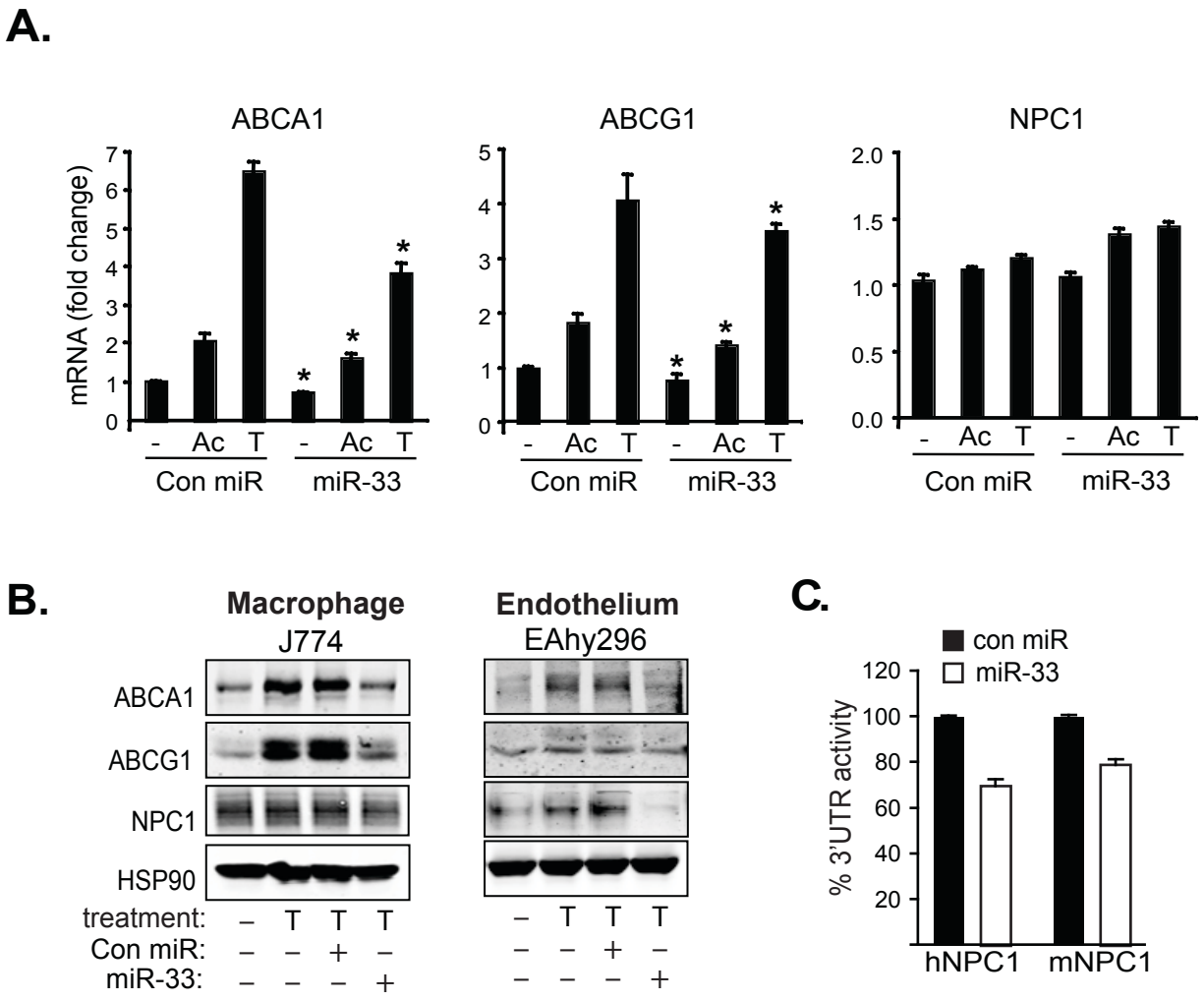


Supplementary Figure 2. Dietary cholesterol alters expression of miR-33.
(A) Relative expression of miR-33 and Abca1 in peritoneal macrophages from 6 month old C57BL6 or Apoe$^{-/-}$ mice. Right panel shows totalcholesterol and cholesterol ester (CE) content of these macrophages. (B) Relative miR-33 expression in macrophage, hepatic or endothelial cell lines. Data are the mean ± s.e.m and are representative of 3 or more experiments. **p≤0.005, *p<0.05.

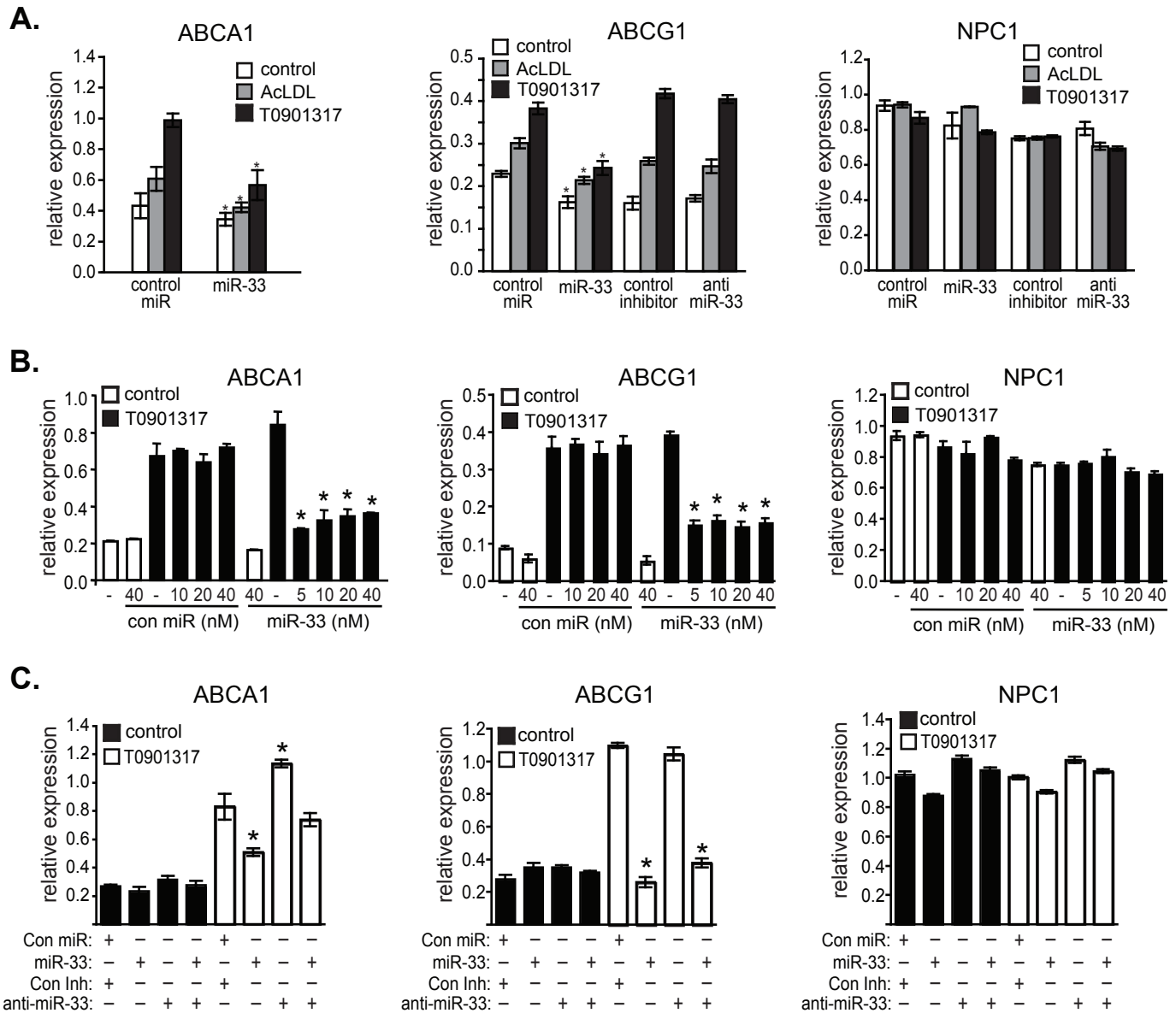


Supplementary Figure 3. Predicted target sites of miR-33 in the 3'UTR regions of ABCA1, ABCG1 and NPC1.

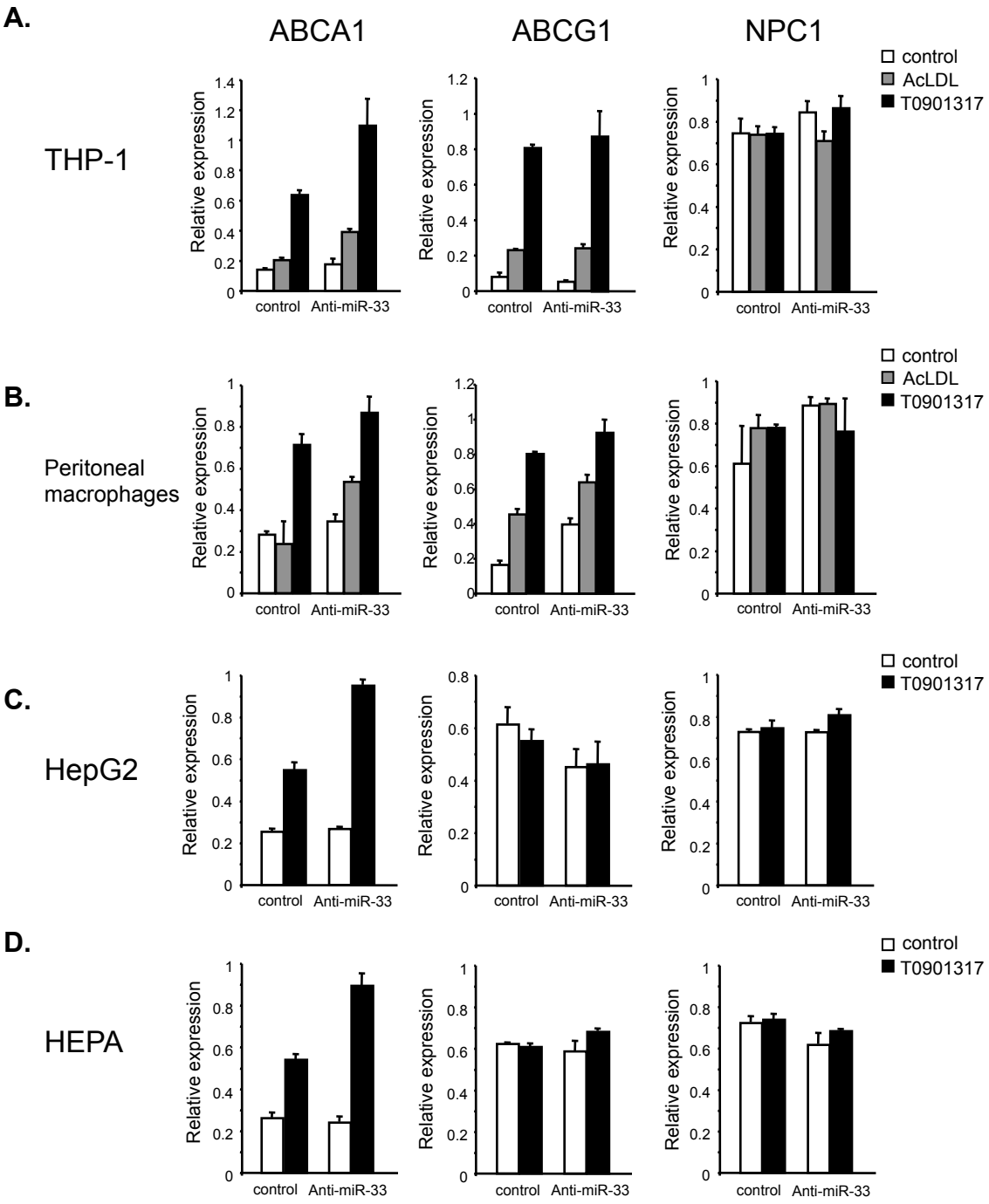
(a) Mouse and human ABCA1 have 3 putative miR-33 binding sites, (b) mouse ABCG1 has 2 miR-33 binding sites, (c) mouse NPC1 has 1 miR-33 binding site, whereas the human NPC1 has 2 sites. Site conservation between species is shown on the right. (Mmu=mouse;Hsa=human; Ptr=chimpanzee; Rno=rat; Cfa=cat). Site prediction based on TargetScan.



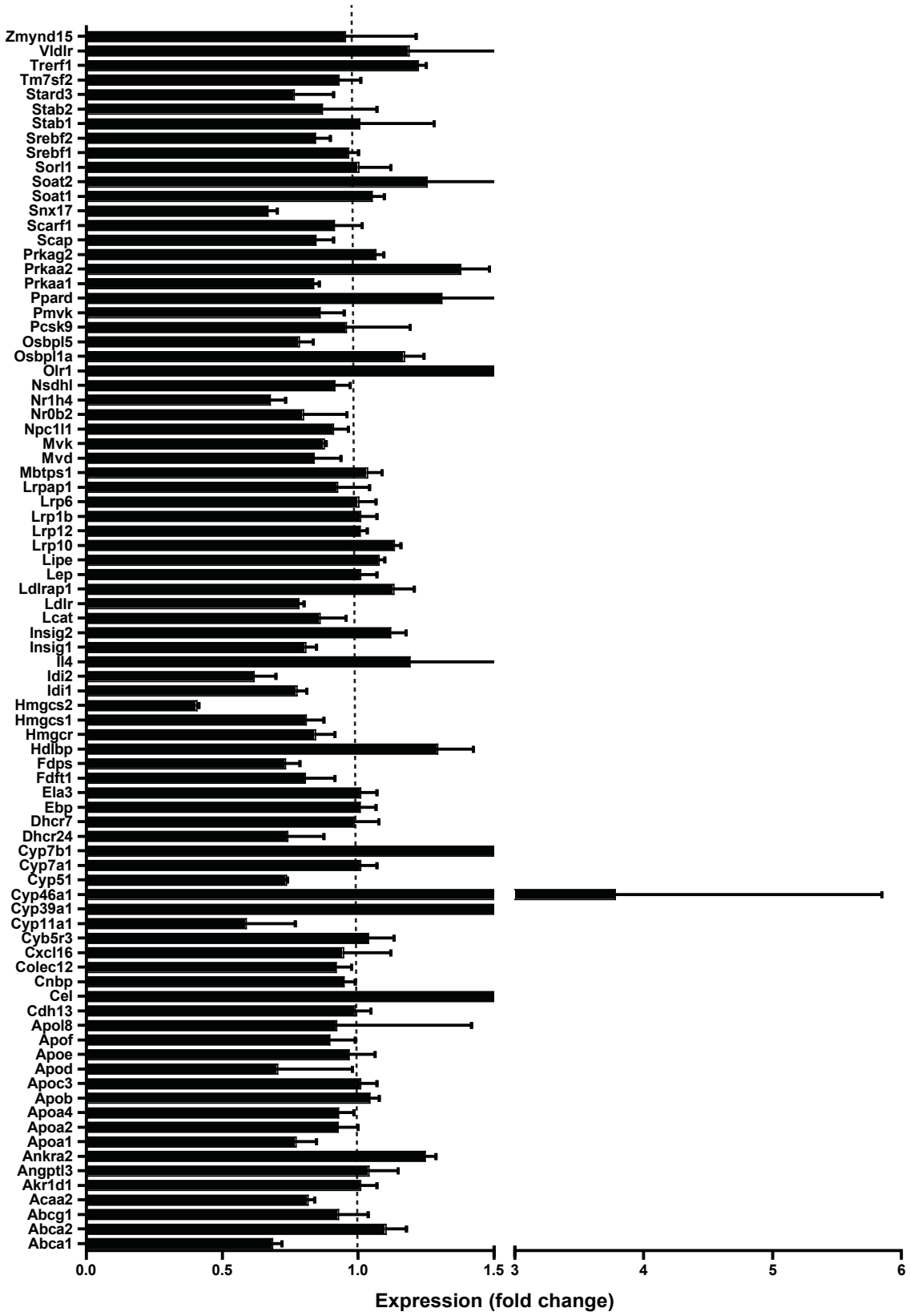
Supplementary Figure 4. miR-33 alters expression of ABCA1, ABCG1 and NPC1
(A) QRT-PCR analysis of ABCA1, ABCG1 and NPC1 mRNA levels in primary mouse macrophages transfected with control miR or miR-33 upon stimulation with AcLDL (Ac) or T0901317 (T).
(B) Expression of ABCA1, ABCG1 and NPC1 expression in human (Eahy296) and mouse (J774) cells of the indicated origin transfected with control miR or miR-33 and treated with or without T0901317 (T).
(C) Comparison of 3'UTR activity of hNPC (with 2 putative miR-33 binding sites) and mNPC1 (with 1 putative miR-33 binding site) in the presence of a control miR or miR-33.



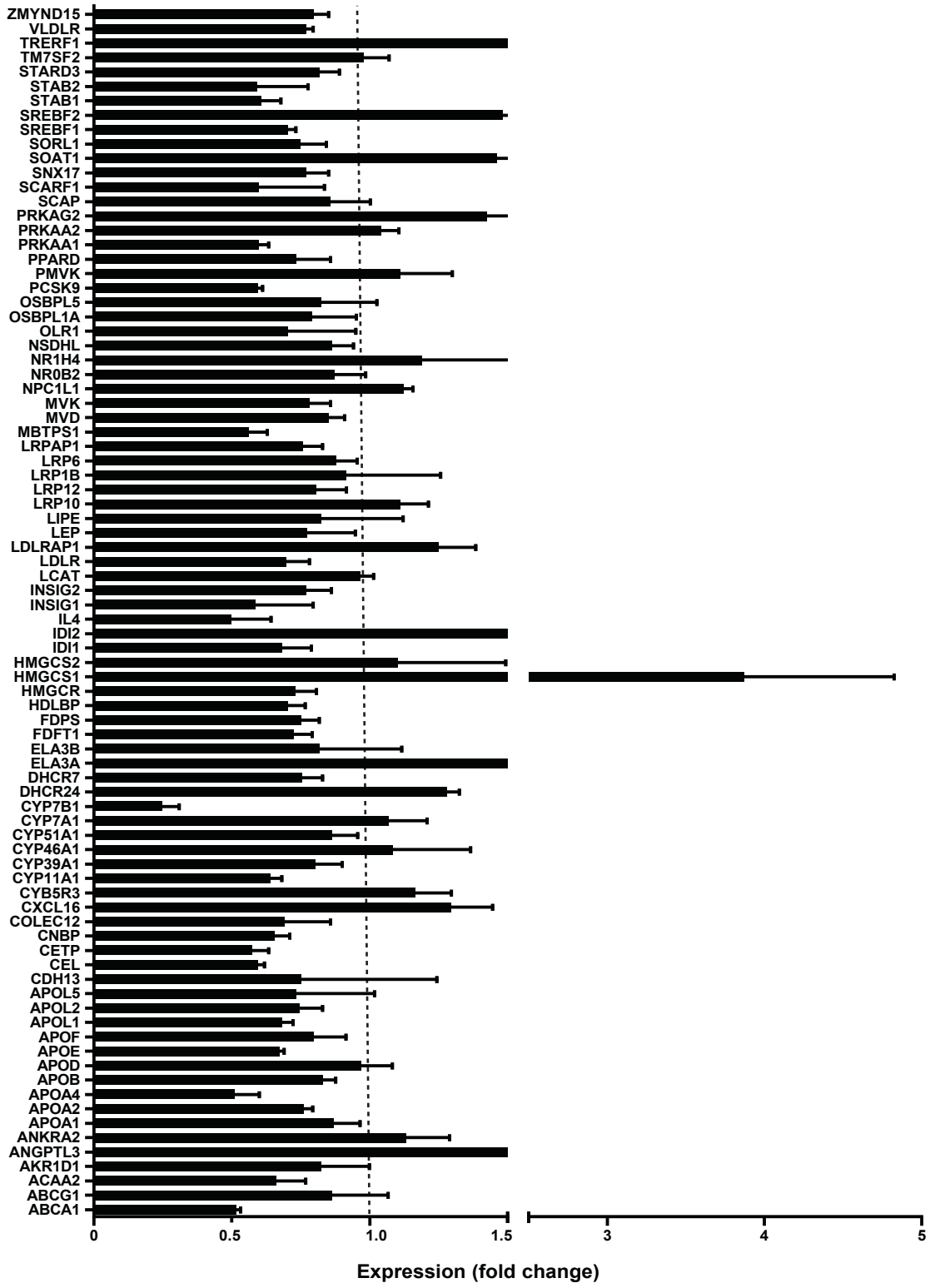
Supplementary Figure 5. miR-33 decreases ABCA1 and ABCG1 expression in mouse macrophages .
Quantification of ABCA1, ABCG1, NPC1 protein (relative to HSP90) in mouse peritoneal macrophages transfected with (a) a control miR or miR-33, (b) increasing concentrations of a control miR or miR-33, or (c) control miR or miR-33 in the presence or absence of a control inhibitor or anti-miR-33.
Macrophages were untreated or treated with AcLDL or TO901317.
Data correspond to Western blots in figure 2 (b-d). $P<0.05$.



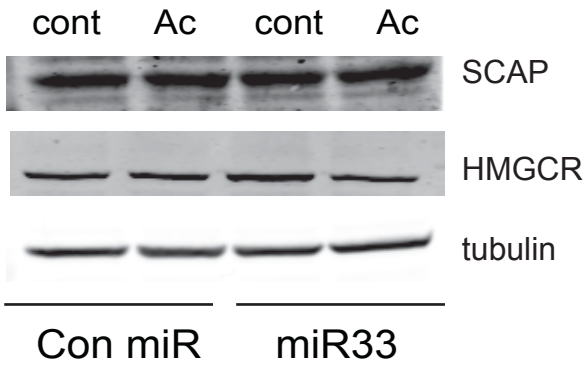
Supplementary Figure 6. Anti-miR-33 increases ABCA1 and ABCG1 gene expression.
Quantification of ABCA1, ABCG1, NPC1 protein (relative to HSP90) in human THP-1 (a) and mouse peritoneal macrophages (b), human HepG2 (c) and mouse HEPA (d) hepatic cells transfected with a control inhibitor or anti-miR33. Cells were untreated or treated with AcLDL or TO901317.
Data correspond to Western blots in figure 2E. $P<0.05$.



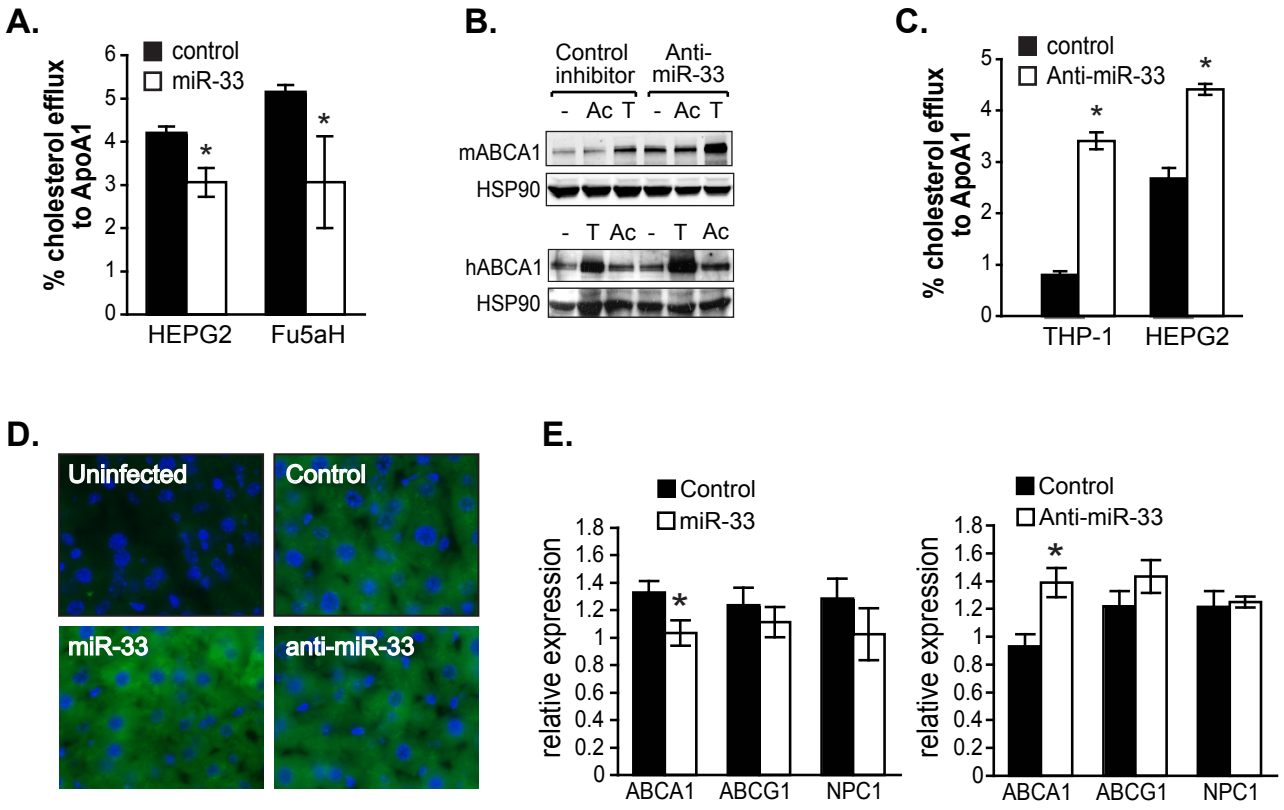
Supplementary Figure 7A. Gene expression analysis in macrophages.
Relative gene expression of 84 lipid-metabolism genes in peritoneal macrophages transfected with a miR-33 mimic. Expression is expressed as fold-change relative to macrophages transfected with a conrol mimic.



Supplementary Figure 7B. Gene expression analysis in hepatocytes.
Relative gene expression of 84 lipid-metabolism genes in HepG2 cells transfected with a miR-33 mimic. Expression is expressed as fold-change relative to cells transfected with a conrol mimic.



Supplementary Figure 8. Expression of lipid-metabolism genes in macrophages expressing miR-33. Expression of genes with putative miR-33 target sites, SCAP and HMGCR, in macrophages transfected with a control mimic or miR-33 mimic in the presence or absence of AcLDL (Ac).



Supplementary Figure 9. Regulation of miR-33 levels alters cholesterol efflux in vitro and ABCA1 levels in vivo.

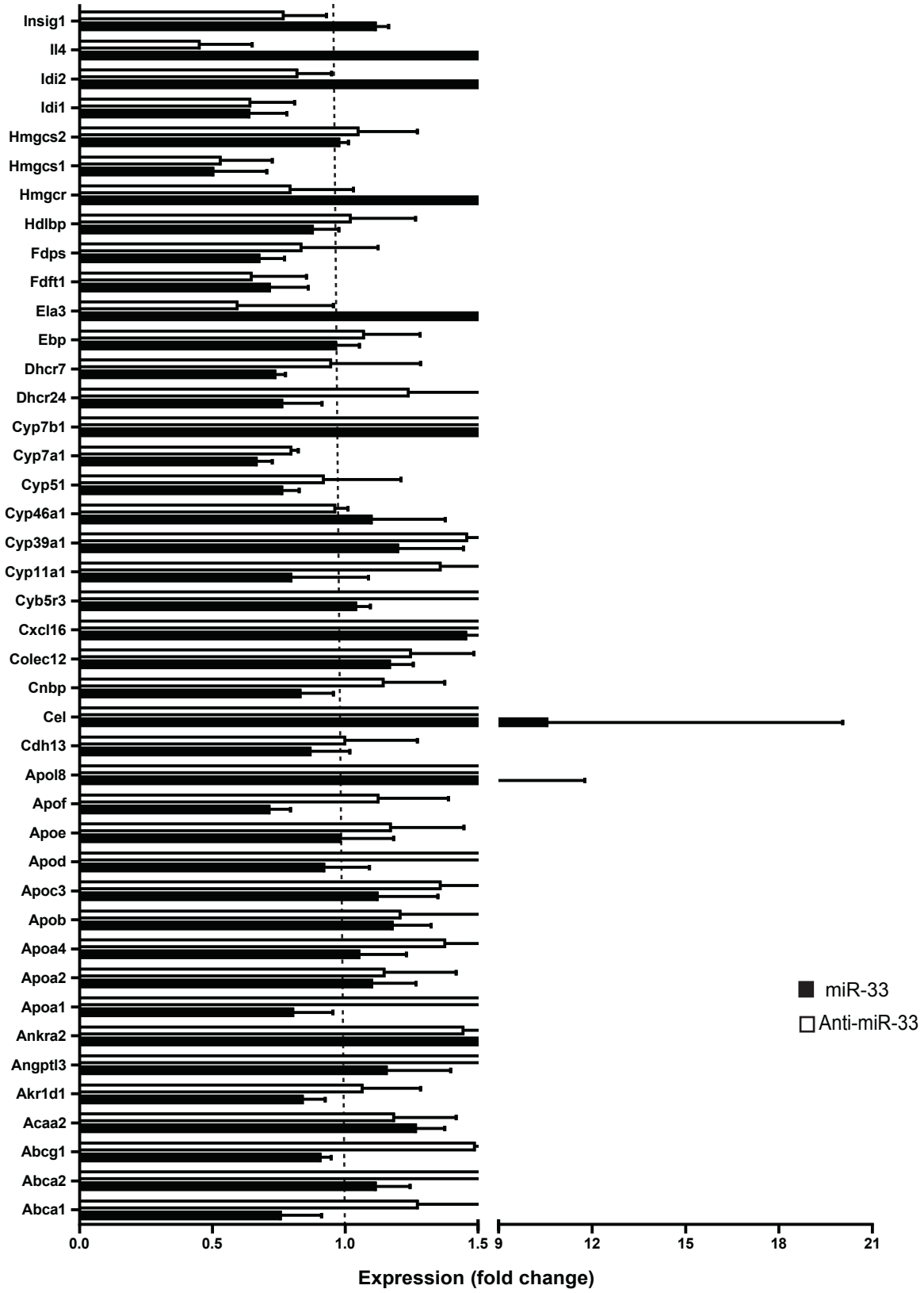
(A) Cholesterol efflux to ApoA1 in hepatic cells expressing a control miR or miR-33.

(B) Western blot of mouse and human ABCA1 in macrophages transfected with control inhibitor or anti-miR-33.

(C) Cholesterol efflux to ApoA1 in THP-1 and HEPG2 cells depleted of cholesterol (condition in which miR-33 rises) and treated with anti-miR-33 or a control inhibitor.

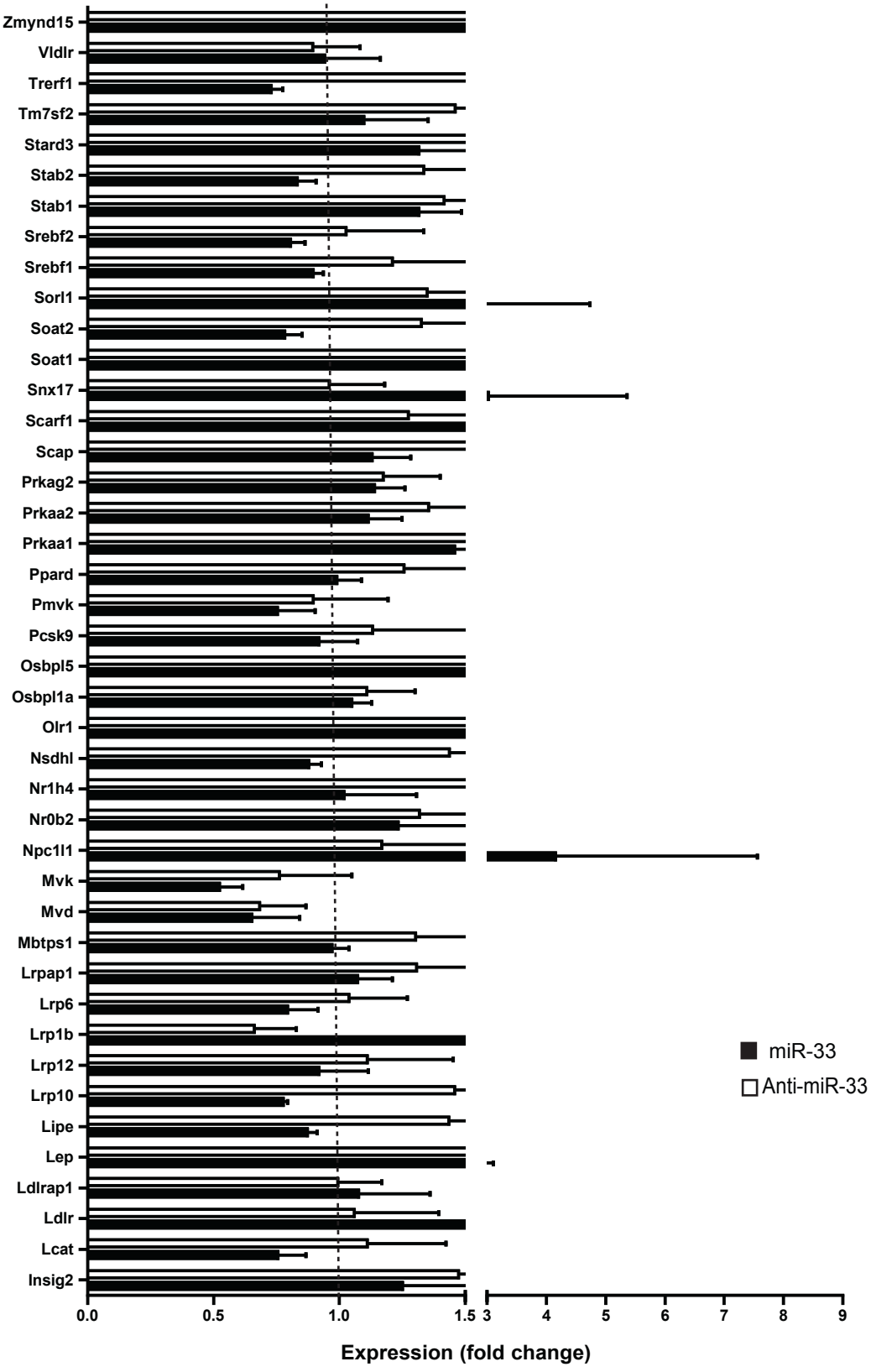
(D) Staining for GFP in livers of mice 6 days after infection with the indicated lentiviral constructs.

(E) Quantification of hepatic ABCA1, ABCG1 and NPC1 expression from mice (n=6) 6 days after infection with control, miR-33 or anti-miR-33 lentiviruses. Data in A-C are the mean ± s.e.m and are representative of 3 experiments.*p≤0.05.



Supplementary Figure 10A. Gene expression analysis in mice treated with miR-33 or anti-miR33 lentiviruses.

Relative gene expression of 84 lipid-metabolism genes in livers from mice treated with a miR-33 (black bars) or anti-miR33 (white bars) lentivirus. Expression is expressed as fold-change relative to mice treated with a control lentivirus.



Supplementary Figure 10B. Gene expression analysis in mice treated with miR-33 or anti-miR33 lentiviruses.

Relative gene expression of 84 lipid-metabolism genes in livers from mice treated with a miR-33 (black bars) or anti-miR33 (white bars) lentivirus. Expression is expressed as fold-change relative to mice treated with a control lentivirus.

Table. Relative miRNA expression in cholesterol loaded vs. depleted macrophages

miRNA ID	Fold change	Gene targets
hsa-let-7c	-3.86	PPARGC1A
hsa-miR-130b	-2.38	LDLR
hsa-miR-29c	-2.27	NPC1,LRP6, LPL, OSBP
hsa-miR-302a	-2.17	VLDLR, ABCA1
hsa-miR-330-3p	2.58	LRP8
hsa-miR-33a	-2.67	ABCA1, NPC1, ABCG1
hsa-miR-342-5p	-2.43	
hsa-miR-369-5p	-2.14	ABCG1, LPL
hsa-miR-376a	-2.02	LRP2
hsa-miR-449a	-2.00	
hsa-miR-489	-2.35	LIPH, LRP11
hsa-miR-502-5p	-2.58	LRP6, APOB48R
hsa-miR-503	-4.44	SRA
hsa-miR-515-3p	2.09	SCARF1
hsa-miR-518c	-3.21	LPIN1
hsa-miR-525-3p	-2.58	FFAR3
hsa-miR-548a-3p	5.16	CAV1
hsa-miR-611	21.11	APOE, OSBP2, MARCO
hsa-miR-615-3p	-2.09	APOB
hsa-miR-629	-2.27	LRP6
hsa-miR-671-5p	-2.09	LRP6

THP-1 cells were treated with AcLDL or Simvastatin for 24h and relative miRNA expression was analyzed by QRT-PCR miRNA PCR Array.
Data are the mean of three independent experiments. Predicted gene targets were identified using Targetscan.