

MicroRNA-33 encoded by an intron of *Srebp2* regulates HDL *in vivo*

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Nonstandard abbreviations used: ABCA1, ATP-binding cassette transporter A1; SREBP-2, sterol regulatory element-binding protein 2; miR, microRNA; apoA, apolipoprotein A

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Abstract

Sterol regulatory element-binding protein 2 (SREBP-2) transcription factor has been identified as a key protein in cholesterol metabolism through the transactivation of the LDL receptor and cholesterol biosynthesis genes. Here, we generated mice lacking microRNA (miR) -33, encoded by an intron of the *Srebp2* gene, and showed that miR-33 repressed the expression of ATP-binding cassette transporter A1 (ABCA1) protein, a key regulator of HDL synthesis by mediating cholesterol efflux from cells to apolipoprotein A (apoA)-I. In fact, peritoneal macrophages derived from miR-33-deficient mice showed a marked increase in ABCA1 levels and higher apoA-I-dependent cholesterol efflux than those from wild-type mice. ABCA1 protein levels in liver were also higher in miR-33-deficient mice than in wild-type mice. Moreover, miR-33-deficient mice had significantly higher serum HDL cholesterol levels than wild-type mice. These data establish a critical role for miR-33 in the regulation of ABCA1 expression and HDL biogenesis *in vivo*.

Key Words: ABCA1, SREBP-2, HDL, macrophage, microRNA

¥body

Introduction

ATP-binding cassette transporter A1 (ABCA1), a 254-kDa cytoplasmic membrane protein, is a pivotal regulator of lipid efflux from cells to apolipoproteins (1). ABCA1 mediates the rate-controlling step in HDL particle formation and plays an important role in reverse cholesterol transfer (2, 3). Mutations in the *ABCA1* gene cause Tangier disease, which is characterized by the near absence of plasma HDL cholesterol associated with storage of cholesterol esters in reticuloendothelial tissues (4-7). *Abca1* mRNA and protein are very unstable with a half life of 1-2 h in murine macrophages (8), which indicates that new transcription and translation are major factors in assuring constant and inducible ABCA1 expression.

SREBPs, including SREBP-1a, -1c, and -2, modulate the transcription of a number of genes involved in the synthesis and receptor-mediated uptake of cholesterol and fatty acids (9-11). In sterol-depleted cells, SREBPs are cleaved by proteases in the Golgi, releasing the N-termini, which translocate into the nucleus and bind to SREs in the enhancers of multiple genes encoding enzymes and proteins involved in cholesterol biosynthesis and lipid uptake (11-13). Results to date support the notion that SREBP-1 primarily activates the fatty acid triglyceride and phospholipid pathways, whereas SREBP-2 is the prominent isoform for cholesterol synthesis and uptake (9, 10, 12).

MicroRNAs (miRs) are small, non-protein-coding RNAs that base pair with specific mRNAs and inhibit translation or promote mRNA degradation. Recent reports have indicated that miR-33 controls cholesterol homeostasis based on knockdown experiments using antisense technology (14-16). Antisense inhibition of miRNA function has been an important tool for elucidating miRNA biology. However, in order

to determine the potential developmental function of specific miRNAs and perform longer term studies, it is necessary to generate mice lacking each miRNA. We generated miR-33-deficient mice, which were born at the expected Mendelian ratio, and show here that miR-33, encoded by an intron16 of the *Srebp2* gene, repressed the ABCA1 protein, which resulted in a reduction in HDL concentration.

Results

miR-33 is encoded by intron16 of the human, mouse, cow, and chicken SREBP2 genes and targets ABCA1. miR-33 is encoded by intron16 of the human, mouse, cow, chicken *SREBP2* genes. The sequence of miR-33 is identical and the stem-loop pre-miRNA is highly conserved in mammals (Fig. 1A). We searched for potential target genes of miR-33 in a public database (TargetScan <http://www.targetscan.org/>), and found that three putative miR-33 binding sites existed in the 3'-untranslated region (UTR) of the *ABCA1* mRNA, and that this region was evolutionarily conserved (Fig. 1A). To test whether the putative miR-33 target sequence in the *Abca1* 3'-UTR could mediate translational repression, we inserted the 3'-UTR of the *Abca1* transcript into a luciferase expression plasmid (luc-*Abca1* 3'-UTR), which we transfected into HEK 293T cells. CMV-driven miR-33 resulted in a decrease in luciferase activity compared with miR-146a or control vector (miR-control). Mutation in the potential binding site in the 3'-UTR abolished the effect of miR-33 (Fig. 1B). Next, we transduced miR-33 into a monocytic cell line, THP-1, and a human primary hepatocyte cell line, HuS-E/2 (17), using lentivirus. The transfection efficiency of the lentivirus was always over 90% (Figure S1A and B). Overexpression of miR-33 resulted in a decrease in ABCA1 protein expression compared with the control vector in both cell lines (Fig. 1C). Because ABCA1 mediates cholesterol efflux from macrophages to lipid-free apoA-I (3), we further examined cholesterol efflux from THP-1-derived macrophages, which were differentiated from THP-1 by stimulation with phorbol 12-myristate 13 acetate (PMA) (100 nM) for 3 days. As shown in Fig. 1D, apoA-I-mediated cholesterol efflux was significantly reduced in miR-33 transduced macrophages compared with miR-control transduced cells.

miR-33 is expressed with Srebp2. We next confirmed that intronic miR-33, expressed synchronously with *Srebp2*, is spliced to target ABCA1. We amplified genomic DNA and cDNA fragments of the mouse *Srebp2* gene that cover the 5' end of exon 16 and 3' end of exon 17, and cloned them into a pcDNA3.1 vector, to make mini-genes that contain exon 16-intron 16-exon 17 and exon 16-exon 17 (Fig. 2A). As shown by RT-PCR, intron 16 was spliced out of the transcript of exon 16-intron 16-exon 17 in HEK 293T cells (Fig. 2B). This *Srebp2* exon 16-intron 16-exon 17 mini-gene significantly suppressed luciferase activity in 293T cells transduced with a luc-*Abca1* 3'-UTR construct compared with the *Srebp2* exon 16-exon 17 mini-gene and pcDNA3.1 empty vector (Fig. 2C). Transfection of the *Srebp2* exon 16-intron 16-exon 17 mini-gene also resulted in a decrease in ABCA1 protein levels compared with the *Srebp2* exon 16-exon 17 mini-gene and pcDNA3.1 empty vector in THP-1 cells (Fig. 2D). These experiments suggested that intronic miR-33 is typically and coordinately expressed with its host gene, as reported for other intronic miRNAs (18, 19)

Sterol depletion activated SREBP2 and increased miR-33 expression. To study the relevance of the miR-33/SREBP/ABCA1 cholesterol regulatory circuit, we cultured THP-1 cells under sterol-depleted conditions by the removal of serum from the culture media (SFM) or in the presence of statin. Under these conditions, THP-1 cells had normal morphology and no cell death was detected. As shown in Fig. 3A, SFM decreased ABCA1 protein levels in THP-1 cells in a time-dependent manner.

LDL-receptor and SREBP2 mRNA expression levels increased in a time-dependent manner after serum depletion (Fig. 3B and C). Expression of miR-33 paralleled *SREBP2* expression (Fig. 3D). The same experiment was conducted in cells treated with simvastatin. Simvastatin reduced the expression of ABCA1 in a dose- and

time-dependent manner (Fig. S2A and B). The expression levels of *LDL-receptor*, *SREBP2*, and miR-33 increased in time-dependent manners as in SFM (Fig. S2C-E). We further suppressed endogenous miR-33 in THP-1-derived macrophages by the transduction of a ‘decoy’ gene, which contained 9-tandem repeats of antisense sequences against miR-33 downstream of the luciferase gene (Fig. S3A). Overexpression of miR-33 along with this decoy gene significantly suppressed luciferase activity (Fig. S3B). The suppression of ABCA1 protein levels in response to sterol depletion was reversed by the miR-33 decoy gene (Fig. S3C), which was consistent with the notion that miR-33 mediates cholesterol-regulated post-transcriptional control of ABCA1 levels.

Generation of miR-33-deficient mice. We deleted the region that encodes the complete pre-miRNA sequence of miR-33 by introducing loxP sites for Cre-mediated recombination into intron 16 of the mouse *Srebp2* gene (Fig. 4A, B, and C). Because disruption of the *Srebp2* gene causes embryonic lethality (20), it was important that the miR-33 targeting strategy did not alter *Srebp2* transcription or splicing. Deletion of miR-33 did not alter the expression of SREBP-2 protein (Fig. 4D) or interfere with *Srebp2* mRNA splicing and expression (Fig. 4E and Fig. S4) in miR-33-deficient mice. *Srebp2* mRNA splicing and expression were not altered even at later time points (Fig. S5). Relative expression levels of miR-33 and *Srebp2* are shown in Fig. S6A. miR-33 appears to be coexpressed with the *Srebp2* host gene as seen in THP-1 cells (Fig. 3). Loss of miR-33 expression in these mice was confirmed in the liver (Fig. 4F) and in other organs (Fig. S6B and C) by real-time PCR. Mice homozygous for the miR-33 deletion were born at the expected Mendelian ratio, were viable, fertile, and did not display obvious abnormalities in size, shape, or structure up to 16 weeks of age.

miR-33 deficiency enhances cholesterol efflux in macrophages. To investigate the role of miR-33 in mice, we first compared the function of peritoneal macrophages in wild-type and miR-33-deficient mice. ABCA1 protein expression levels were considerably higher in macrophages of miR-33-deficient mice than those of wild-type mice (Fig. 5A). We measured apoA-I-mediated cholesterol efflux from peritoneal macrophages and found that macrophages in miR-33-deficient mice had higher apoA-I-mediated cholesterol efflux than wild-type mice (Fig. 5B).

miR-33 deletion enhances HDL levels. Overexpression of hepatic ABCA1 raises HDL cholesterol levels (21), and liver-specific deletion of ABCA1 results in a substantial (~80%) decrease in plasma HDL cholesterol in chow-fed mice (22). Therefore, we measured ABCA1 protein expression levels in the liver. Fig. 5C and D indicate that ABCA1 protein levels were higher in miR-33-deficient mice liver than in wild-type mice liver of both genders. High-performance liquid chromatography (HPLC) with gel permeation columns was used for classifying and quantifying lipoproteins on the basis of differences of particle size (23, 24). Figure 5E and F show representative results of the HPLC elution profile of serum from wild-type (black) and miR-33-deficient mice (red) at the age of 16 weeks. HDL from miR-33-deficient mice showed a broader peak with a slight shift to the left consistent with cholesterol enrichment. Serum lipid profiles of mice at the age of 16 weeks are summarized in Table 1. miR-33-deficient mice had significantly higher total cholesterol and HDL cholesterol levels than wild-type mice, whereas triglyceride levels were unchanged. Elevation of HDL was prominent in female mice. In fact, the expression level of miR-33 showed an approximately 1.5 fold increase in the liver of wild-type female mice compared with wild-type male mice (Fig. S6D). Moreover, the increased levels of HDL were mainly composed of very large, large, and

medium HDL (mature HDL). Because *Abcg1*, which is required for cholesterol efflux to HDL and contributes to form mature HDL, is a potential target of miR-33 in rodents (not human) (14, 16) (Fig. S7A), we also inserted the 3'-UTR of the *Abcg1* transcript into a luciferase expression plasmid (luc-*Abcg1* 3'-UTR). CMV-driven miR-33 resulted in a decrease in luciferase activity compared with miR-146a or control vector (miR-control) in HEK 293T cells. Mutation in the potential binding site in the 3'-UTR abolished the effect of miR-33 (Fig.S7B). We further measured the protein levels of ABCG1 in liver. However, the levels were the same in wild-type and miR-33-deficient mice of both genders (Fig. S7C and D).

Discussion

Recent studies have shown that miR-33 helps to regulate the homeostasis of HDL cholesterol, suggesting that it might be a possible target for the treatment of cardiovascular and metabolic disorders (14-16), which reported similar or complementary findings. Expression of miR-33 was found in various cells and tissues, including macrophages, hepatic cells, endothelial cells, brain, liver, colon, small intestine and skeletal muscle. The predominant target identified for miR-33 was the gene encoding *ABCA1*. It was suggested that miR-33 antisense approaches resulted in augmented HDL-cholesterol levels in mice. One report indicated that three injections of locked nucleic acid (LNA) antisense over 5 days elevated plasma HDL-cholesterol levels by about 35% with only modest effects of miR-33a LNA-antimiR on hepatic *ABCA1* mRNA/protein levels as compared with control mice fed on a high fat diet (15). Another report showed that overexpression of antisense miR-33 using lenti-virus showed a 50% increase in hepatic *ABCA1* protein levels and a concomitant 25% increase in plasma HDL levels after 6 days (14). Marquart *et al.* indicated that injection of an anti-miR-33 oligonucleotide resulted in a substantial increase in *ABCA1* expression and HDL levels (16).

Antisense inhibition of miRNA function has been an important tool for elucidating miRNA biology. However, in order to determine the potential developmental functions and perform longer term studies, it is necessary to completely ablate the miRNA under investigation. We generated mice lacking miR-33, encoded by an intron of the *Srebp2* gene. The major findings obtained by the analysis of miR-33-deficient mice were that 1) mice homozygous for the miR-33-deletion were born at the expected Mendelian ratio, were viable, fertile, and did not display obvious

abnormalities in size, shape, or structure up to 16 weeks of age; 2) Complete loss of miR-33 enhanced liver ABCA1 protein levels remarkably, and serum HDL levels were elevated by about 22% in male and 39% in female mice, probably because the expression level of miR-33 was 1.5-fold higher in the liver of female mice compared with male mice; 3) the increased levels of HDL were mainly composed of very large, large, and medium HDL (mature HDL), which was consistent with the results obtained in hABCA1 transgenic mice (25); and 4) although the *in vitro* experiment suggested that *Abcg1* is also a potential target of miR-33, the depletion of miR-33 did not alter the expression levels of ABCG1 protein in liver. Rayner *et al.* could also not see the difference in hepatic ABCG1 protein expression levels between mice 6 days after injection with control and anti-miR-33 lentivirus (14). Thus, our results unambiguously indicated that miR-33 regulates plasma HDL levels through the repression of ABCA1 *in vivo*.

Gene regulation via miRNAs is a strongly conserved mechanism found in nearly all multicellular organisms, including animals and plants (26). Mammalian genomes encode more than 500 known miRNA genes. Approximately 50% are expressed from non-protein coding transcripts, whereas the rest are located mostly in the introns of genes (27). Intronic miRNAs are generally transcribed coincidentally with their host genes (28). In the present study we showed the presence of miR-33 within the intronic sequence in the *Srebp2* gene and examined whether miR-33 is expressed with its host gene. *In vitro* studies demonstrated that *Srebp2* intron 16 suppressed ABCA1 protein expression in the same way as miR-33. The co-regulation of a miRNA with its host gene typically exhibits one of two main functions; 1) an antagonistic effect by miRNA mediated knock-down of genes with perturbing effects on a pathway or a biological

process activated by the host gene, or 2) a synergistic effect by miRNA-mediated fine tuning of a target gene generating a positive effect on the host gene. In this case, miR-33 has a synergistic effect on ABCA1 via its host gene *Srebp2* because SREBP-2 is known to suppress the transcription of *Abca1* in vascular endothelial cells (29). SREBPs are activated in sterol-depleted conditions and serve as transcription factors for lipid/cholesterol synthesis, uptake, storage, and efflux (11-13, 29). In peripheral cells intracellular cholesterol homeostasis is precisely regulated and depends on the balance between cholesterol synthesis, degradation, cholesterol ester formation, influx, and efflux (30, 31). Because SREBPs are activated by HMG-CoA reductase inhibitor accompanied by an increased expression of miR-33, ABCA1 expression and cholesterol efflux are presumably suppressed by miR-33 in these conditions.

In humans, *SREBP1* and *SREBP2* encode miR-33b and miR-33a, respectively(15). It is well known that hypertriglycemia in metabolic syndrome is caused by the insulin-induced increase in *SREBP1c* mRNA and protein levels(32, 33). Low HDL often accompanies this situation and it is possible that the reduction in HDL is caused by a decrease in ABCA1, because of the increased production of miR-33b from the insulin-induced induction of *SREBP1c*. Although it is impossible to prove this in animal models that lack miR-33b, antagonizing miR-33 could be a promising way to raise HDL levels when the transcription of both *SREBPs* is upregulated. Thus, our study suggests that a combination of silencing of endogenous miR-33 and statins may be a useful therapeutic strategy for raising HDL and lowering LDL levels especially for metabolic syndrome subjects.

Materials and Methods

Cells and plasmids

Immortalized human primary hepatocyte HuS-E/2 cells were described previously (17). Other cells and plasmids used in this experiment are summarized in Supplemental Materials and Methods.

Generation of miR-33 deficient mice

A targeting vector was constructed by modifying bacterial artificial chromosome RP24-291F2 (Invitrogen) using defective prophage λ -Red recombination system (34, 35). As a selection marker, a neomycin resistance cassette flanked by loxP sites (loxP-PGK-gb2-neo-loxP cassette; Gene Bridges, Germany) was inserted at the pre-miR-33 site. The targeting vector was electroporated into C57BL/6 mouse ES cells (DS Pharma Biomedical) using a Nucleofector system (Lonza). Positive clones were selected by incubating cells with 200 mM geneticin (Invitrogen) for 5 days, and homologous recombination was confirmed by Southern blotting. Successfully recombined ES cells were injected into blastocysts from ICR strain mice supplied by Unitech Inc. (Japan), and chimeric mice were bred with C57BL/6 mice to generate F1 mice. The genotype of F1 mice was confirmed by Southern blotting. The neomycin resistance cassette was removed in the mouse germ line by breeding heterozygous mice with *Ayu-1 Cre* knockin mice, which express Cre recombinase in multiple tissues, including the germ line (36). Descendant miR-33 heterozygous mice without the *Ayu-1 Cre* allele were crossed with each other to generate miR-33-deficient mice. All experiments were carried out in C57BL/6 background mice and wild-type littermates were used as a control. Primer sequences for the probe (865 bp) for Southern blotting

and genotyping (WT: 385 bp, KO: 491 bp) are indicated in the Supplemental Materials and Methods.

Lentivirus production and DNA transduction

We produced lentiviral stocks in 293FT cells in accordance with the manufacturer's protocol (Invitrogen). Cells were used for analyses two days after transduction.

Luciferase assay

To create wild-type or mutant 3'UTR luciferase reporter constructs, a fragment of the 3'UTR of the *Abca1* and *Abcg1* genes (Supplemental Materials and Methods) was subcloned downstream of a CMV-driven firefly luciferase cassette in a pMIR-REPORT vector (Ambion). Luciferase activities were measured as described previously (37).

Cellular cholesterol efflux from macrophages

Cellular cholesterol efflux via apoA-I was determined as described previously (38).

Serum lipid profiling

Lipoproteins were analyzed by high-performance liquid chromatography at Skylight Biotech (Akita, Japan), in accordance with the procedure described by Usui *et al.* (24).

Statistics

Data are presented as means $\pm$ S.E. Statistical comparisons were performed using unpaired two-tailed Student's *t*-tests or one-way analysis of variance with Bonferonni

post hoc test where appropriate, with a probability value of <0.05 taken to indicate significance.

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Figure legends

Fig. 1. ABCA1 is a target of miR-33.

- A. Schema and sequence alignment of *SREBP2*, miR-33, and *ABCA1* 3'UTR. miR-33 is located in intron16 of *SREBP2*. There are 3 potential conserved miR-33 binding sites in the *ABCA1* 3'UTR.
- B. 293T cells were transfected with wild-type or mutant *Abca1* 3'UTR luciferase constructs, along with expression plasmids for miR-control (negative control), miR-33, and miR-146a. Values are the means $\pm$ S.E. of 4 independent experiments. * $p < 0.01$ compared with other columns.
- C. Western analysis of ABCA1 in THP-1-derived macrophages and HuS-E/2 (human primary hepatocytes) transfected with miR-control and miR-33, using a lentivirus vector.
- D. Cholesterol efflux was measured in the presence or absence of apoA-I (15 μ g/ml) for 24 hours in THP-1-derived macrophages transfected with miR-control and miR-33. Values are the means $\pm$ S.E. of 3 independent experiments. * $p < 0.01$.

Fig. 2. Analysis using the *Srebp2* mini-gene.

- A. Schema of the *Srebp2* mini-gene used in this report. Arrows indicates RT-primers.
- B. RT-PCR analysis of 293T cells transfected with the *Srebp2* mini-gene. Note that there is no band around 2000 bp, indicating that intron 16 was correctly spliced.
- C. 293T cells were transfected with the wild-type *Abca1* 3'UTR luciferase construct, along with the expression plasmids. Values are the means $\pm$ S.E. of 4 independent experiments. * $p < 0.05$ compared with empty vector (pcDNA3.1) or the *Srebp2* mini-gene (exon 16-exon 17).
- D. Western analysis of ABCA1 in THP-1 macrophages transfected with the *Srebp2* mini-gene using lentivirus vectors. GAPDH was used as a loading control.

Fig. 3. The effect of serum starvation in THP-1 macrophages.

- A. Western analysis of ABCA1 protein levels under serum starvation conditions for the indicated time periods in THP-1 macrophages. GAPDH was used as a loading control.
- B. Quantitative real-time PCR analysis of *LDL receptor* expression levels under serum starvation conditions for the indicated time periods in THP-1 macrophages. Values are the means $\pm$ S.E. of 6 independent experiments with normalization using *GAPDH* expression. * $p < 0.05$ compared with 10% FBS.
- C. Quantitative real-time PCR analysis of *SREBP2* expression levels under serum starvation conditions for the indicated time periods in THP-1 macrophages. Values are the means $\pm$ S.E. of 6 independent experiments with normalization using *GAPDH* expression. * $p < 0.05$ compared with 10% FBS.
- D. Quantitative real-time PCR analysis of miR-33 under serum starvation conditions for the indicated time periods in THP-1 macrophages. Values are the means $\pm$ S.E. of 6 independent experiments with normalization using U6 snRNA. * $p < 0.05$ compared with 10% FBS.

Fig. 4. Generation of miR-33-deficient mice.

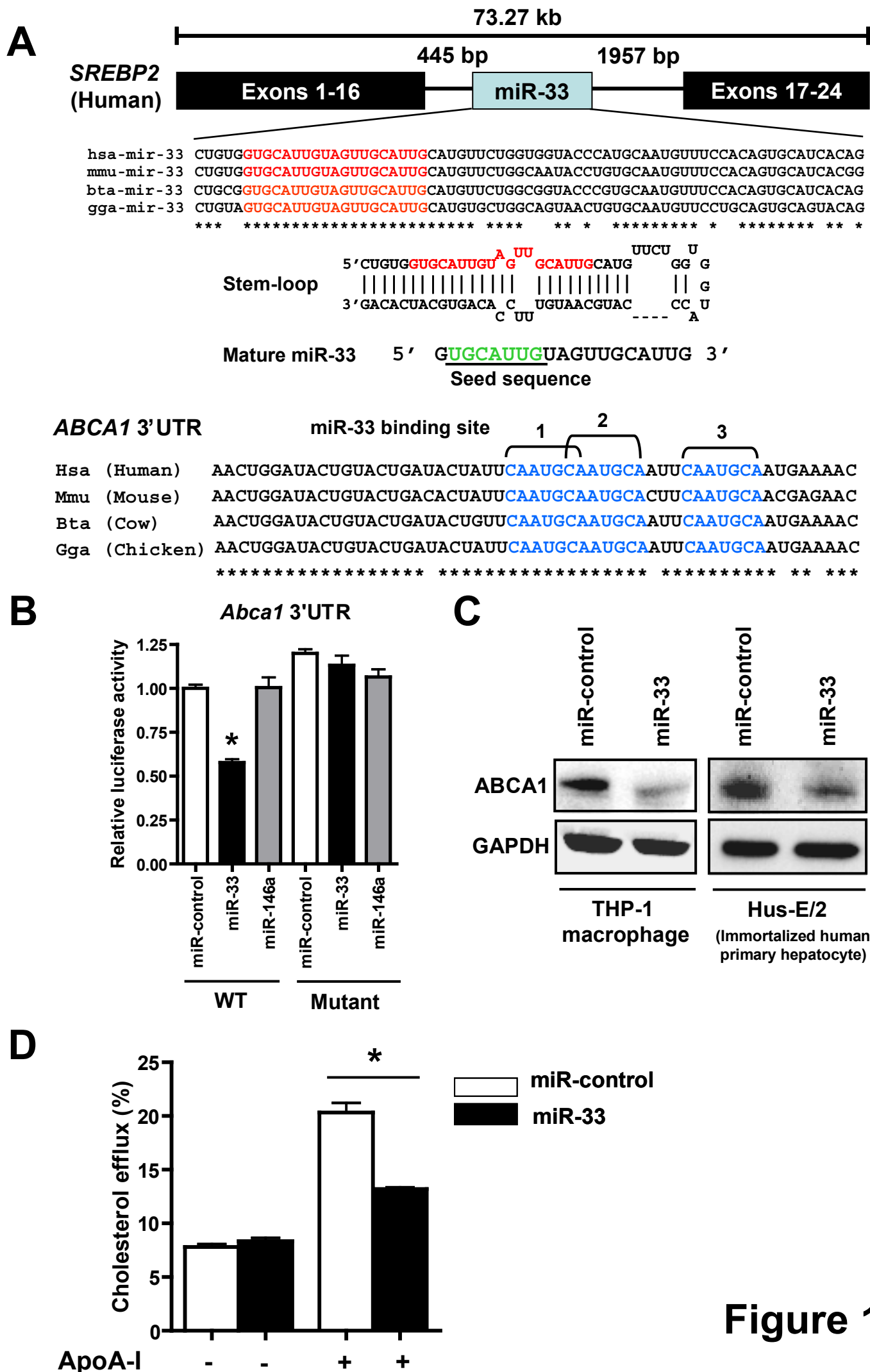
- A. Strategy to generate miR-33-deficient mice by homologous recombination. The

pre-miR-33 sequence was replaced with a neomycin resistance cassette (pgk-gb2-Neo) flanked by loxP sites. The neomycin resistance cassette was removed in the mouse germ line by breeding heterozygous mice with *Ayu-1 Cre* mice, which express Cre recombinase in multiple tissues, including the germ line.

- B. Southern blotting of mice tail genome.
- C. PCR analysis of mice tail genome.
- D. Western analysis of SREBP-2 in the liver of 8-week-old male mice (Precursor: 126 kDa, Mature: 55 kDa). GAPDH was used as a loading control.
- E. RT-PCR analysis of *Srebp2* in the liver of 8-week-old male mice. The sense primer was designed in exon 16 of *Srebp2* and the antisense primer was designed in exon 17 of *Srebp2*. *Gapdh* was used as a control.
- F. Quantitative real-time PCR analysis of miR-33 in the liver of 8-week-old male mice, using a Taqman microRNA assay (N.D.: not determined).

Fig. 5. ABCA1 expression of peritoneal macrophages and the liver in wild-type and miR-33-deficient mice.

- A. Western analysis of ABCA1 in peritoneal macrophages. Thioglycolate-elicited peritoneal macrophages were isolated from 8-week-old wild-type and miR-33-deficient mice. GAPDH was used as a loading control.
- B. Cholesterol efflux from peritoneal macrophages was measured in the presence or absence of apoA-I (15 μ g/ml) for 6 hours. Values are the means $\pm$ S.E. of 6 independent experiments. * $p < 0.01$.
- C. Western analysis of hepatic ABCA1 and SREBP-2 in 16-week-old male mice. GAPDH was used as a loading control.
- D. Western analysis of hepatic ABCA1 and SREBP-2 in 16-week-old female mice. GAPDH was used as a loading control.
- E. Representative HPLC analysis of serum cholesterol from male wild-type and miR-33-deficient mice.
- F. Representative HPLC analysis of serum cholesterol from female wild-type and miR-33-deficient mice.



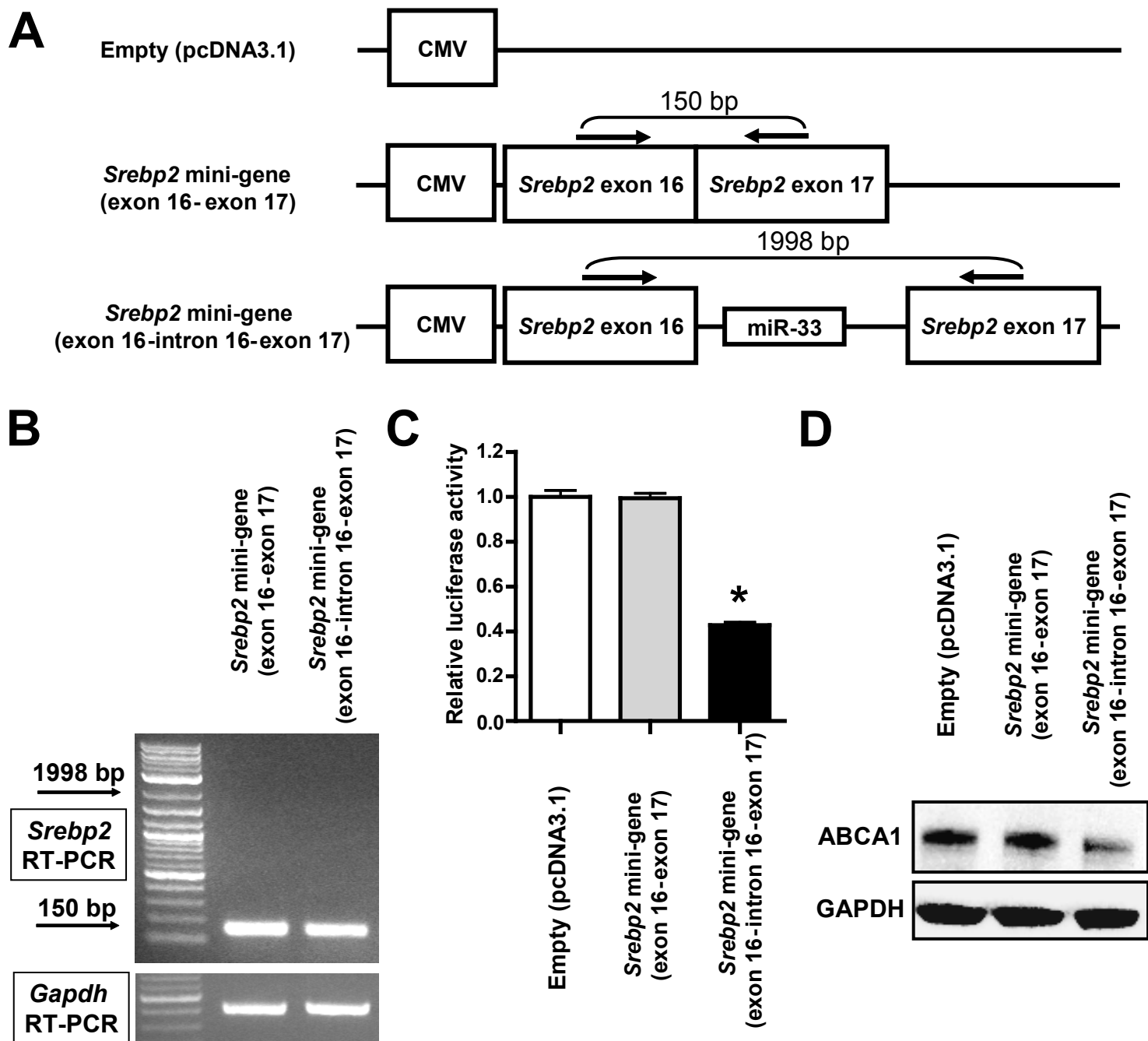
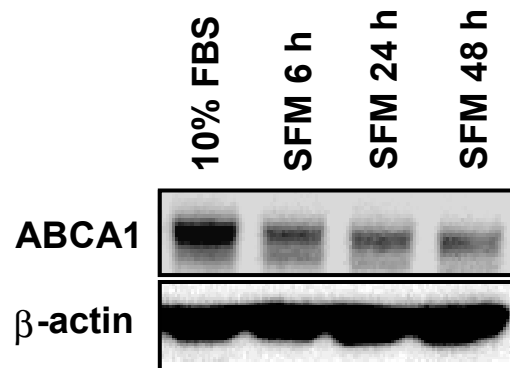
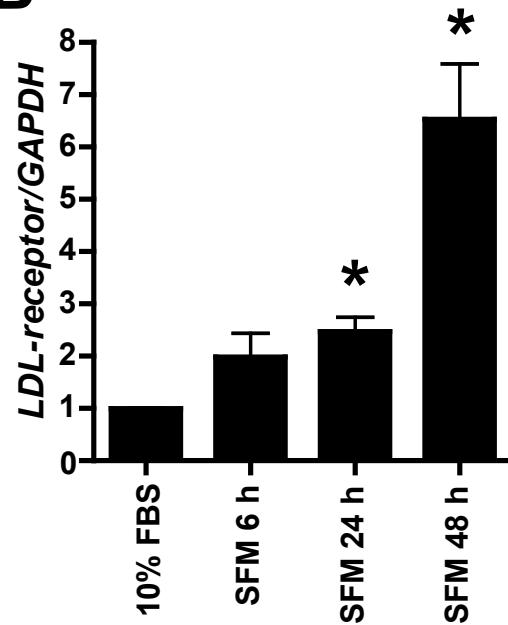
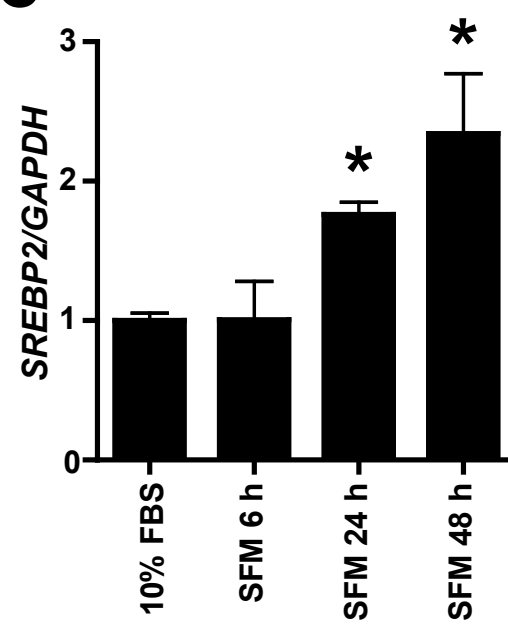
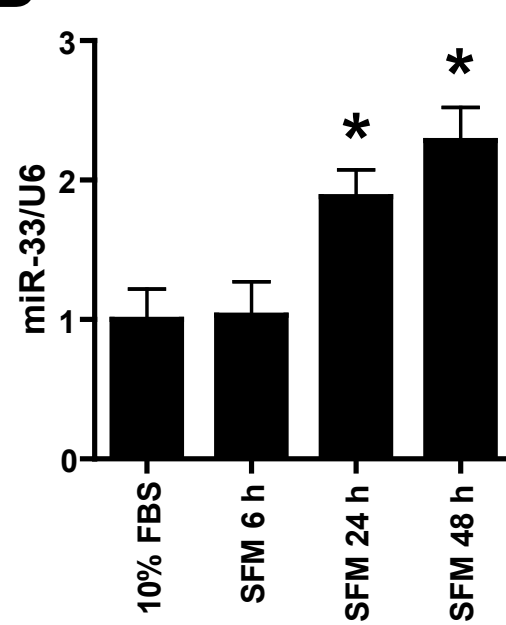


Figure 2

A**B****C****D****Figure 3**

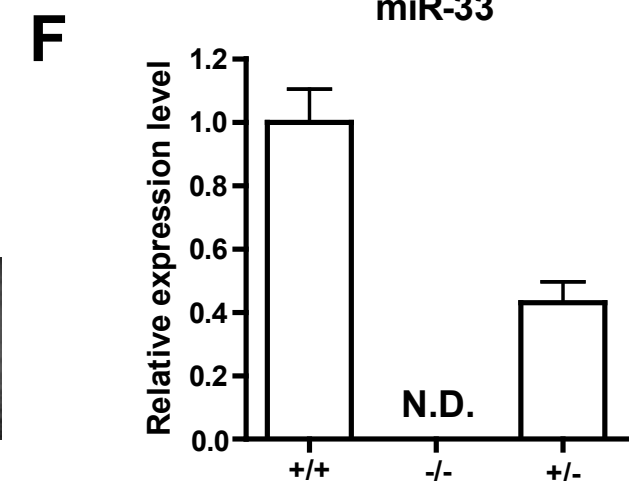
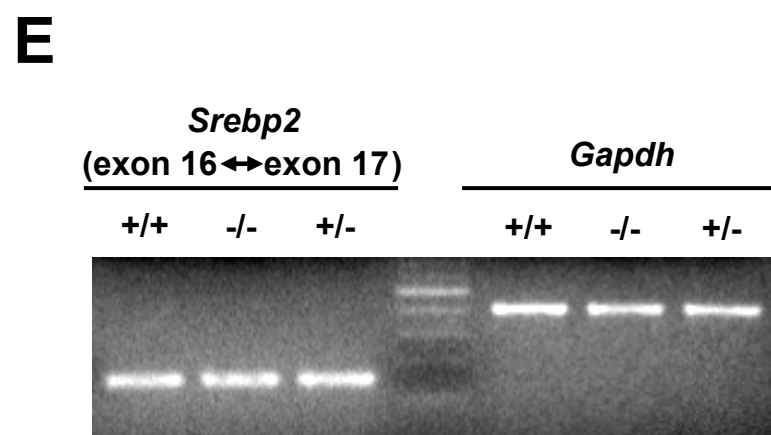
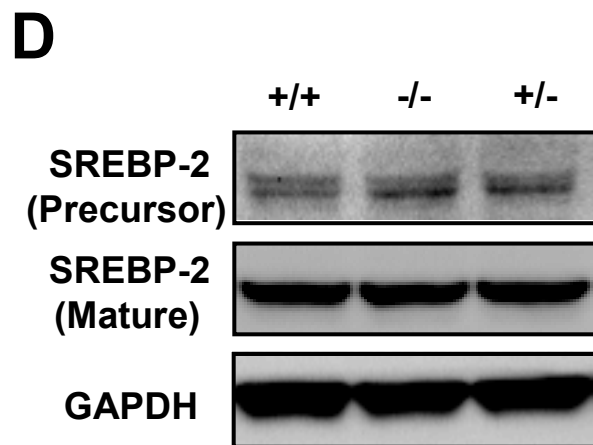
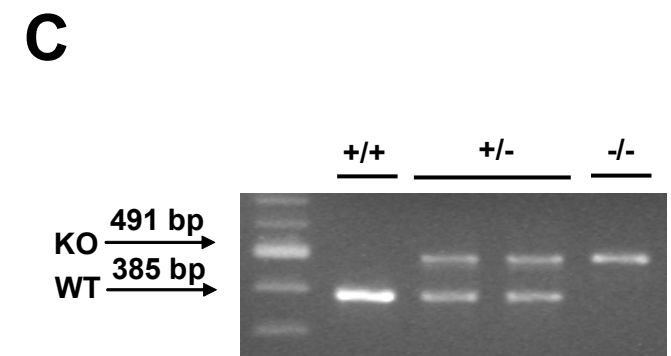
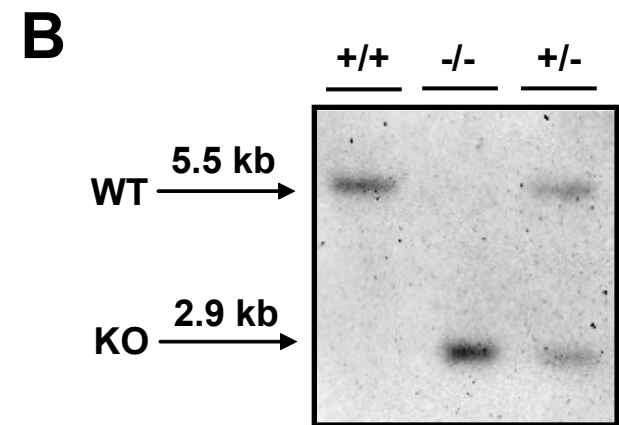
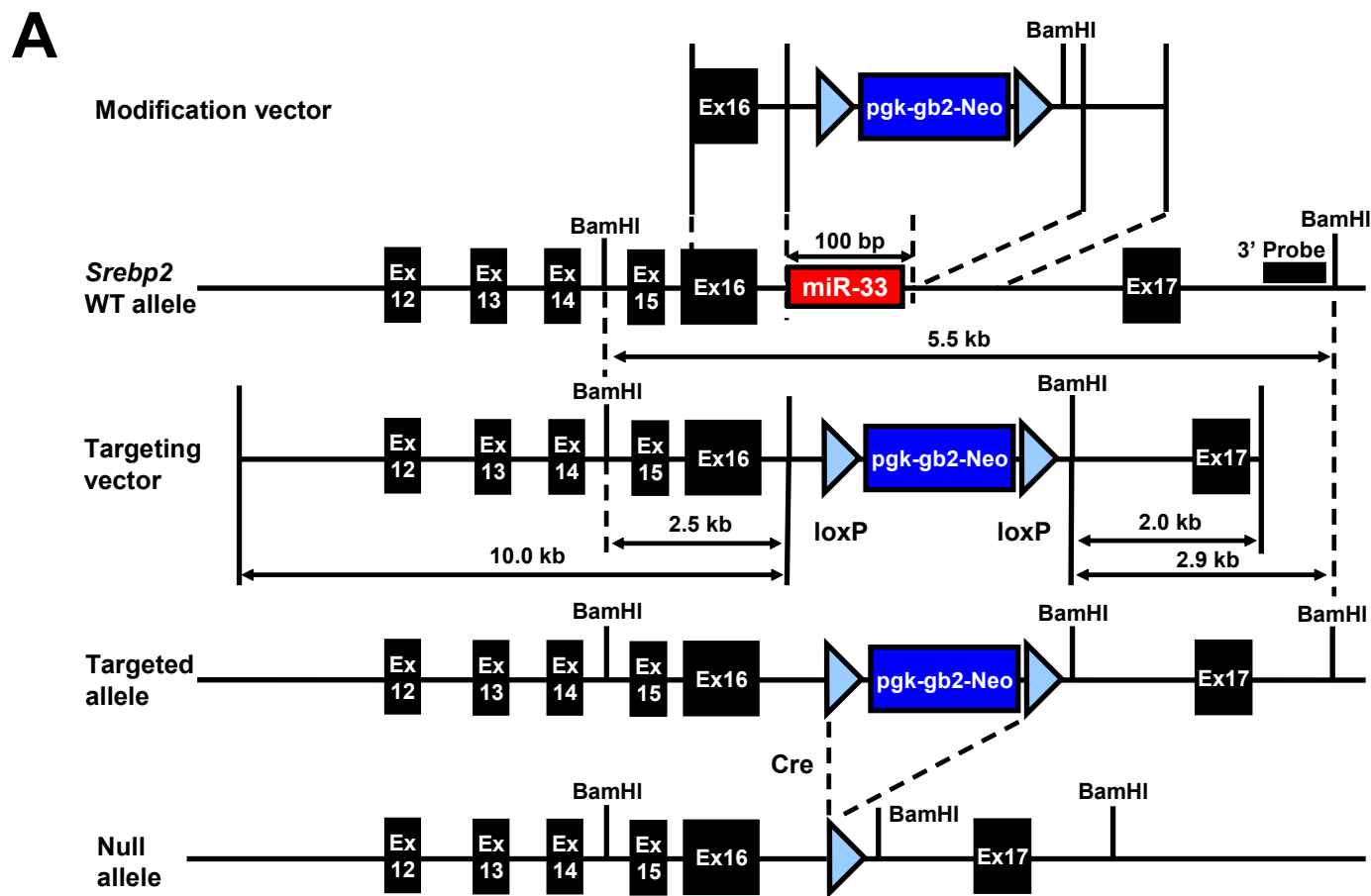


Figure 4

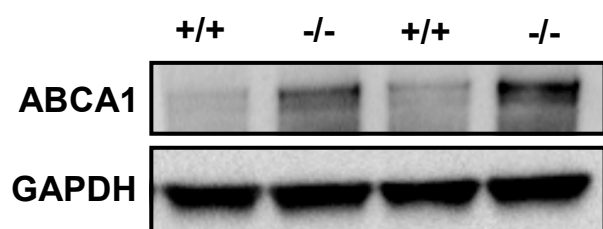
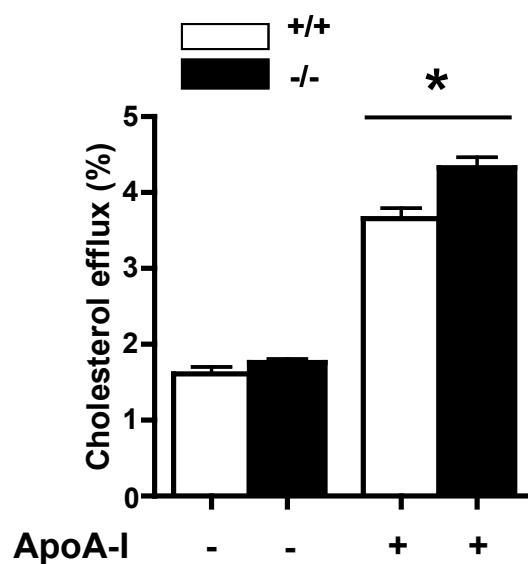
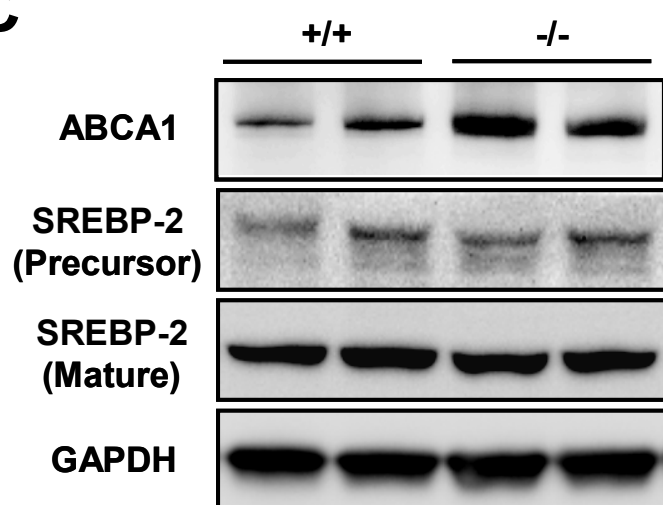
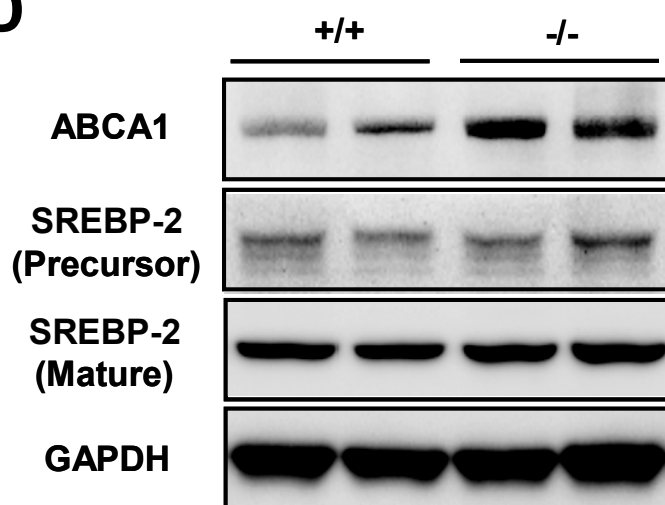
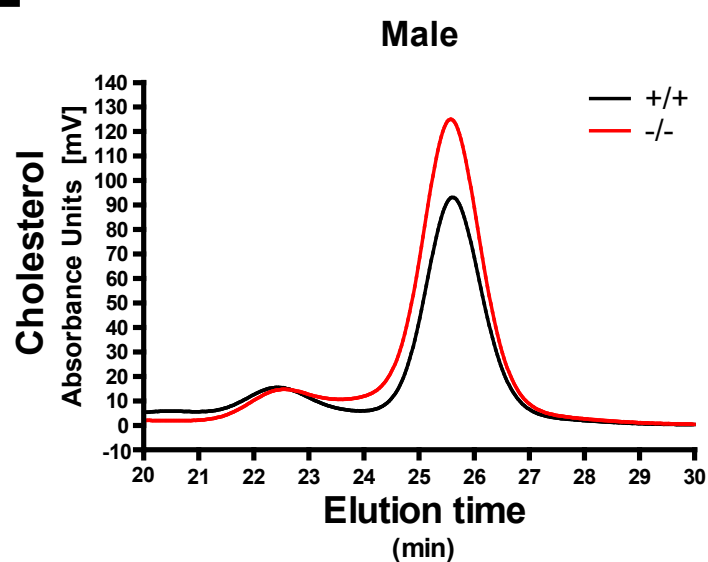
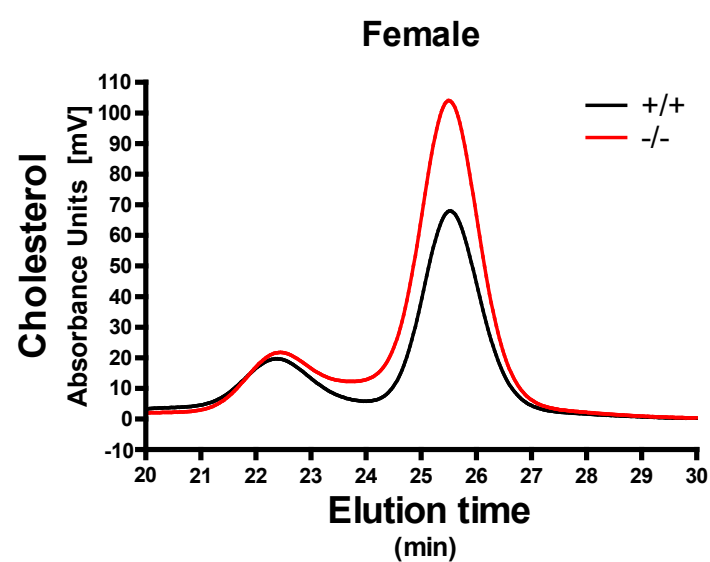
A**B****C****D****E****F****Figure 5**

Table 1

Serum lipid profiling of wild-type and miR-33-deficient mice

Lipoprotein		Males		Females	
Major (Fraction No.) diameter	Sub-class (Fraction No.)	(+/+) n=6	(-/-) n=5	(+/+) n=6	(-/-) n=5
TC (mg/dl)		73.70 ± 3.39	85.90 ± 4.11*	58.56 ± 3.37	78.19 ± 4.41**
CM (1-2) >80 nm		0.13 ± 0.03	0.21 ± 0.07	0.09 ± 0.02	0.12 ± 0.03
VLDL (3-7) 30-80 nm		4.89 ± 0.75	4.04 ± 0.75	3.82 ± 0.37	3.49 ± 0.24
LDL (8-13) 16-30 nm		13.25 ± 0.48	13.31 ± 0.90	13.78 ± 0.53	17.89 ± 1.57*
	Large LDL (8)	2.16 ± 0.13	2.31 ± 0.29	2.76 ± 0.23	3.10 ± 0.32
	Medium LDL (9)	4.32 ± 0.15	4.05 ± 0.22	4.90 ± 0.23	5.95 ± 0.68
	Small LDL (10)	3.27 ± 0.22	2.86 ± 0.16	3.12 ± 0.12	4.02 ± 0.40*
	Very small LDL (11-13)	3.51 ± 0.20	4.09 ± 0.51	2.99 ± 0.31	4.82 ± 0.46*
HDL (14-20) 8-16 nm		55.42 ± 2.82	67.55 ± 4.09*	40.88 ± 3.22	56.69 ± 3.82*
	Very large HDL (14-15)	3.85 ± 0.24	5.77 ± 0.64*	3.60 ± 0.66	5.97 ± 0.71*
	Large HDL (16)	16.50 ± 0.88	22.15 ± 1.35**	14.81 ± 1.79	21.04 ± 1.81*
	Medium HDL (17)	22.01 ± 1.18	25.65 ± 1.39	14.50 ± 0.73	19.57 ± 1.19**
	Small HDL (18)	9.24 ± 0.52	9.61 ± 0.63	5.30 ± 0.05	6.70 ± 0.33**
	Very small HDL (19-20)	4.75 ± 0.19	5.12 ± 0.30	3.24 ± 0.09	3.60 ± 0.42
TG (mg/dl)		45.87 ± 6.20	37.26 ± 4.90	20.70 ± 2.64	20.64 ± 1.67

Values are mean ± S.E. Blood was obtained from chow-fed 16-week-old mice after fasting for 4 hours.

Serum was analysed by HPLC, as described in the Materials and Methods.

TC, total cholesterol; TG, Triglyceride; CM, chylomicrons; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high density lipoprotein.

*p<0.05, **p<0.01 compared with wild-type mice