MicroRNA-33 encoded by an intron of sterol regulatory element-binding protein 2 (Srebp2) regulates HDL in vivo.

AUTHOR(S):

Horie, Takahiro; Ono, Koh; Horiguchi, Masahito; Nishi, Hitoo; Nakamura, Tomoyuki; Nagao, Kazuya; Kinoshita, Minako; ... Yokode, Masayuki; Kimura, Takeshi; Kita, Toru

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Supplemental Materials and Methods

Cell culture and reagents

THP-1 cells were obtained from the American Type Cell Collection (Rockville, MD, USA). THP-1 cells were transformed into macrophages by incubation for 3 days with 100 nM PMA (Nacalai Tesque, Kyoto, Japan). An immortalized primary human hepatocyte (HuS-E/2) cell line was kindly given by Makoto Hijikata (Kyoto University). Peritoneal macrophages were obtained from the peritoneal cavity of wild-type and miR-33-deficient mice 4 days after intra-peritoneal injection of 1mL of 10 % thioglycolate. The cells obtained were washed with RPMI1640 (Nacalai Tesque), spun at 1000 rpm for 10 min, and plated at a density of 106 cells/mL. Cells were washed 1h later and incubated for 2 days, then, used for experiments. The antibodies used were a polyclonal anti-ABCA1 antibody (Novus Biologicals, Littleton, CO, USA), a polyclonal anti-ABCG1 antibody (Novus Biologicals), a polyclonal anti-SREBP-2 antibody (Cayman Chemical, Ann Arbor, MI, USA), an anti-GAPDH antibody (Cell Signaling Technology, Beverly, MA, USA), and an anti-β actin antibody (Sigma-Aldrich Co, St. Louis, MO, USA). Simvastatin was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Acetylated LDL (AcLDL) was purchased from Biomedical Technologies Inc. (Stoughton, MA, USA). ApoA-I was from Sigma-Aldrich Co. [1, 2-^{3}H (N)]-cholesterol was from Perkin Elmer (Boston, MA, USA).

Plasmids

Expression vectors for the negative control (miR-control) and microRNAs were generated using BLOCK-iT^{TM} Pol Ⅱ miR RNAi Expression Vector Kits in accordance with the manufacturer's protocol (Invitrogen). The miR-control vector contains a hairpin structure just as for a regular pre-miRNA, but which is predicted not to target any known vertebrate gene (pcDNA6.2-GW/EmGFP-miR-neg control plasmid). In order to create an anti-miR-33 (decoy) vector, the luciferase 3'UTR was modified to include 3-9 tandem sequences complementary to miR-33, separated by a single nucleotide space. The sequences of all constructs were analyzed using an ABI 3100 genetic analyzer. All of these constructs were correctly inserted into a pLenti6/V5-D-TOPO vector (Invitrogen) driven by a CMV promoter to stably express genes in THP-1 and HuS-E/2 cells.

Southern blotting

Southern blotting was performed using DIG High Prime DNA Labeling and Detection Starter Kit II in accordance with the manufacturer's protocol (Roche).

Primer sequences for the Southern blotting probe and genotyping

Primer sequences for the probe (865 bp) for Southern blotting and genotyping (WT: 385 bp, KO: 491 bp) were as follows.

Southern probe primer sense; AATGCAGTGAGCAGGTGGAGTTTG

Southern probe primer antisense; ACTGCACTTGAGTTCAGACGCTAC

WT/KO sense; GGCACTACTTCTGATCCTTC

WT antisense; CAACTACAATGCACCACAGCTG

KO antisense; TTGGGATCCAGAATTCGTGATTAA

Western blotting

Cell lysates were prepared and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by standard western blotting procedure.

Quantitative PCR for microRNA

Measurement of miR-33 was performed in accordance with the TaqMan MicroRNA Assays (Applied Biosystems) protocol and the products were analyzed using a thermal cycler (ABI Prism®7900HT sequence detection system). Values were normalized using U6 snRNA expression.

Quantitative PCR for mRNA

Total RNA was isolated using TRIzol® reagent (Invitrogen). cDNA was synthesized using Transriptor First Strand cDNA Synthesis Kit (Roche) and PCR was performed with a SYBR Green PCR master mix (Applied Biosystems), normalized with GAPDH. An ABI Prism® 7900HT sequence detection system was used as a thermal cycler. Gene-specific primers were as follows:

ABCA1 sense (human); 5'GTCCTCTTTCCCGCATTATCTGG3'

ABCA1 antisense (human); 5'AGTTCCTGGAAGGTCTTGTTCAC3'

SREBP2 sense (human); 5'AGGAGAACATGGTGCTGA3'

SREBP2 antisense (human); 5'TAAAGGAGAGGCACAGGA3'

LDL-receptor sense (human); 5'CAGATATCATCAACGAAGC3'

LDL-receptor antisense (human); 5'CCTCTCACACCAGTTCACTCC3'

GAPDH sense (human and mouse); 5'TTGCCATCAACGACCCCTTC3'

GAPDH antisense (human and mouse); 5'TTGTCATGGATGACCTTGGC3'

Srebp2 sense (mouse); 5'GTGGAGCAGTCTCAACGTCA3'

Srebp2 antisense (mouse); 5'TGGTAGGTCTCACCCAGGAG3

Oligonucleotide sequences used for the construction of wild-type or mutant Abca1

and Abcg1 3'UTR luciferase reporter constructs

WT *Abca1*:

GAACAAACTGGATACTGTACTGACACTATTCAATGCAATGCACTTCAATGC

AACGAGAACACAATTCCATTAC

Mutant *Abca1*:

GAACAAACTGGATACTGTACTGACACTATTCA***TACGTTACGT***CTTCA***TACGT***

ACGAGAACACAATTCCATTAC

WT *Abcg1*:

CTAGTACACCAGCTGCGCTGGGGCAGCAGGGACTAACGCAACGCAATGCAA

CGCAATGCAGACAGTGCTGGGGTACTTA

Mutant *Abcg1*:

CTAGTACACCAGCTGCGCTGGGGCAGCAGGGACTAACGCAACGCA***TACGT***A

CGCA***TACGT***GACAGTGCTGGGGTACTTA

Supplemental Figure Legends

Fig. S1. Microscopy images of THP-1 and HuS-E/2 cells.

A. miRNAs were transduced into THP-1 cells using lentivirus vectors. The transduction efficiency, which was shown using GFP, was always over 90%. THP-1 cells were induced to differentiate into macrophages by PMA stimulation (100 nM) for 3 days.
B. miRNAs were transduced into HuS-E/2 cells, which are immortalized human primary hepatocytes. The transduction efficiency, which was shown using GFP, was always over 90%.

Fig. S2. The effect of simvastatin in THP-1 macrophages.

A. Western analysis of ABCA1 protein levels following stimulation with simvastatin for 24 h at the indicated concentrations in THP-1 macrophages. GAPDH was used as a loading control.
B. Western analysis of ABCA1 protein levels following stimulation with simvastatin (10 μM) for the indicated time periods in THP-1 macrophages. GAPDH was used as a loading control.
C. Quantitative real-time PCR analysis of *LDL receptor* expression levels following stimulation with simvastatin (10 μM) for the indicated time periods in THP-1 macrophages. Values are the means ± S.E. of 6 independent experiments with normalization using *Gapdh* expression. *$p<0.05$ compared with 0 h.
D. Quantitative real-time PCR analysis of *Srebp2* expression levels following stimulation with simvastatin (10 μM) in THP-1 macrophages. Values are the means ± S.E. of 6 independent experiments with normalization using *GAPDH* expression. *$p<0.05$ compared with 0 h.
E. Quantitative real-time PCR analysis of miR-33 expression levels following stimulation with simvastatin (10 μM) for the time indicated in THP-1 macrophages. Values are the means ± S.E. of 6 independent experiments with normalization using U6 snRNA expression. *$p<0.05$ compared with 0 h.

Fig. S3. Silencing of endogenous miR-33 using a decoy gene *in vitro*.

A. Schema of the decoy gene. The luciferase 3'UTR was modified to include 3-9 tandem sequences complementary to miR-33 each separated by a single nucleotide spacer.
B. 293T cells were transfected with control-luc or decoy-luc (x3, x6, and x9) constructs,

along with the expression vector of miR-33. Luciferase activity was measured 48 h after transfection. The reduction in luciferase activity indicates the effect of the decoy gene. Values are the means ± S.E. of 3 independent experiments. ***p<0.001. *p<0.05.

C. Western analysis of ABCA1. THP-1 cells were transfected with control-luc or decoy (anti-miR-33 x9)-luc using a lentivirus vector. Cells were cultured in RPMI 1640 with 10% FBS, otherwise cells were cultured without FBS or treated with simvastatin (10 μM) for 24 h. GAPDH was used as a loading control.

Fig. S4. Comparison of *Srebp2* expression in 8-week-old mice.

Quantitative real-time PCR analysis of *Srebp2* in the liver of 8-week-old male and female mice. Values are the means ± S.E. of 3-4 mice with normalization using *Gapdh* expression. The value for wild-type male mice was set at 1.0.

Fig. S5. Comparison of *Srebp2* expression in 16- and 24-week-old mice.

A. RT-PCR analysis of *Srebp2* in the liver of 16-week-old male (upper) and female (lower) mice. The sense primer was designed in exon 16 of *Srebp2* and the antisense primer was designed in exon 17 of *Srebp2*. *Gapdh* expression was used as a control.
B. Quantitative real-time PCR analysis of *Srebp2* in the liver of 16-week-old male and female mice. Values are the means ± S.E. of 3-4 mice with normalization using *Gapdh* expression. The value for wild-type male mice was set at 1.0.
C. RT-PCR analysis of *Srebp2* in the liver of 24-week-old male (upper) and female (lower) mice. The sense primer was designed in exon 16 of *Srebp2* and the antisense primer was designed in exon 17 of *Srebp2*. *Gapdh* expression was used as a control.
D. Quantitative real-time PCR analysis of *Srebp2* in the liver of 24-week-old male and female mice. Values are the means ± S.E. of 3-4 mice with normalization using *Gapdh* expression. The value for wild-type male mice was set at 1.0.

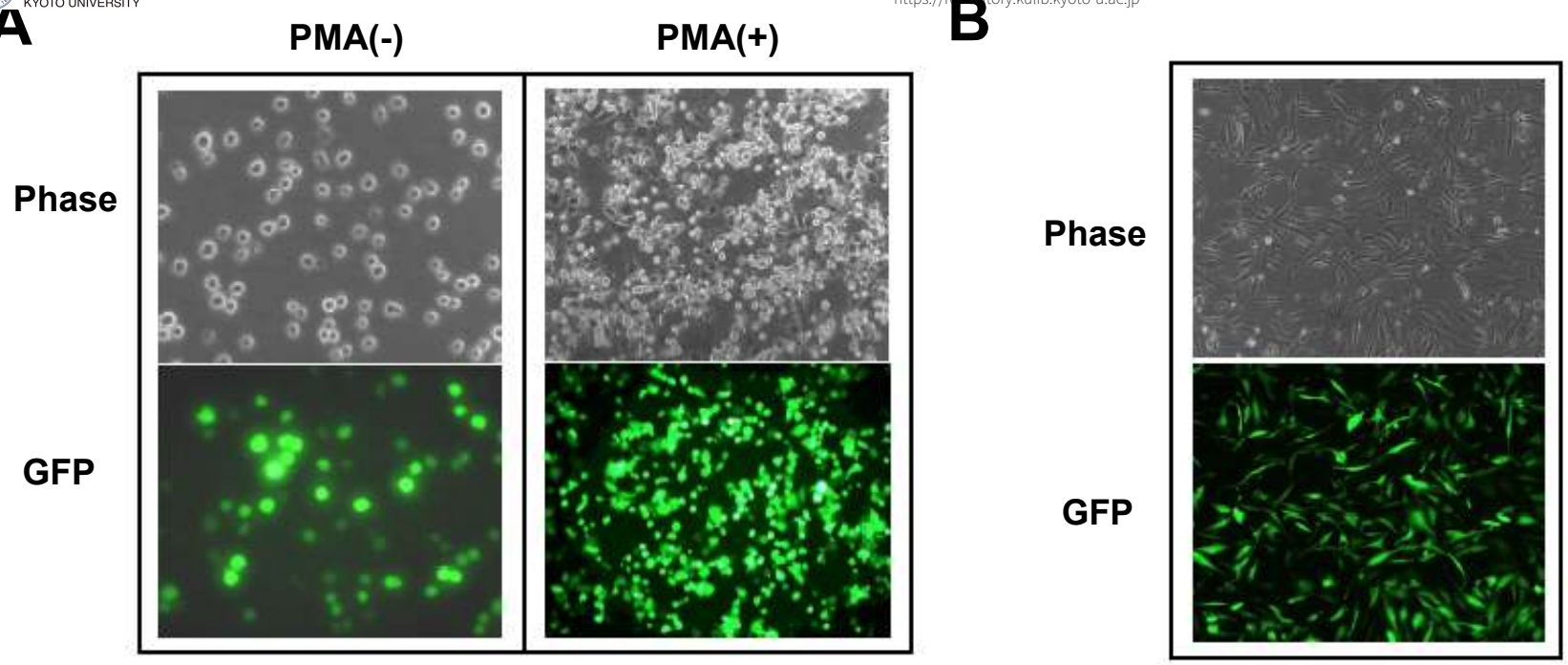
Fig. S6. The expression level of miR-33 in mice.

A. Quantitative real-time PCR analysis of miR-33 and *Srebp2* in 8-week-old wild-type male mice with normalization using U6 snRNA or *Gapdh* expression. The values for the liver were set at 1.0.
B. Quantitative real-time PCR analysis of miR-33 in the kidney of 8-week-old male mice (N.D.: not determined).
C. Quantitative real-time PCR analysis of miR-33 in the brain of 8-week-old male mice (N.D.: not determined).

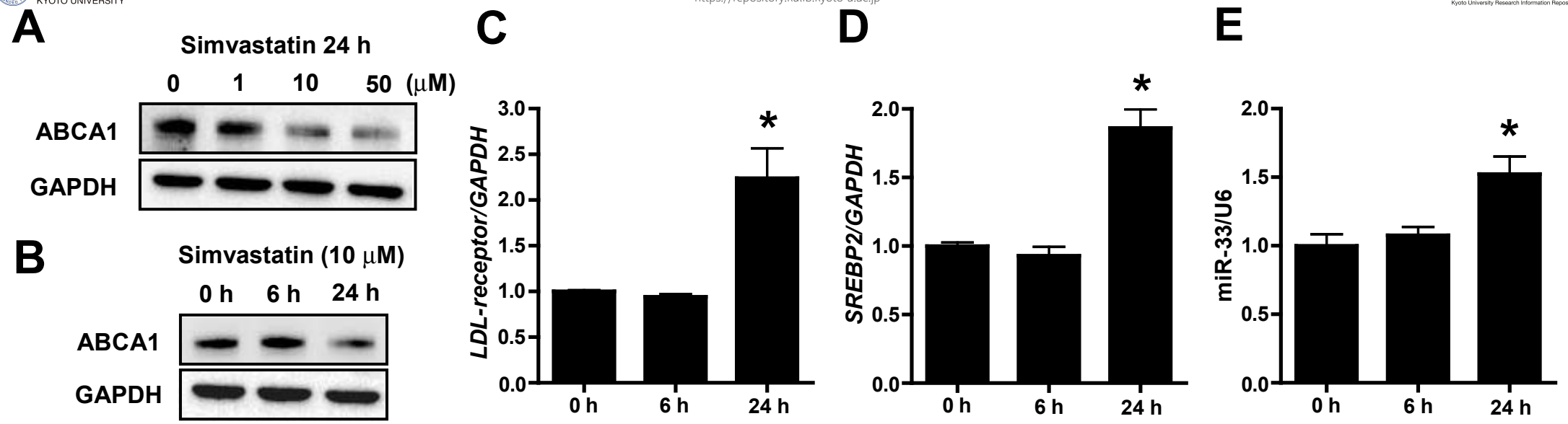
D. Quantitative real-time PCR analsysis of miR-33 in the liver of 16-week-old male and female mice (N.D.: not determined)

Fig. S7 Analysis of ABCG1 *in vitro* and *in vivo*.

A. Sequence alignment of *Abcg1* 3'UTR. There are 2 potential miR-33 binding sites in the *Abcg1* 3'UTR; however, these were conserved only in rodents (not human).
B. 293T cells were transfected with wild-type or mutant *Abcg1* 3'UTR luciferase constructs, along with the expression plasmids for miR-control (negative control), miR-33, and miR-146a. Values are the means ± S.E. of 4 independent experiments. $^{*}p<0.05$ compared with other columns.
C. Western analysis of hepatic ABCG1 in 16-week-old male mice. GAPDH was used as a loading control.
D. Western analysis of hepatic ABCG1 in 16-week-old female mice. GAPDH was used as a loading control.



Supplemental Figure 1



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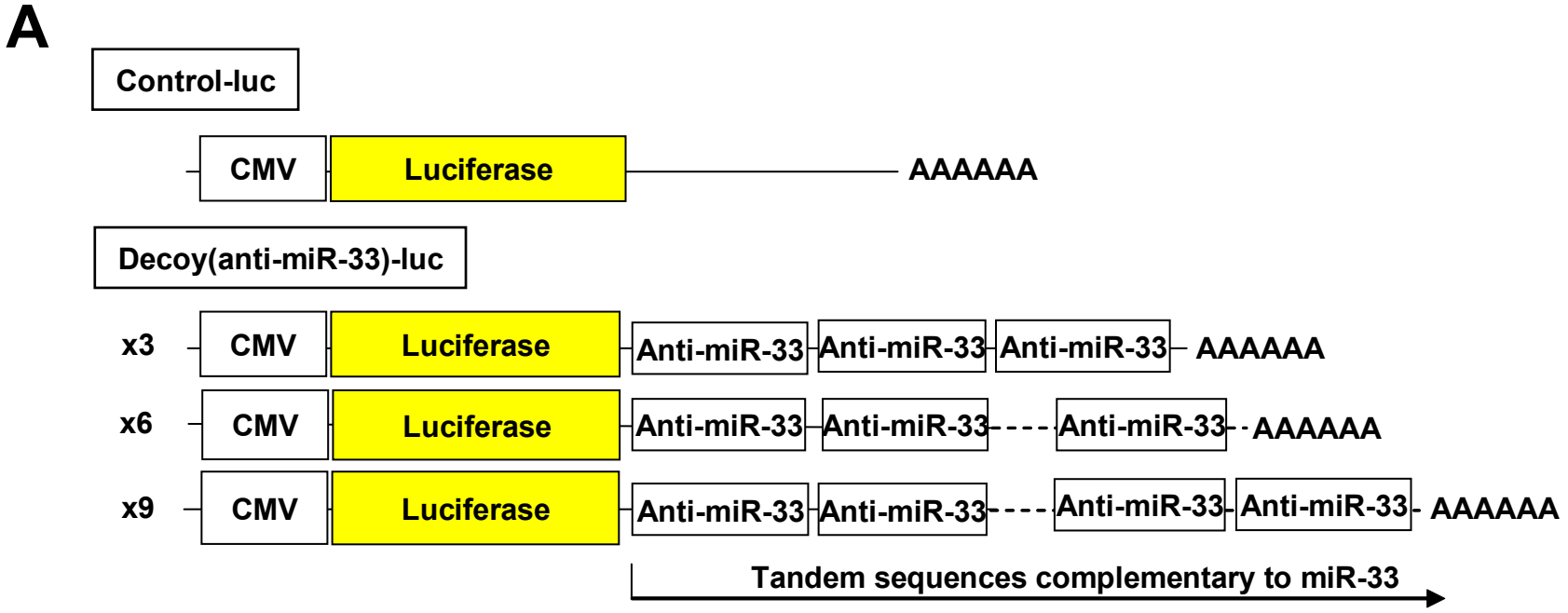

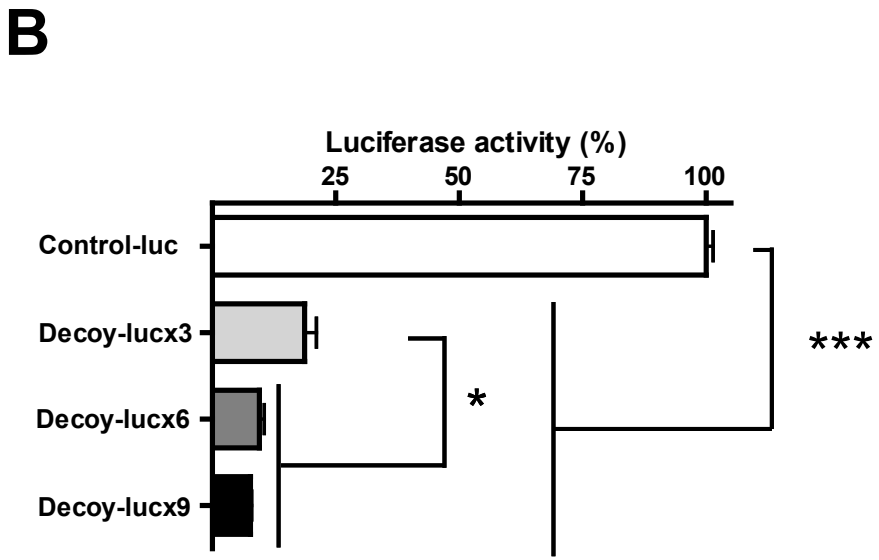

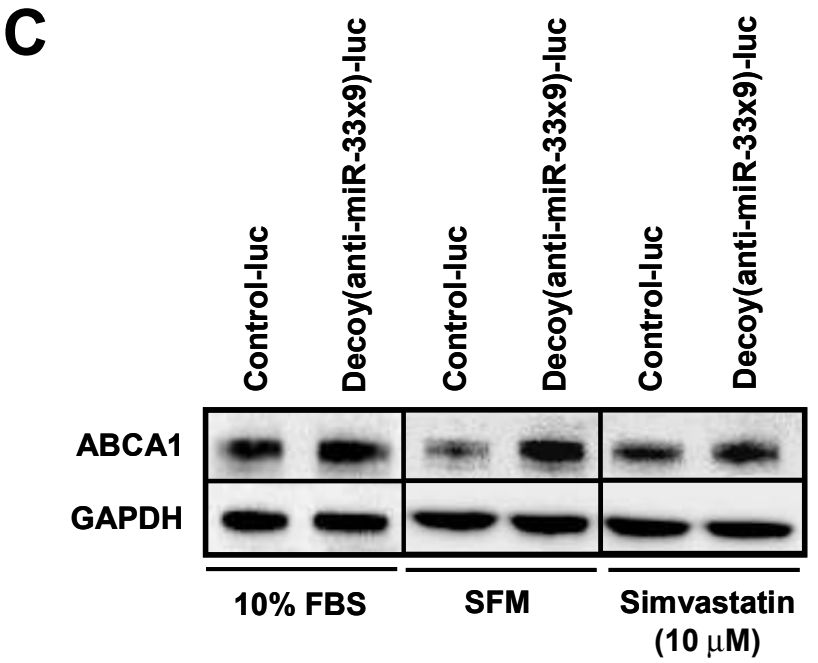

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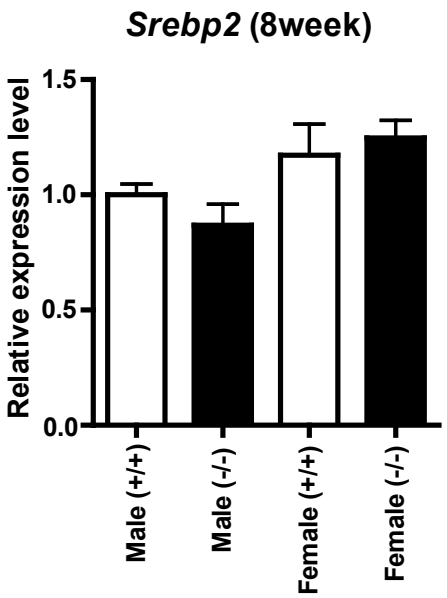

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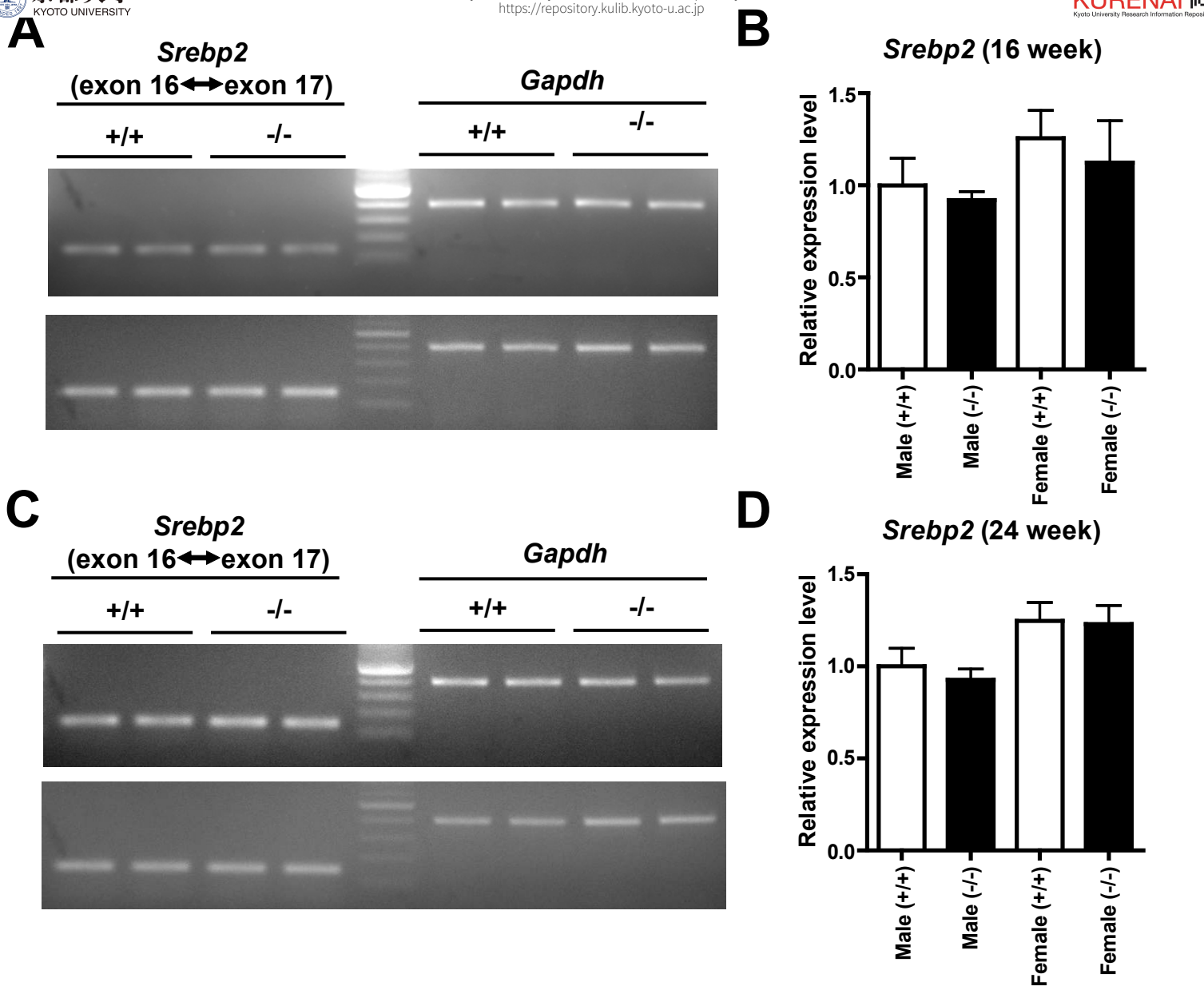

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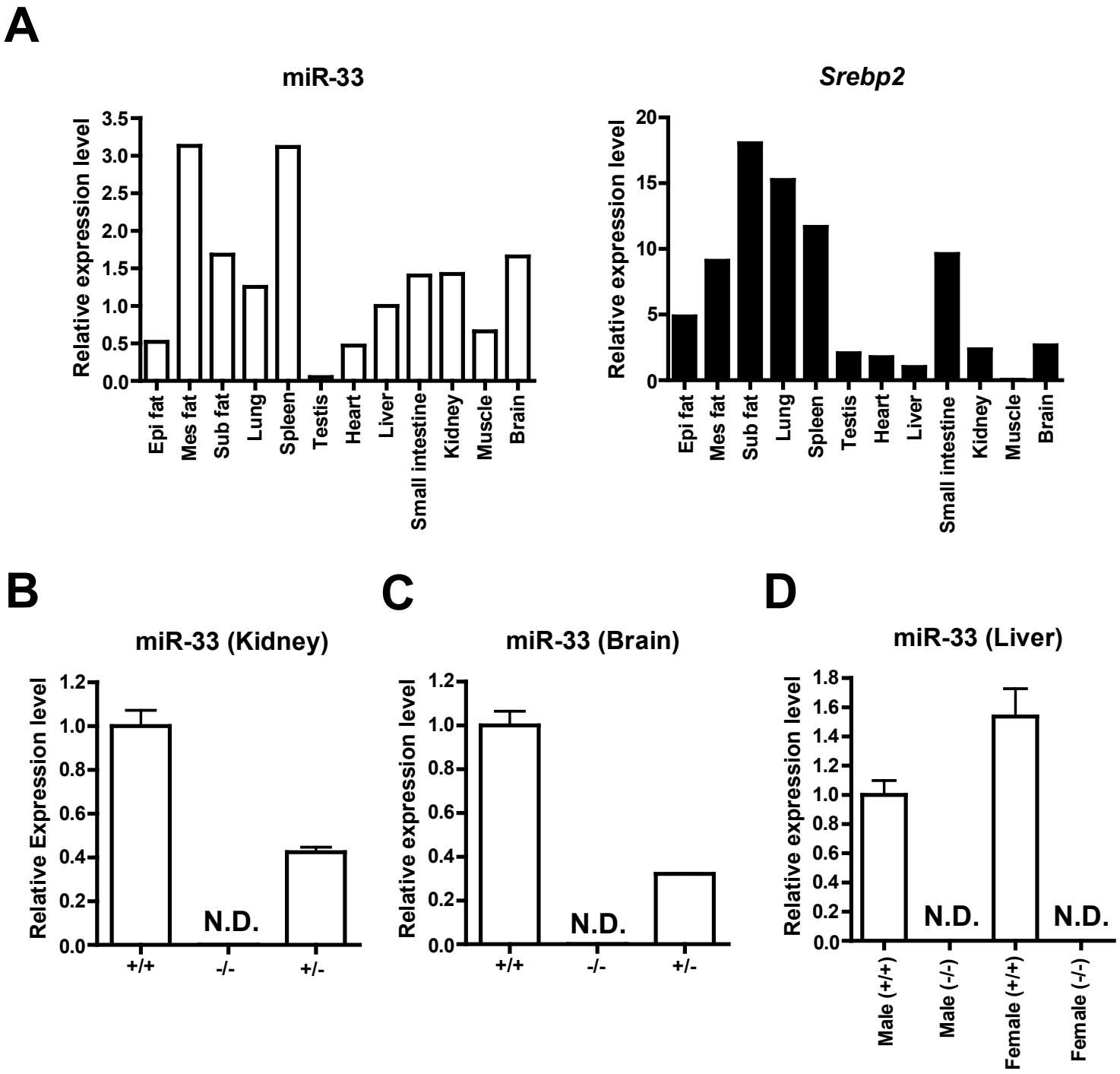

Supplemental Figure 6

A

***Abcg1* 3'UTR**

miR-33 binding site

```
                                                        1                 2
Mmu (Mouse)  CUGCGCUGGGGCAGCAGGGACUAACGCAACGCAAUGCAACG-----CAAUGCAGACAGUGCUGGGG
Rno (Rat)    CUGCGCUGGGCCAGCAGGGACU---------CAAUGCAAUGCAACACAAUGCAGACAGUGCUGGGG
             ********** ***********          ******** *     ********************
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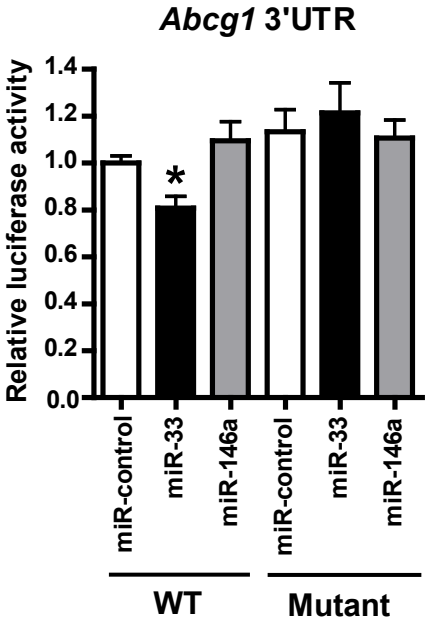
B



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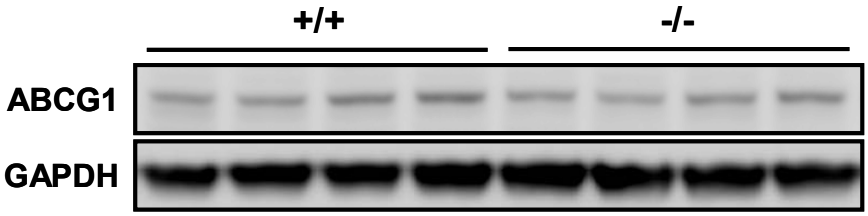

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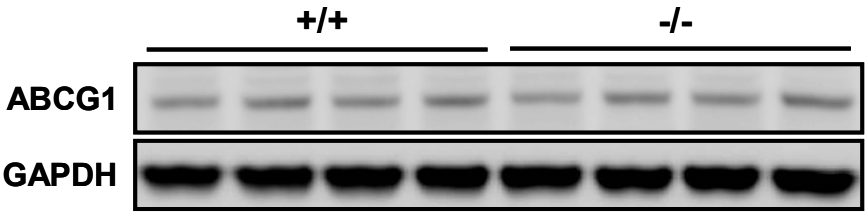

Supplemental Figure 7