SUPPLEMENTAL MATERIAL

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ONLINE DETAILED MATERIALS AND METHODS

Materials

Chemicals were obtained from Sigma unless otherwise noted. Human lipoproteins (acetylated LDL) were obtained from Biomedical Technologies Inc. The synthetic LXR ligand T090 is from Cayman Chemical. Human ApoA1 was obtained from Meridian Life Sciences. A mouse monoclonal antibody against ABCA1 was purchased from Abcam and a mouse monoclonal HSP90 antibody was purchased from BD Bioscience. Rabbit polyclonal antibodies against ABCG1 and SR-B1 were obtained from Novus. Secondary fluorescently labeled antibodies were from Molecular Probes (Invitrogen).

Cell Culture

Human monocytic (THP-1), human hepatic (HepG2 and Huh-7), mouse macrophages (J774), mouse hepatic (HEPA), human endothelial (EAhy296) and monkey kidney fibroblast (COS-7) cells were obtained from American Type Tissue Collection (ATTC). 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% CO_2. THP-1 differentiation into macrophages was induced using 100 nM phorbol-12-myristate acetate (PMA) for 72 h. HepG2, HEPA and COS-7 cells 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 male C57BL/6J mice were harvested by peritoneal lavage four days after intraperitoneal injection of thioglycollate (3% w/v). Bone marrow derived macrophages were isolated from WT and LXR-deficient mice. The cells were maintained in culture as an adherent monolayer in medium containing DMEM, 10% FBS, and 20% L-cell-conditioned medium. Cells were stimulated with 120 µg/ml Ac-LDL or 3 µmol/L T090 for 48 h.

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 iScript RT Supermix (Bio-Rad), following the manufacturer's protocol. Quantitative real-time PCR was performed in triplicate using iQ SYBR green Supermix (BioRad) on Real-Time Detection System (Eppendorf). The mRNA level was normalized to GAPDH as a house keeping gene. The human primer sequences used were: ABCA1, 5'-GGTTTGGAGATGGTTATACAATAGTTGT-3' and 5'-CCCGGAAACGCAAGTCC-3; ABCG1, 5'-TCACCCAGTTCTGCATCCTCTT-3' and 5'-GCAGATGTGTCAGGACCGAGT-3'; LXRβ, 5'-TTCGCTAAGCAAGTGCCTGGTTTC-3' and 5'-AGTCGTCCTTGCTGTAGGTGAAGT-3'; GAPDH, 5'-AACTTTGGCATTGTGGAAGG-3' and 5'-ACACATTGGGGGTAGGAACA-3'; RXRβ, 5'-GCTTGGCTGCCTGAGGGCAA-3' and 5'-TGGCAAACCGTCCCTGCTGC-3', PPARα, 5'-AAGAGTAGCTTGGAGCTCGGCG-3' and 5'-GCTGGTGAAAGCGTGTCCGTG-3'. The mouse primers sequences used were: ABCA1, 5'-GGTTTGGAGATGGTTATACAATAGTTGT-3' and 5'-CCCGGAAACGCAAGTCC-3'; ABCG1, 5'-TCACCCAGTTCTGCATCCTCTT-3' and 5'- GCAGATGTGTCAGGACCGAGT-3'; RXRβ, 5'-

TCTCAGGGGATCCGTCCGTC-3' and 5'-CGACACTGTGGAGTTGATCTGAG -3'; LXRβ, 5'-CAGCTGCAGTGCAACAAACGATCT-3' and 5'-ACCTGCTTGGCAAAGTCCACAATC-3'; CYP7A1 5'-AGCAACTAAACAACCTGCCAGTACTA-3´ and 5'-GTCCGGATATTCAAGGATGCA-3'; Actin 5'-TGAGAGGGAAATCGTGCGTGAC-3' and 5'-CCGCTCGTTGCCAATAGTGATG-3', and PPARα, 5'- AAGAACCTGAGGAAGCCGTTCTGT-3' and 5'-GCAGCCAAACAGGGAAATGTCA-3'. For miRNA quantification, total RNA was reverse transcribed using the RT^2 miRNA First Strand kit (SABiosciences). Primers specific for human and mouse miR-144 (SABiosciences) were used and values normalized to SNORD38B as a housekeeping gene. For pri-miRNA quantification, cDNA was synthesized using TaqMan® reverse transcription reagents (Applied Biosystems), following the manufacturer's protocol. Quantitative real-time PCR was performed in triplicate using TaqMan Universal Master Mix (Applied Biosystems) on a Real-Time PCR System (Applied Biosystems). The pri-miRNA level was normalized to 18S as a housekeeping gene.
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. 1 µg of total RNA was reverse transcribed using the RT^2 miRNA First Strand kit (SABiosciences) for miR-144 quantitation and normalized to SNORD66 using quantitative PCR as described above.

Western blot Analysis
Cells 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, 1 mM orthovanadate and 1 mg/ml of protease inhibitor cocktail (Roche) and 0.25 mg/ml AEBSF (Roche). Cell lysates were rotated at 4ºC for 1 h before the insoluble material was 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/).

miR-144/451 Promoter- Luciferase Reporter Assay
The pGL3-miR-CRE1 promoter-luciferase vector was kindly provided by M. Weiss. Huh-7 cells were plated into 12-well plates and cotransfected using Lipofectamine® LTX & Plus Reagent (Invitrogen), following manufacturer's instructions. Cells were transfected with 1µg of pGL3-miR-CRE1 promoter vector or the pGL3-promoter vector (Promega) as a control, and also cotransfected with 1µg of *Renilla* plasmid, which was used to normalize transfections. At 24h post transfection, cells were treated with 3 µmol/L T090 or vehicle. After 6h of treatment, cells were lysed with Passive Lysis Buffer (Promega) and 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 pGL3-promoter. Experiments were performed in triplicate, and at least 3 independent experiments were performed.

3'UTR Luciferase Reporter Assays
cDNA fragments corresponding to the entire 3'UTR of human and mouse *Abca1* were amplified by RT-PCR from total RNA extracted from HepG2 or Hepa cells with XhoI and NotI linkers. The PCR product was directionally cloned downstream of the *Renilla* luciferase open reading frame of the psiCHECK2™ vector (Promega) that also contains a constitutively expressed firefly luciferase gene, which is used to normalize transfections. Point mutations in the seed region of

the predicted miR-144 sites within the 3'UTR of *Abca1* 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 and co-transfected with 1 μg of the indicated 3'UTR luciferase reporter vectors and the miR-144 mimic or control mimic (Con-miR) (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.

ONLINE FIGURE LEGENDS

Online Figure I. **Sequence and conservation of the miR-144 binding sites in the human *Abca1* 3'UTR**. (**A**) Sequence alignment of the human *Abca1* 3'UTR. Relative position of the binding sites is indicated. (**B**) Sequence alignment of the seven human (Hsa) miR-144 binding sites and the indicated species [Pan troglodytes (Ptr), Mus musculus (Mmu), Rattus norvegicus (Rno), and Oryctolagus cuniculus (Ocu)].

Online Figure II. LXR ligands induce miR-144 and miR-451 expression in macrophages and hepatic cell lines. (**A**) qRT-PCR analysis of miR-144 expression in THP-1 (left panel) and Huh-7 cells (right panel) treated with GW3965. Data are the mean ± SEM of 3 independent experiments in triplicate. *$P \leq 0.05$. (**B**) qRT-PCR analysis of miR-144 expression in mouse peritoneal macrophages treated with Ac-LDL. Data are the mean ± SEM of 3 independent experiments in triplicate. *$P \leq 0.05$. (**C** and **D**) qRT-PCR analysis of miR-144 expression in THP-1 (left panel) and Huh-7 cells (right panel) treated with different doses of T090 (**C**) or GW3965 (**D**). Data are the mean ± SEM of 2 independent experiments in duplicate. (**E**) qRT-PCR analysis of miR-451 expression in mouse peritoneal macrophages (left panel), THP-1 cells (middle panel) and Huh-7 cells (right panel) treated with T090. Data are the mean ± SEM of 3 independent experiments in triplicate. *$P \leq 0.05$.

Online Figure III. Distribution and dietary regulation of miR-144. (**A**) qRT-PCR analysis of miR-144 expression in selected mouse tissues. (**B**) Representative pictures of hepatocyte (Hep) and Kupffer (Kupff) cells isolated by isopycnic centrifugation (upper left panels). The purity of each cell population was confirmed by Western blot (bottom left panel). miR-144 relative expression assessed by qRT-PCR is represented in the right panel. Data are the mean ± SEM of three independent cellular isolations. qRT-PCR analysis of miR-144 expression in the liver of C57BL6 mice (=5 per group) fed in a chow diet or high-fat diet (HFD) for 5 weeks. Graphs (left to right) represent body weight, cholesterol plasma levels, triglycerides plasma levels and miR-144 expression. Data are expressed as relative expression and correspond to mean±SEM of three independent experiments. *Significantly different from mice fed with chow diet, $P \leq 0.05$.

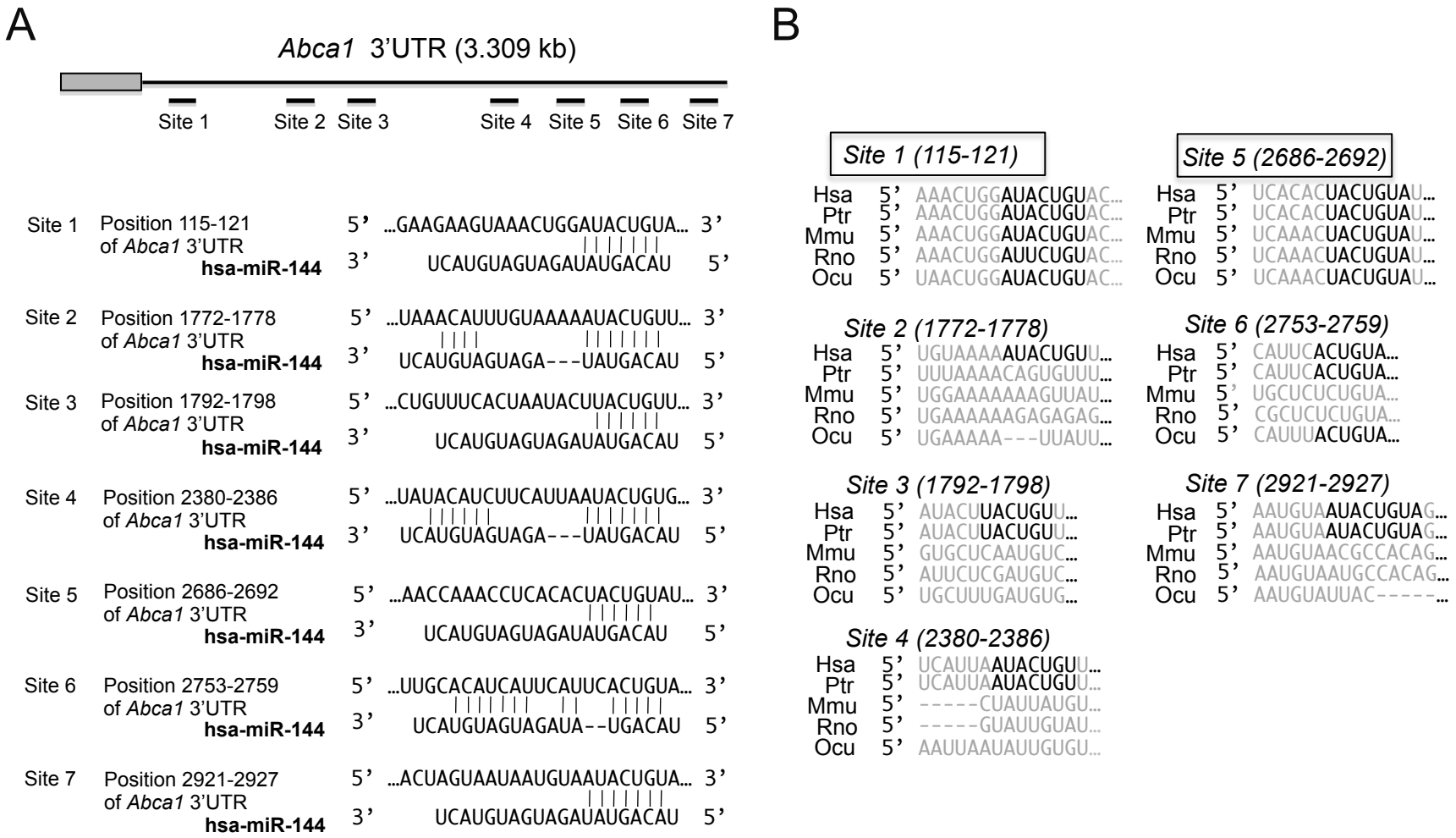
Online Figure IV. miR-144 over-expression reduces ABCA1 expression in HepG2, Hepa and EAhy296 cells. (**A**) qRT-PCR analysis of *ABCA1* in HepG2 transfected with miR-144 mimics. Data are the mean ± SEM of 3 independent experiments in triplicate. *$P \leq 0.05$. (**B**) Western blot analysis of ABCA1 and HSP90 expression in HepG2, Hepa and EAhy296 cells transfected with miR-144 mimics and treated or untreated with T0901317. Data correspond to a representative blot among two that gave similar results.

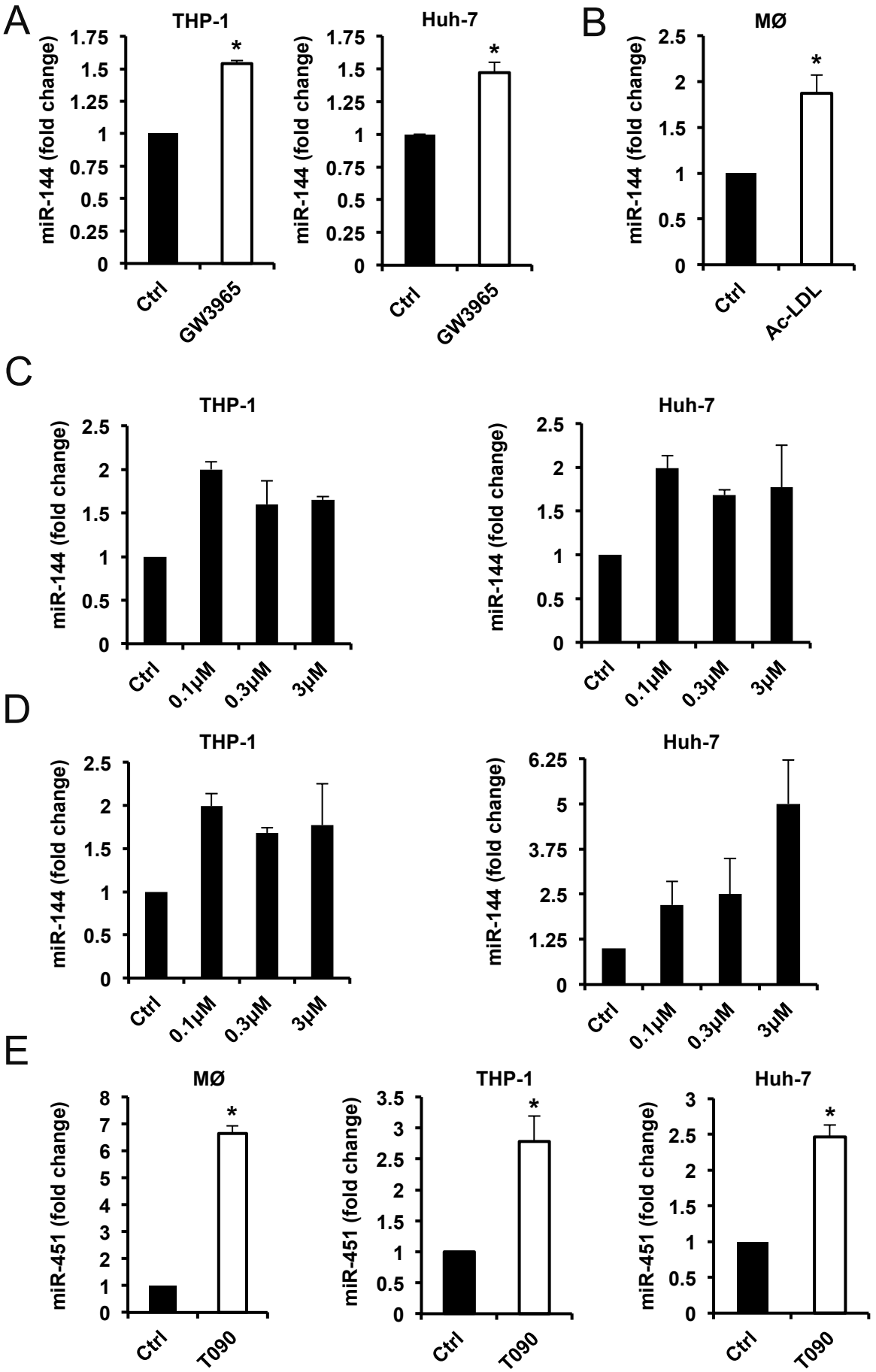
Online Figure V. **miR-144 levels regulates mouse *Abca1* 3'UTR activity**. Luciferase-reporter activity in COS-7 cells transfected with Con-miR or miR-144 mimic and mouse *Abca1* 3'UTR

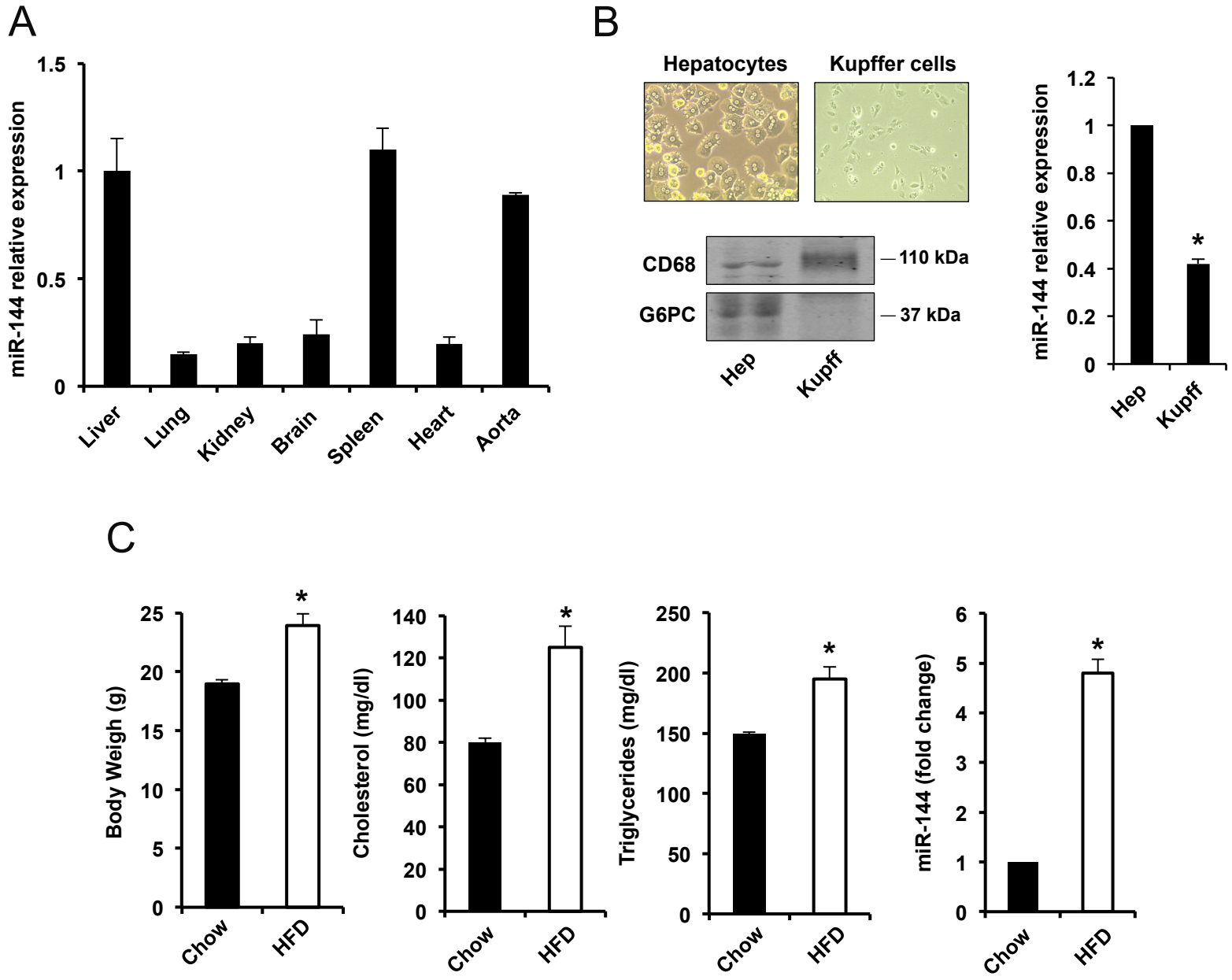
containing or not the point mutations (PM) in the miR-144 target sites. Data are expressed as mean % of 3'UTR activity of Con-miR ±SEM and are representative of ≥3 experiments. *Significantly different from cells cotransfected with Con-miR and wild-type (WT) 3'UTR. $P \leq 0.05$.

Online Figure VI. **miR-33 and miR-144 inhibit ABCA1 expression in Huh-7 cells independently of the endogenous levels of each miRNA.** (**A**) qRT-PCR analysis of miR-33 (left panel) and miR-144 (right panel) expression levels in Huh-7 cells transfected with miR-33 inhibitor (Inh-miR-33) or miR-144 inhibitor (Inh-miR-144 panel) compared with cells transfected with control inhibitor (Con-inh). Data are the mean ± SEM of 2 independent experiments in duplicate. (**B**) Western blot analysis of ABCA1 expression in Huh-7 cells transfected with miR-144, miR-33, Inh-miR-33 and Inh-miR-144 and treated or not with T0901317 (T090). Data correspond to a representative blot among two that gave similar results. HSP-90 was used as a loading control. Values of the band densitometry analysis are shown.

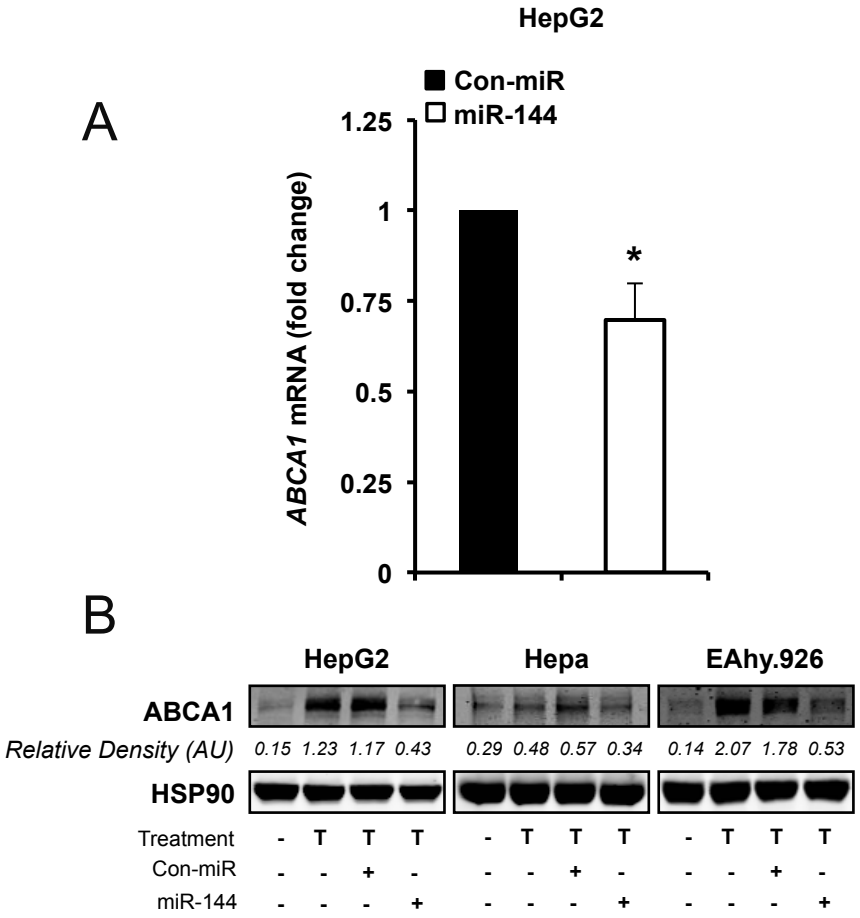
Online Figure I



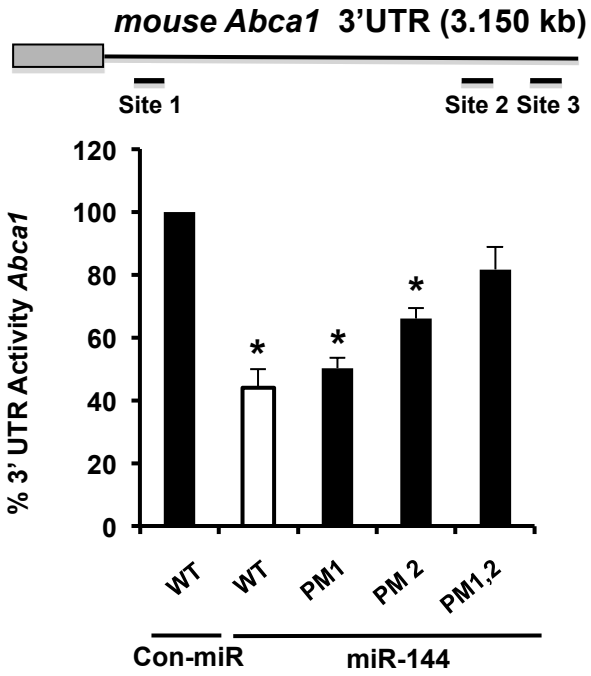

A
THP-1
Huh-7
miR-144 (fold change)
Ctrl
GW3965
B
MØ
Ac-LDL
C
0.1μM
0.3μM
3μM
D
E
miR-451 (fold change)
T090

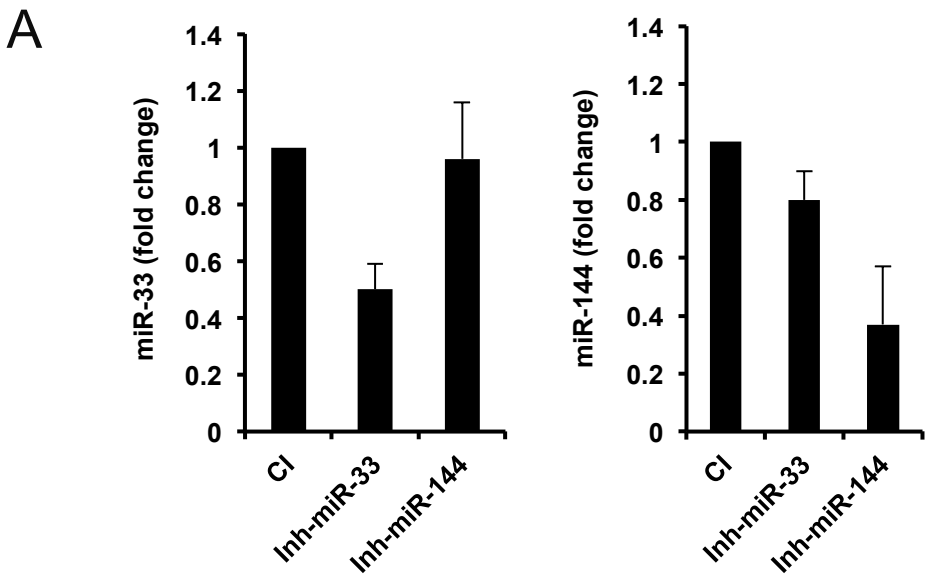

A
miR-144 relative expression
1.5
1
0.5
0
Liver
Lung
Kidney
Brain
Spleen
Heart
Aorta
B
Hepatocytes
Kupffer cells
CD68
G6PC
110 kDa
37 kDa
Hep
Kupff
miR-144 relative expression
1.2
1
0.8
0.6
0.4
0.2
0
*
Hep
Kupff
C
Body Weigh (g)
25
20
15
10
5
0
*
Chow
HFD
Cholesterol (mg/dl)
140
120
100
80
60
40
20
0
*
Chow
HFD
Triglycerides (mg/dl)
250
200
150
100
50
0
*
Chow
HFD
miR-144 (fold change)
6
5
4
3
2
1
0
*
Chow
HFD

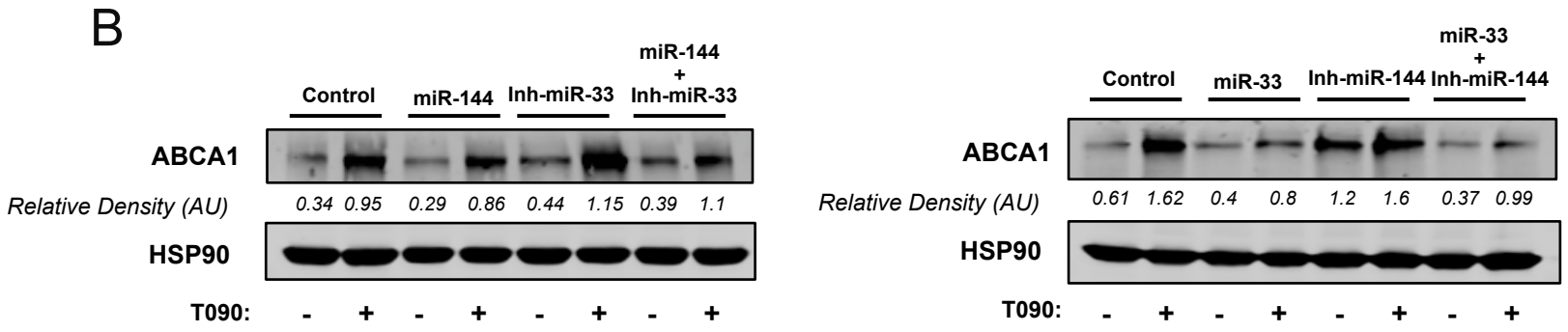
Online Figure IV



Online Figure V




A
miR-33 (fold change)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Cl
Inh-miR-33
Inh-miR-144
miR-144 (fold change)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Cl
Inh-miR-33
Inh-miR-144


B
Control
miR-144
Inh-miR-33
miR-144 + Inh-miR-33
ABCA1
Relative Density (AU)
0.34 0.95 0.29 0.86 0.44 1.15 0.39 1.1
HSP90
T090: - + - + - + - +
Control
miR-33
Inh-miR-144
miR-33 + Inh-miR-144
ABCA1
Relative Density (AU)
0.61 1.62 0.4 0.8 1.2 1.6 0.37 0.99
HSP90
T090: - + - + - + - +