

MicroRNA-33 Controls Adaptive Fibrotic Response in the Remodeling Heart by Preserving Lipid Raft Cholesterol

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ABSTRACT

Rationale: Heart failure (HF) and atherosclerosis share the underlying mechanisms of chronic inflammation followed by fibrosis. A highly conserved microRNA (miR), miR-33 is considered as a potential therapeutic target for atherosclerosis because it regulates lipid metabolism and inflammation. However, the role of miR-33 in HF remains to be elucidated.

Objective: To clarify the role of miR-33 involved in HF.

Methods and Results: We first investigated the expression levels of miR-33a/b in human cardiac tissue samples with dilated cardiomyopathy. Increased expression of miR-33a was associated with improving hemodynamic parameters. To clarify the role of miR-33 in remodeling hearts, we investigated the responses to pressure overload by transverse aortic constriction (TAC) in miR-33-deficient (KO) mice. When mice were subjected to TAC, miR-33 expression levels were significantly up-regulated in wild-type (WT) left ventricles. There was no difference in hypertrophic responses between WT and miR-33KO hearts, whereas cardiac fibrosis was ameliorated in miR-33KO hearts compared with WT hearts. Despite the ameliorated cardiac fibrosis, miR-33KO mice showed impaired systolic function after TAC. We also found that cardiac fibroblasts (CFs) were mainly responsible for miR-33 expression in the heart. Deficiency of miR-33 impaired CF proliferation, which was considered to be caused by altered lipid raft cholesterol content. Moreover, CF-specific miR-33-deficient mice also showed decreased cardiac fibrosis induced by TAC as systemic miR-33KO mice.

Conclusions: Our results demonstrate that miR-33 is involved in cardiac remodeling and it preserves lipid raft cholesterol content in fibroblasts and maintains adaptive fibrotic responses in the remodeling heart.

Keywords:

microRNA, cardiac fibroblast, heart failure, lipid metabolites.

Nonstandard Abbreviations and Acronyms:

ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
ACEI/ARB	angiotensin-converting enzyme inhibitor and/or angiotensin receptor II blocker
ANOVA	analysis of variance
BrdU	bromodeoxyuridine
CF	cardiac fibroblast
Colla1	Collagen type 1 alpha 1
CTB	cholera toxin subunit B
EF	ejection fraction
ERK	Extracellular signal-regulated kinase
FS	fractional shortening
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLS	global longitudinal strain
HDL-C	high-density lipoprotein cholesterol
HF	heart failure
KO	knock-out
LV	left ventricle
LSR	longitudinal strain rate
M β CD	methyl- β -cyclodextrin
MEF	mouse embryonic fibroblast
miR	microRNA
NASH	non-alcoholic steatohepatitis
NPC1	Niemann-Pick C1
NPPB	B-type natriuretic peptide
PCWP	pulmonary capillary wedge pressure
Postn	Periostin
SEM	standard error of the mean
SD	standard deviation
SREBF	Sterol regulatory element-binding factor
TAC	transverse aortic constriction
Tgfb	Transforming growth factor beta
WT	wild-type



INTRODUCTION

Despite numerous improvements in therapeutic options, heart failure (HF) is still the leading cause of death worldwide, indicating the need for an in-depth understanding of its etiology and the development of innovative treatment strategies.^{1, 2}

HF is characterized by diverse molecular, cellular, and physiological changes in the myocardium, resulting in adverse cardiac remodeling.³⁻⁵ Remodeling of the adult myocardium involves multiple processes including myocyte hypertrophy, immune cell activation, and fibrosis.⁵⁻⁸ The cellular and molecular processes underlying cardiac remodeling are considered as a type of inflammation.^{9, 10} Chronic inflammation followed by fibrosis can also be seen in other disorders such as atherosclerosis,¹¹ renal dysfunction,¹² non-alcoholic steatohepatitis (NASH),¹³ and cancer.¹⁴

Both cardiac myocytes and non-myocytes play essential roles in the processes involved in cardiac remodeling. In non-myocytes, cardiac fibroblasts (CFs) are cells that have received the most attention,^{15, 16} because CFs produce a variety of growth factors and humoral factors in addition to extracellular matrix proteins and these factors are essential for pathological cardiac hypertrophy and also for normal heart development.^{5, 17-19} When the myocardium is exposed to pathological conditions such as pressure overload, CFs are activated and proliferate rapidly. Recently, it has been reported that the functions of CFs during cardiac remodeling have both adaptive and detrimental sides.^{20, 21} Activated CFs promote pathological remodeling, and at the same time, CFs play essential roles in the adaptive response to pathological stimuli.

MicroRNAs (miRs) constitute a class of small non-coding RNAs that inhibit protein expression by base pairing to complementary sequences located within the 3' untranslated region of target mRNAs. A highly conserved microRNA, miR-33 is considered as a potential therapeutic target for atherosclerosis, because recent reports, including ours,²²⁻²⁵ indicated miR-33 has atherogenic effects by reducing high-density lipoprotein cholesterol (HDL-C) levels.^{22, 23, 26, 27} MiR-33 (or miR-33a in primates) is encoded by intron 16 of sterol regulatory element-binding factor 2 (*SREBF2*), a master regulator of sterol and fatty acid synthesis, and suppresses genes involved in cholesterol metabolism such as ATP-binding cassette transporter A1 (*ABCA1*) and G1 (*ABCG1*) as target genes (primates including *Homo sapiens* also have miR-33b in the intron of *SREBF1* in addition to miR-33a²⁸). It was also reported that miR-33 is involved in inflammatory processes in atherosclerotic lesions²⁹ and fibrotic responses in the liver.³⁰ However, the functions of miR-33 in cardiac hypertrophy and/or heart failure, which are also chronic inflammatory and fibrotic processes as mentioned above, remain to be elucidated.

MiR-33 is expressed ubiquitously, but its expression level in the heart varies depending on the report in question, and varies from 0.5- to 10-fold of its expression in the liver.^{22, 26, 27} MiR-33 can, at least, be detected in the heart at a similar level to that in the liver or in immune cells, which have been investigated intensively. However, there are no reports regarding the function of miR-33 in the heart.

In the present study, we investigated the functions of miR-33 in the remodeling heart using clinical samples and genetic mouse models. We first investigated the correlation between expression levels of miR-33a/b in myocardial biopsy samples and hemodynamic parameters in patients with dilated cardiomyopathy. Next, we used miR-33-deficient (KO) mice (both whole body KO and fibroblast-specific KO) to examine

the role of miR-33 in cardiac hypertrophy and fibrosis. We applied transverse aortic constriction (TAC), a model of left ventricular pressure overload characterized by cardiac myocyte hypertrophy and interstitial fibrosis.

METHODS

Detailed methods are provided in the Online Supplement.

Myocardial biopsy.

We analyzed the expression levels of miR-33a/b in the tissues of endomyocardial biopsies from left ventricles (LVs) and examined the relationship between their expression levels and hemodynamic parameters in 33 patients who were diagnosed with dilated cardiomyopathy at Osaka Red Cross Hospital.³¹ Hemodynamic parameters were obtained by catheterization. All patients provided written informed consent for the procedure and gene expression analyses. The ethics committee of Osaka Red Cross Hospital approved the study protocol.

Mice.

MiR-33 KO mice were generated as reported previously.²² MiR-33-floxed mice were generated by homologous recombination in a C57BL/6J background as described in the Online Supplement.³² Periostin-Cre (Pn-Cre) mice were generated by Dr. Conway and colleagues.^{21, 33} Mice were maintained in specific pathogen-free conditions at the Institute of Laboratory Animals of Kyoto University Graduate School of Medicine. This study was approved by the Kyoto University Ethics Review Board. The primer sequences for genotyping are listed in Online Table II. All the in vivo experiments were performed using male mice in a C57BL/6J background.

Pressure-overload model.

TAC was performed as described previously.³⁴⁻³⁶

Primary neonatal rat cardiac myocytes and fibroblasts

Neonatal rat cardiac myocytes and cardiac fibroblasts were isolated from 1-day-old Sprague-Dawley rats, as described previously.³⁶ After enzymatic digestion with pancreatin (Sigma, P3292), cardiac myocytes and fibroblasts were purified by Percoll density gradient centrifugation (Sigma, P4937).

Culture of fibroblasts.

For isolation of adult CFs, hearts from 8-week-old mice were digested with collagenase/ dispase solution (Roche, 11097113001) and plated for 2 h.¹⁸ We used adult CFs at passage 3-4. For isolation of tail-tip fibroblasts, the tails from 8-week-old mice were peeled, minced into 1 cm pieces, placed on gelatin-coated culture dishes, and incubated for 5 days.³⁷ Cells that migrated out of the graft pieces were transferred to new plates (passage 1). We used tail-tip fibroblasts at passage 4-5. Mouse embryonic fibroblasts (MEFs) were isolated from wild-type (WT) and miR-33KO embryos at E14.5. We used MEFs at passage 5-6.

Quantitative real-time PCR.

To evaluate mRNA expression, single-strand cDNA was synthesized from 1 µg of total RNA by means of reverse transcriptase reaction and quantitative PCR (qPCR) was performed using a LightCycler®96 (Roche Diagnostics) with THUNDERBIRD® SYBR qPCR Mix (TOYOBO, QPS-201). Expression levels were normalized by housekeeping genes as indicated. The primer sequences are listed in Online Table III.

Quantitative real-time PCR for microRNAs.

Expression levels of miR-33a and miR-33b were measured using TaqMan® MicroRNA Assays (Applied Biosystems) and a StepOnePlus™ Real-Time PCR System (Applied Biosystems) in accordance with the manufacturer's protocol. Expression levels of miRs were normalized by *U6* small nuclear RNA and calculated by the $2^{-\Delta\Delta C_t}$ method.

Lipid raft staining.

We used fluorescence-conjugated cholera toxin subunit B (CTB) for lipid raft labeling. To obtain images of lipid raft staining, fibroblasts were plated onto chamber slides and stained with Vybrant® Alexa Fluor®594 Lipid Raft Labeling Kit (Molecular Probes™, V34405). To quantify the signals from CTB-labeled lipid rafts on the cell surface, flow cytometric analyses were performed. Fibroblasts were plated on 10-cm culture dishes and cultured overnight. The cells were dissociated using trypsin EDTA, and stained with 1 µg/ml FITC-conjugated CTB (Sigma, C1655) at 4°C for 15 min, and then analyzed on a FACSARIAII™ (Becton Dickinson). To make control samples with decreased lipid rafts, some samples were incubated with 5 mmol/L methyl-β-cyclodextrin (MβCD) for 1 h before labeling with CTB.

Echocardiography.

To analyze the cardiac function of mice, we performed echocardiography (Vevo®2100, VISUALSONICS) at the indicated time points after TAC.

Statistical analysis.

Measurements except for clinical biopsy data are presented as means ± standard error of the mean (SEM). Measurements for clinical samples are presented as median ± interquartile range or mean ± standard deviation (SD) as indicated. To compare gene expression levels and the hemodynamic parameters in Figure 1A, linear-regression analysis was performed using R version 3.2.3 (The R Project). For Gene Ontology analysis, the clusterProfiler package in R/Bioconductor was used for data sets with differentially expressed genes. P values were adjusted by FDR correction. For other statistical comparisons, unpaired Student's t-test (two groups, parametric), Mann-Whitney test (two groups, non-parametric), or one-way analysis of variance (ANOVA) with Sidak's post-hoc test (three or more groups) were used as indicated in the figure legends. A p value of <0.05 was considered as statistically significant. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc.).

RESULTS

MicroRNA-33a expression levels in cardiac tissue are associated with improving hemodynamic parameters in patients with dilated cardiomyopathy.

To analyze the expression pattern of miR-33a/b in human remodeling hearts, we measured their levels using Taqman qPCR in the tissues from endomyocardial biopsies of LVs and examined the relationships between miR-33a/b expression levels and hemodynamic parameters obtained by catheterization in patients with dilated cardiomyopathy.

A total of 33 patients were included in the analysis (Online Table I). The cardiac expression level of miR-33a was modestly but significantly correlated with ejection fraction (EF) and inversely correlated with pulmonary capillary wedge pressure (PCWP), whereas the expression level of miR-33b was not correlated with these parameters (Figure 1A). Thus, we focused on miR-33a, which is highly conserved across species unlike miR-33b.

When the patients were categorized by Forrester classification (there were no patients with Forrester II HF in this cohort) (Online Table I), miR-33a expression levels in patients with high-stage HF (Forrester III or IV) were significantly lower than its expression in patients with low-stage HF (Forrester I) (Figure 1B), whereas the expression of *SREBF2*, whose intron 16 has the coding region of miR-33a, did not differ among the groups (Figure 1C).

These data from the clinical samples indicated that decreased miR-33a was associated with worsened cardiac function and miR-33a had function in the development of HF.

MicroRNA-33 plays an important role in pressure overload-induced cardiac fibrosis.

Because rodents have only miR-33a, but not miR-33b, unlike human or non-human primates, miR-33KO mice are good tool to analyze miR-33a functions. As we previously reported, miR-33KO mice show normal growth and displayed no obvious abnormalities in heart development.²² To analyze the function of miR-33 in response to pathological stimuli, mice were subjected to pressure overload-induced cardiac hypertrophy by TAC.

To analyze hypertrophic and fibrotic responses to TAC, we performed gene expression analyses in sham- or TAC-operated WT and miR-33KO LVs at 2 weeks after operation and also histological analyses at 4 weeks after operation. The expression level of miR-33 was significantly up-regulated in response to TAC in WT LVs (Figure 2A). Heart weight and cardiac myocyte size were increased similarly in WT and miR-33KO mice after TAC (Figure 2B and 2C; Online Figure IA-D), which showed no significant difference in TAC-induced cardiac myocyte hypertrophy between WT and miR-33KO mice.

On the other hand, histological analysis showed that TAC-induced cardiac fibrosis was ameliorated in miR-33KO mice (Figure 2D and 2E). Moreover, TAC-induced up-regulation of fibrosis-related genes such as *Col1a1*, *Postn*, and *Tgfb3* in LVs was significantly suppressed in miR-33KO mice compared with WT mice, in spite of the similar expression level of a hypertrophic marker, *Nppb* (Figure 2F).

To analyze gene expression more comprehensively, we performed microarray analysis. The microarray data detected 114 up-regulated genes and 148 down-regulated genes in miR-33KO hearts versus WT hearts after TAC (fold change > 2). Gene ontology analysis using the down-regulated genes showed that several biological processes related to fibrosis and extracellular matrix were enriched (Online Figure II). The heatmap data showed suppressed up-regulation of fibrosis-related genes by TAC in miR-33KO mice (Online Figure III; orange cluster in A, red and orange cluster in C, and red cluster in D), whereas the genes involved in cardiac muscle contraction showed only small changes (Online Figure IIIB).

These data showed that miR-33 deficiency did not affect the hypertrophic response to TAC but alleviated the fibrotic response to TAC. Therefore, miR-33 was considered to play an important role in TAC-induced cardiac fibrosis.

Deficiency of microRNA-33 worsened systolic function after chronic pressure overload.

As shown above, miR-33 deficiency alleviated fibrotic response in the heart after TAC. To investigate whether the alterations by miR-33 deficiency improved cardiac function in vivo, we performed echocardiography of WT and miR-33KO mice up to 8 weeks after TAC. During the follow-up, no WT mice died, and only one miR-33KO mouse died at 8 weeks after TAC. Echocardiographic parameters including EF and fractional shortening (FS) did not differ among WT and miR-33KO mice in the period from preoperation to 4 weeks after TAC. However, contrary to our expectations, both EF and FS were deteriorated in miR-33KO mice compared with WT mice at 8 weeks after TAC (Figure 3A, 3B, 3C, and 3E). The wall thickness stopped increasing at 4-8 weeks after TAC in miR-33KO mice whereas it continued to increase in WT mice (Figure 3D). To analyze LV function more precisely, we measured global longitudinal strain (GLS) and longitudinal strain rate (LSR) using speckle-tracking strain imaging (Figure 3F and Online Figure V). We found that GLS, a sensitive parameter for systolic function, also deteriorated in miR-33KO mice at 8 weeks after TAC. There were no significant differences in early diastolic LSR, a parameter for diastolic function.

In addition, the activation of Protein kinase B (Akt), which is known to be protective in the remodeling heart,^{38,39} was inhibited in miR-33KO LVs (Figure 3G and 3H).

These data showed that deficiency of miR-33 deteriorated systolic function after chronic pressure overload, which was consistent with the human sample data shown in Figure 1.

Cardiac fibroblasts are responsible for microRNA-33 expression in the heart, and microRNA-33-deficient fibroblasts have impaired proliferative capacity.

To clarify which cell type is the source of miR-33, we measured the expression level of miR-33 in Percoll-isolated neonatal rat cardiac myocytes and fibroblasts. The expression level of miR-33 in CFs was about 3.4-fold higher than cardiac myocytes (Figure 4A; Online Figure VI). Thus, CFs were considered to be responsible for miR-33 in the heart, which was consistent with the difference between the hypertrophic response and fibrotic response after TAC in miR-33KO mice as shown in Figure 2.

Therefore, we focused on the function of miR-33 in fibroblasts. We isolated three types of fibroblasts from miR-33KO and control (WT) mice. Because proliferation and increased density of CFs are involved in cardiac fibrosis during pathological remodeling,^{15, 20} we analyzed proliferative capacity of miR-33KO fibroblasts. MTT assays showed that all the three types of miR-33KO fibroblasts had impaired proliferative capacity in vitro, compared with WT fibroblasts (Figure 4B). Moreover, flow cytometric analysis showed impaired anti-apoptotic capacity in miR-33KO fibroblasts against hydrogen peroxide (Figure 4C and 4D). MiR-33KO fibroblasts also showed impaired activation of Akt when stimulated with fetal bovine serum (Figure 4G and 4H). This was consistent with the impaired proliferation of miR-33 KO fibroblasts because Akt is a one of the most important protein kinases that regulate cellular proliferation and survival.⁴⁰

To analyze cell proliferation in vivo, bromodeoxyuridine (BrdU) incorporation analysis was performed. Because CFs proliferate rapidly immediately after TAC, we analyzed BrdU incorporation in LVs on day 7 after TAC. By using confocal microscopy, we found that all the BrdU⁺ cells were also positive for vimentin, and we could not detect any BrdU⁺ cardiac myocytes in LVs. The number of vimentin⁺ CFs positive for BrdU after 7-days TAC was significantly smaller in miR-33KO LVs than WT (Figure 4E and 4F). It confirmed impaired proliferative capacity of miR-33KO fibroblasts in vivo.

Taken together, miR-33 was considered to be necessary to maintain appropriate proliferation of fibroblasts in vitro and also in vivo.

Deficiency of microRNA-33 increased the expression level of ABCA1 and decreased lipid raft content in cardiac fibroblasts.

Recent studies have shown that miR-33 has atherogenic and proinflammatory effects, because it regulates cholesterol metabolism in the liver and immune cells via inhibition of genes involved in cholesterol efflux and transport, such as ABCA1, ABCG1, and Niemann-Pick C1 (NPC1).^{22, 23, 29, 30, 41} Among these genes, we confirmed the up-regulation of ABCA1 even in miR-33KO CFs (Figure 5A and Online Figure VIIA-C).

Because of the altered proliferative capacity and altered gene expression involved in cholesterol metabolism in miR-33KO fibroblasts, we speculated that altered lipid rafts in miR-33KO fibroblasts were the underlying mechanism of the reduced fibrosis of miR-33KO hearts. To analyze lipid raft content in miR-33KO fibroblasts, we stained lipid rafts by fluorescence-conjugated cholera toxin subunit B (CTB), which binds to ganglioside GM1 on the plasma membrane and is a marker to identify lipid rafts. Analyses by confocal laser scanning microscopy showed reduced lipid raft content in miR-33KO fibroblasts compared with WT fibroblasts (Figure 5B). To quantify the signal intensity by CTB binding which marked lipid rafts, we used flow cytometry. The mean fluorescence intensity of miR-33KO fibroblasts marked by FITC-CTB was significantly lower than that of WT fibroblasts (Figure 5C and 5D). The altered lipid rafts in miR-33KO fibroblasts were consistent with the impaired activation of Akt because it has been reported that lipid rafts are involved in the phosphatidylinositol-3 kinase/Akt signaling pathway and that Akt is phosphorylated in lipid rafts on the plasma membrane (Figure 4G and 4H).^{42, 43}

To investigate whether ABCA1 is involved in the impaired proliferation of miR-33KO fibroblasts, we performed siRNA-mediated knockdown of ABCA1 (siABCA1). Both WT and miR-33KO fibroblasts

showed similar proliferation in the condition of siABCA1 (Figure 5E and Online Figure VIID). The results indicated that both ABCA1 and miR-33 are involved in cellular proliferation.

These data indicated that miR-33 maintained lipid raft content by regulating genes involved in cholesterol metabolism.

Cardiac fibroblast-specific deletion of microRNA-33 ameliorated cardiac fibrosis after pressure overload.

To eliminate the effect of increased HDL-C and the altered phenotype of immune cells in miR-33KO mice, we decided to analyze fibrotic responses in CF-specific miR-33 deficient mice. We generated miR-33 floxed mice, in which loxP sites flanked the pre-miR sequence of miR-33 (Online Figure VIIIA-D). We confirmed that the expression of miR-33 was hardly detected in miR-33-deleted mice after crossed with Ayu1-Cre mice, which express Cre recombinase ubiquitously (Online Figure IXA), and also that the expression and splicing of *Sreb2* mRNA was not altered in both miR-33-floxed mice and miR-33-deleted mice (Online Figure IXB-C).

To generate CF-specific knockout mice, we used a transgenic mouse line in which Cre-recombinase was driven by a 3.9-kb promoter of mouse Periostin (Pn-Cre), which is not normally expressed in adult hearts but induced specifically in CFs by pathological stimuli such as pressure overload.^{8, 18, 33}

When we generated CF-specific KO mice of miR-33 (FBKO, Pn-Cre⁺miR-33^{flx/flx}), FBKO mice showed an approximately 30% reduction in miR-33 in LVs compared with littermate control (miR-33^{flx/flx}) mice after TAC (Figure 6A). The cell type in which the expression of miR-33 increased after TAC was thought to be CFs because the expression levels of miR-33 in FBKO mice were the same as at baseline (Figure 2A and 6A). FBKO mice did not show higher HDL-C levels than littermate controls, whereas miR-33^{Δ/Δ} mice showed higher HDL-C levels than control mice (Online Figure XA-B). This result suggested that miR-33 in fibroblasts did not contribute to HDL-C elevation.

Heart weight after TAC did not differ among FBKO and control mice (Figure 6B), whereas histological analysis showed that the fibrotic area of FBKO LVs was smaller than control mice (Figure 6C and 6D), like whole-body KO mice. Gene expression analysis showed that fibrosis-related genes tended to be down-regulated in FBKO LVs (Online Figure XC). Furthermore, FBKO mice showed smaller numbers of Ki-67-positive fibroblasts at 2 weeks after TAC (Figure 6E and 6F). Therefore, miR-33 was considered to contribute to CF proliferation and fibrotic responses after pathological stimuli such as pressure overload.

DISCUSSION

Here, by using genetic models, we demonstrate that deficiency of miR-33 resulted in reduced fibrotic response to pressure overload in vivo. In spite of the reduction in fibrosis, cardiac function deteriorated in miR-33KO hearts. We also found that miR-33 in the heart was predominantly expressed in CFs, and deficiency of miR-33 impaired cell proliferation in fibroblasts both in vitro and in vivo, and that miR-33 preserved lipid raft cholesterol content in fibroblasts by regulating genes involved in cholesterol metabolism. Moreover, in clinical samples, miR-33a expression correlated with improving hemodynamic parameters including EF. These data indicate that miR-33 promotes fibrotic responses in the heart by maintaining lipid raft content in fibroblasts and the response has protective effects on cardiac function (Online Figure XIII).

It has been said that miRs play critical roles in normal heart development and also in HF by fine-tuning gene expression, because cardiac-specific deletion of *Dicer*, a gene that is essential for miR processing, leads to poorly developed myocardium, rapidly progressive HF, and postnatal lethality.⁴⁴⁻⁴⁶ In our clinical samples, miR-33a expression was correlated with EF and inversely with mean PCWP, and its expression was down-regulated in high-stage HF patients compared with low-stage HF patients. The results were consistent with our echocardiographic data using miR-33KO mice, because EF and FS were significantly lower in miR-33KO mice compared with WT mice after chronic pressure overload. The wall thickness stopped increasing at 4-8 weeks after TAC in miR-33KO mice, and this indicated insufficient hypertrophic adaptation. Additionally, the up-regulation of miR-33 after TAC was only in the early phase (Online Figure IE). These data indicated that miR-33 had protective effects on developing HF.

MiR-33a (or miR-33 in rodents) is transcribed from intron 16 of *SREBF2*, which is a master regulator of lipid synthesis.²² Expression of *SREBF2* mRNA did not differ between low-stage HF patients and high-stage HF patients, whereas expression of miR-33a was different between the groups. It seemed that this was because of post-transcriptional regulation of miR-33a such as processing by DROSHA and DICER.

Next, gene expression and histological analyses using miR-33KO mice after TAC showed that deficiency of miR-33 did not affect hypertrophic responses but alleviated fibrotic responses to pressure overload. We also found that the expression of miR-33 was predominant in CFs. Moreover, the phenotype of the reduced fibrotic response was also reproduced in CF-specific miR-33-deficient mice. These data were consistent with recent reports that inhibition of miR-33 reduces liver fibrosis in a NASH model, and that expression of miR-33 in hepatic stellate cells, which are the primary source for activated fibroblasts in liver, is involved in the fibrotic processes in NASH.^{30, 47} Therefore, miR-33 is considered as a profibrotic microRNA in pathological conditions.

Cardiac fibrosis is a characteristic of failing hearts.^{5, 8, 15, 20} Our data is paradoxical in that deficiency of miR-33 reduced systolic function after chronic pressure overload in spite of ameliorated cardiac fibrosis. Recently, however, it has been said that CFs and fibrosis have both adaptive and maladaptive sides, and some reports showed inconsistency between cardiac fibrosis and hypertrophy (or LV function) in the remodeling heart.^{5, 20, 21, 48} For example, it has been reported that deficiency of *Smad3*, which is essential for canonical TGF- β signaling, showed increased heart weight and worsened heart failure after TAC despite decreased cardiac fibrosis in mice.⁴⁸ Although persistent cardiac fibrosis is detrimental, fibroblasts also play a protective role in the heart by producing a necessary acute wound healing response. Additionally, there

are two types of cardiac fibrosis; reactive and reparative.⁴⁹ Reactive fibrosis in the early phase of remodeling is different from the reparative (replacement) fibrosis that follows cardiac myocyte death. In our data, proliferation of fibroblasts was impaired in vivo in the acute phase (1 week after TAC) in miR-33KO mice (and also in vitro), and fibrotic responses in the early phase (2-4 weeks) were suppressed without affecting cardiac myocyte hypertrophy, and LV function worsened in the chronic phase (8 weeks). Therefore, we can explain that impaired proliferation of fibroblasts in miR-33KO mice was primary and it resulted in inadequate “reactive” fibrosis and insufficient adaptive responses, such as protective paracrine factors from CFs (Online Figure XIII).

There may be additional cells that affect the phenotype of miR-33KO mice, because the phenotype observed in FBKO mice was not so prominent as in systemic KO mice. Macrophages are also important for cardiac fibrosis and remodeling. Although the expression of *Tgfb3* was suppressed in peritoneal macrophages in vitro, we did not detect significant differences in macrophage infiltration between WT and miR-33KO mice in vivo (Online Figure IV).

Cholesterol metabolism is tightly regulated because cholesterol is a critically important component in cellular membranes.⁵⁰ It is enriched in microdomains on the plasma membrane, called lipid rafts, which function as platforms that concentrate and segregate proteins such as growth factor receptors and regulates cellular processes.⁵¹ In our data, deficiency of miR-33 up-regulated its target genes involved in cholesterol efflux and transfer even in fibroblasts, as shown previously in the liver and immune cells.^{22-24, 29} As a result, miR-33KO fibroblasts have altered lipid raft contents and impaired proliferative capacity. Because fibroblasts are cells that can proliferate rapidly depending on the situation, it is reasonable that an insufficient content of cholesterol in miR-33KO fibroblasts resulted in impaired proliferative capacity. This is considered as an underlying mechanism of the reduced fibrotic response in miR-33KO hearts. In the experiment with siRNA-mediated knockdown of ABCA1 (Figure 5E and Online Figure VIID), we need to consider a problem that knockdown of ABCA1 in WT fibroblasts resulted in impaired proliferation compared with the controls. Overexpression of miR-33 also inhibited proliferation (Online Figure VIIE). Alteration of cholesterol homeostasis, regardless of excess or deficiency, may inhibit the proliferation of fibroblasts.

There are some reports that suggest miR-33 slows down the cell cycle by inhibiting the expression of genes associated with the cell cycle such as Cyclin-dependent kinase 6 (CDK6) and Cyclin-D1.⁵² We found that CDK6 was not increased in miR-33KO fibroblasts (Online Figure XI). The result was probably due to the difference in cell types. Although CDK6 is preferentially expressed in hematopoietic cells, CDK4 is predominant over CDK6 in fibroblasts.⁵³

The importance of miRs in cardiac hypertrophy and fibrosis in pathological conditions has been demonstrated.^{44-46, 54} Notably, with regard to fibroblasts, several miRs in CFs have been investigated intensively, including miR-21, miR-29, and miR-30.⁵⁵⁻⁵⁸ Interestingly, members of the miR-30 family are involved both in lipid metabolism and cardiac fibrosis,^{58, 59} like miR-33 in our data. These studies screened differentially expressing miRs in HF samples and in CFs. The changes of miR-33 expression in HF are not so prominent as these CF-enriched miRs, probably because *Srebf2* and miR-33 are ubiquitously expressed and cholesterol homeostasis is tightly regulated. The present study is notable in that it showed the relationship between cellular lipid homeostasis and fibrotic responses.

Several limitations of this study should be acknowledged. There might be some variations in the quality of clinical biopsy samples. We did not use isolated cardiac myocytes from adult mice or human samples to analyze cell-type specific expression levels of miR-33, but instead used Percoll-isolated neonatal rat cardiac myocytes and fibroblasts, because isolation of fresh cardiac myocytes from adult mice or human sample is technically difficult. To generate CF-specific KO mice, we used a Pn-Cre line, which express Cre-recombinase only in a subset of fibroblasts in the heart, and there is still no truly specific marker of CFs.

Finally, inhibition of miR-33 is considered to be a potent therapeutic strategy for atherosclerosis, because miR-33 has atherogenic effect by reducing HDL-C.^{23, 41, 60} It is considered that inhibition of miR-33 results in altered cellular cholesterol content because of the target genes involved in cholesterol efflux and transfer such as ABCA1, ABCG1 and NPC1. In our data, deficiency of miR-33 reduced the lipid raft cholesterol content in fibroblasts. Statins also reduce cellular cholesterol content, and they are reported to have pleiotropic effects on various diseases such as prostate cancer,⁶¹ Alzheimer's disease^{62, 63} and HIV infection⁶⁴ through modulation of lipid rafts.⁵¹ Therefore, our data implicated the possibility of pleiotropic effects of miR-33 inhibition. On the other hand, our data suggest that inhibition of miR-33 may cause cardiac dysfunction. It has also been reported that long-term silencing of miR-33 resulted in an unexpected increase in circulating triglyceride levels and lipid accumulation in the liver⁶⁵. Because our genetic KO mice were equivalent to permanent inhibition, long-term inhibition of miR-33 can cause harmful side-effects. To utilize the protective effect of miR-33 inhibition, further research is necessary.

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DISCLOSURES

None.

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ONLINE FIRST

FIGURE LEGENDS

Figure 1. Association between miR-33a expression and hemodynamic parameters in patients with dilated cardiomyopathy. **A**, Linear-regression analysis of miR-33a/b expression in myocardial biopsy samples versus hemodynamic parameters obtained by heart catheterization (n=33). Regression lines, r values, and p values are shown with the plots. EF, ejection fraction; PCWP, pulmonary capillary wedge pressure. **B** and **C**, Expression levels of miR-33a/b (**B**) and *SREBF2* (**C**) in myocardial biopsy samples in patients with low-stage heart failure (HF) (Forrester I, n=17) and high-stage HF (Forrester III or IV, n=11). The patient characteristics are shown in Online Table I. Expression levels of miR-33a/b and *SREBF2* were normalized by *U6* snRNA or *18S* ribosomal RNA respectively. P values between low-stage and high-stage HF patients by two-tailed Mann-Whitney tests are shown. Data are presented as median $\pm$ interquartile range.

Figure 2. Deficiency of miR-33 ameliorated cardiac fibrosis induced by pressure overload in vivo. **A**, MiR-33 expression levels in left ventricles (LVs) of WT mice at 2 weeks after sham- or TAC-operation (n=4). Expression of *U6* snRNA was used as an internal control. **P<0.01, by unpaired two-tailed Student's t-test. **B**, Representative images of wheat germ agglutinin staining in LVs at 4 weeks after operation. White bar indicates 50 μ m. **C**, Quantification of cardiac myocyte cross-sectional area in sham- or TAC-operated WT and miR-33KO mice at 4 weeks after TAC (sham: n=4, TAC: n=8-10). **D**, Representative images of Masson's trichrome staining and picrosirius red staining in LVs at 4 weeks after operation. Black bars indicate 200 μ m (Masson's trichrome) and 500 μ m (picrosirius red) respectively. **E**, Quantification of the fibrotic area in whole LVs and the perivascular fibrosis 4 weeks after TAC. (sham: n=4, TAC: n=8-12). **F**, Quantitative real-time PCR analysis in LVs 2 weeks after TAC. (n=7-9). Expression of *Gapdh* mRNA was used as an internal control. *P<0.05, **P<0.01 vs sham, #P<0.05 vs WT, n.s. not significant, by ANOVA with Sidak correction. Data are presented as mean $\pm$ SEM.

Figure 3. Deficiency of miR-33 impaired systolic function after chronic pressure overload.

A, Representative images of M-mode echocardiography of WT and miR-33KO mice at 8 weeks after TAC. **B-E**, Echocardiographic data of WT and miR-33KO up to 8 weeks after TAC. Ejection fraction (EF) (**B**), fractional shortening (FS) (**C**), interventricular septum (IVS) (**D**), and LV diameters (**E**) were measured at the indicated time points after TAC (n=9-12). **F**, Global longitudinal strain (GLS) measured using speckle-tracking strain imaging (n=9-12). **G**, Western blotting analysis of phosphorylation of Akt (p-Akt) and Extracellular signal-regulated kinase (p-ERK) in LVs. **H**, Densitometry of p-Akt and p-ERK in LVs 2 weeks after TAC (n=9). GAPDH was used as an internal control. *P<0.05 vs WT, by unpaired two-tailed Student's t-test. Data are presented as mean $\pm$ SEM.

Figure 4. Deficiency of miR-33 in fibroblasts impaired proliferative capacity. **A**, MiR-33 expression levels in neonatal rat cardiac myocytes and cardiac fibroblasts isolated by Percoll gradient separation (n=5). Expression of *U6* snRNA was used as an internal control. **P<0.01, by unpaired two-tailed Student's t-test. **B**, Growth-curve analyses of three types of fibroblasts; MEFs, cardiac fibroblasts, and tail-tip fibroblasts (MTT assay, n=5-8). *P<0.05, by unpaired two-tailed Student's t-test. **C**, Flow cytometric analysis of apoptotic cell death in WT and miR-33KO MEFs. Cells were treated with 1 mmol/L hydrogen peroxide (H_2O_2) for 4 h and stained with AnnexinV and propidium iodide (PI). **D**, Quantification of the early apoptotic cells (Q4: AnnexinV⁺ PI⁺) (n=4). **P<0.01 vs untreated control, ##P<0.01 vs WT, by ANOVA

with Sidak correction. **E**, Representative images of in vivo bromodeoxyuridine (BrdU) incorporation in WT and miR-33KO LVs at 7 days after TAC. White bars indicate 100 μ m and 30 μ m. **F**, Quantification of BrdU⁺vimentin⁺ fibroblasts in the BrdU incorporation assay (sham: n=4, TAC: n=9-10). *P<0.05, **P<0.01 vs sham, #P<0.05 vs WT, by ANOVA with Sidak correction. **G**, Western blotting analysis of phosphorylation of Akt (p-Akt) in CFs. WT or miR-33KO CFs cultured on 6-well plates were serum-starved for 2 h and stimulated with 3% fetal bovine serum (FBS) for 3 min. **H**, Densitometry of p-Akt in CFs stimulated with FBS in Figure 4G (n=12). Expression of GAPDH was used as an internal control. *P<0.05, by unpaired two-tailed Student's t-test. Data are presented as mean $\pm$ SEM.

Figure 5. MiR-33KO fibroblasts showed reduced lipid raft content. **A**, Expression of *Abca1* in miR-33KO cardiac fibroblasts (n=4). Expression of β -actin was used as an internal control. *P<0.05, **P<0.01, by unpaired two-tailed Student's t-test. **B**, Representative images of lipid raft staining by cholera toxin subunit B (CTB). Tail-tip fibroblasts from WT and miR-33KO mice were stained by Alexa Fluor[®]594-conjugated CTB. White bar indicates 50 μ m. **C**, Flow cytometric analysis of lipid raft staining. Tail-tip fibroblasts were stained using FITC-conjugated CTB after treated with or without 5 mmol/L methyl- β -cyclodextrin (M β CD) for 1 h. The grey curve indicates the unstained control. **D**, Quantification of lipid raft content labeled with CTB. Mean fluorescence intensity (MFI) of FITC-CTB were obtained using flow cytometry as shown in Figure 5C (n=3). *P<0.05 vs M β CD(-) control, #P<0.05 vs WT, by ANOVA with Sidak correction. **E**, Fibroblast proliferation in the knockdown of ABCA1. WT and miR-33KO MEFs were transfected with lentiviral small interfering RNA vectors against ABCA1 (siABCA1) and control (siControl). MTT assay was performed 1 day or 7 days after transfection (n=11). **P<0.01 vs WT, ##P<0.01 vs siControl, by ANOVA with Sidak correction. Data are presented as mean $\pm$ SEM.

Figure 6. Cardiac fibroblast-specific deficiency of miR-33 reduced cardiac fibrosis after TAC.

A, Expression levels of miR-33 in LVs of cardiac fibroblast-specific deficient mice (FBKO, Pn-Cre⁺miR-33^{flox/flox}) at 2 weeks after TAC (n=13-15). MiR-33^{flox/flox} littermates were used as controls. Mean expression level of five sham-operated miR-33^{flox/flox} mice was defined as 1.0. Expression of *U6* snRNA was used as an internal control. **B**, Heart weight of control and FBKO mice at 2 weeks after TAC (n=18). Heart weight was normalized by body weight. **C**, Representative images showing picrosirius red staining of LVs of control and FBKO mice. Black bars indicate 200 μ m. **D**, Quantification of fibrotic area determined by picrosirius red staining in LVs of control and FBKO mice 2 weeks after TAC (n=18). **E**, Representative images of immunostaining of Ki-67 in control and FBKO LVs 2 weeks after TAC. White bars indicate 100 μ m and 30 μ m. **F**, Quantification of Ki-67 positive fibroblasts in LVs 2 weeks after TAC (n=15). *P<0.05, n.s., not significant, by unpaired two-tailed Student's t-test. Data are presented as mean $\pm$ SEM.

NOVELTY AND SIGNIFICANCE

What Is Known?

- Multiple processes such as myocyte hypertrophy, immune cell activation, and fibrosis are involved in heart failure.
- MicroRNAs play critical roles in heart failure by fine-tuning gene expression.
- MicroRNA-33 (miR-33) regulates lipid metabolism and inflammation.

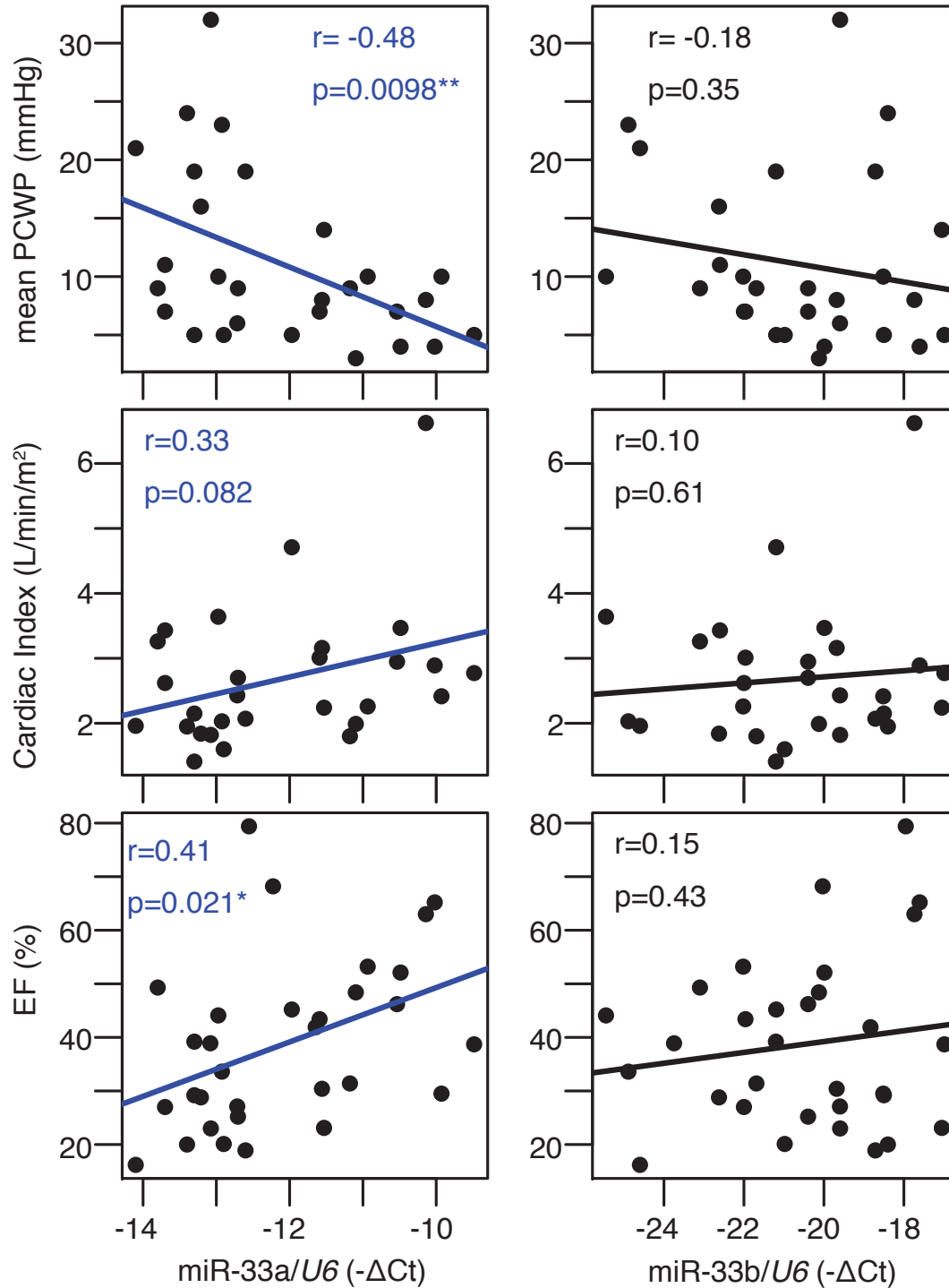
What New Information Does This Article Contribute?

- MiR-33 is involved in cardiac remodeling, especially in cardiac fibrosis.
- MiR-33 regulates fibroblast proliferation by maintaining lipid raft cholesterol content.

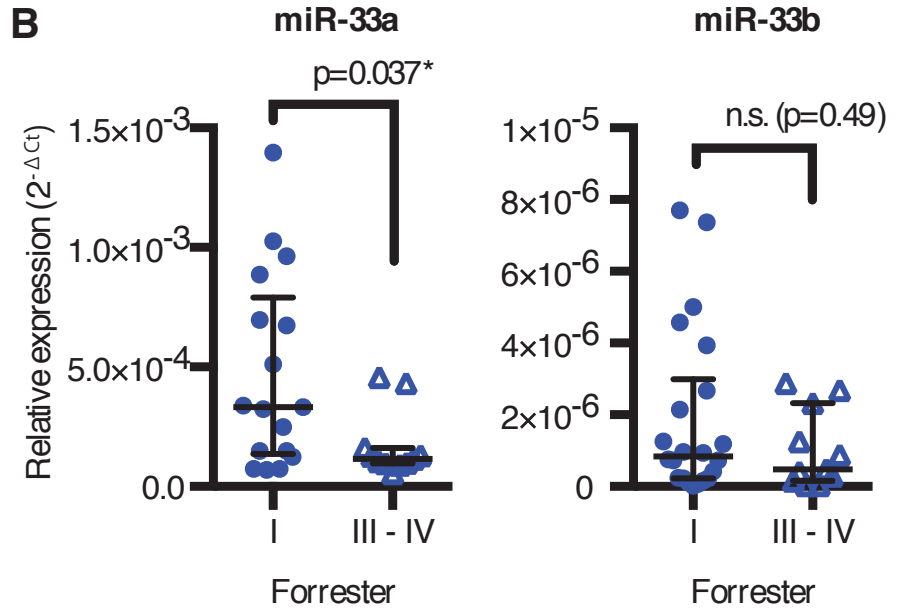
Chronic inflammation followed by fibrosis is an important underlying mechanism of heart failure. The similar processes can be seen in other disorders including atherosclerosis. Inhibition of miR-33 is a potent therapeutic strategy for atherosclerosis because miR-33 reduces high-density lipoprotein cholesterol levels and promotes chronic inflammation. However, the role of miR-33 in the heart is unknown. Here, we showed that miR-33 was involved even in cardiac remodeling, especially in fibrosis, using human samples and genetic mouse models. In human samples, the expression of miR-33 was down-regulated in high-stage heart failure patients. In the mouse models, deficiency of miR-33 reduced cardiac fibrosis but worsened systolic function after pressure overload in vivo. Deficiency of miR-33 impaired fibroblast proliferation due to decreased lipid raft cholesterol in vitro. Our data showed new roles of miR-33 in cardiac remodeling and fibroblast proliferation. This study is notable in that it showed the relationship between cellular lipid homeostasis and fibrotic responses. This study also implicated the possibility of pleiotropic effects of miR-33 inhibition, regardless of protective or harmful effects, because it may modulate lipid rafts. It will be necessary to consider these effects in order to translate the silencing of miR-33 to humans.



A



B



C

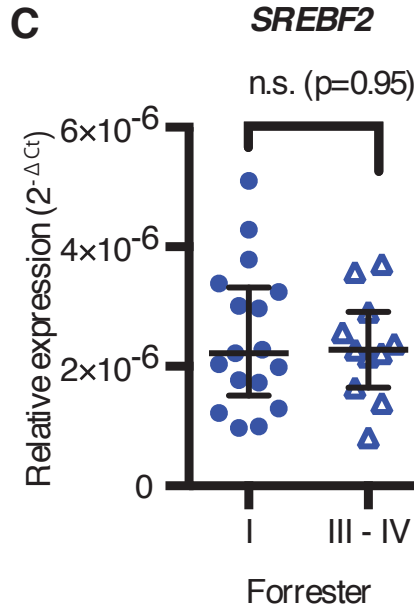


Figure 1.

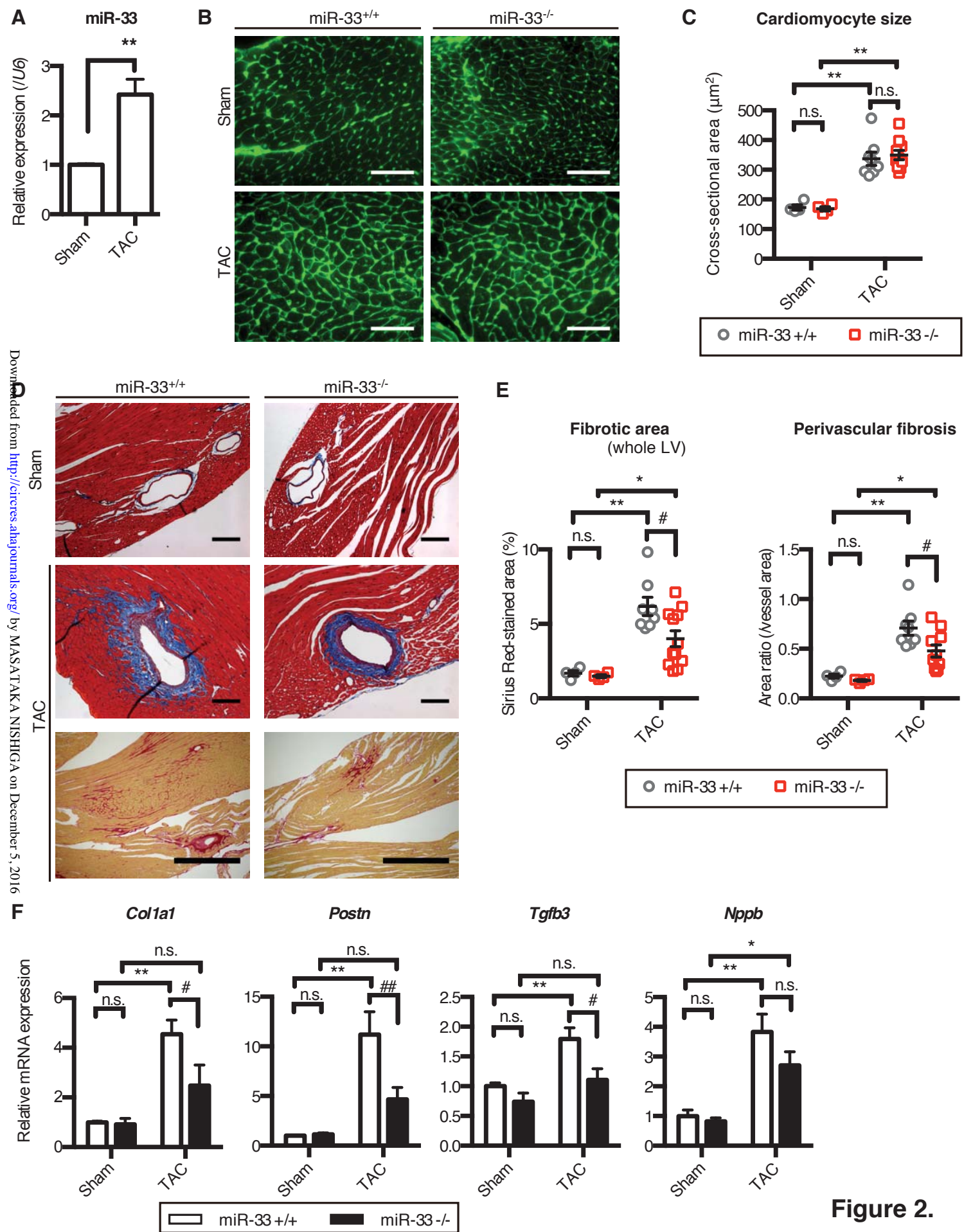


Figure 2.

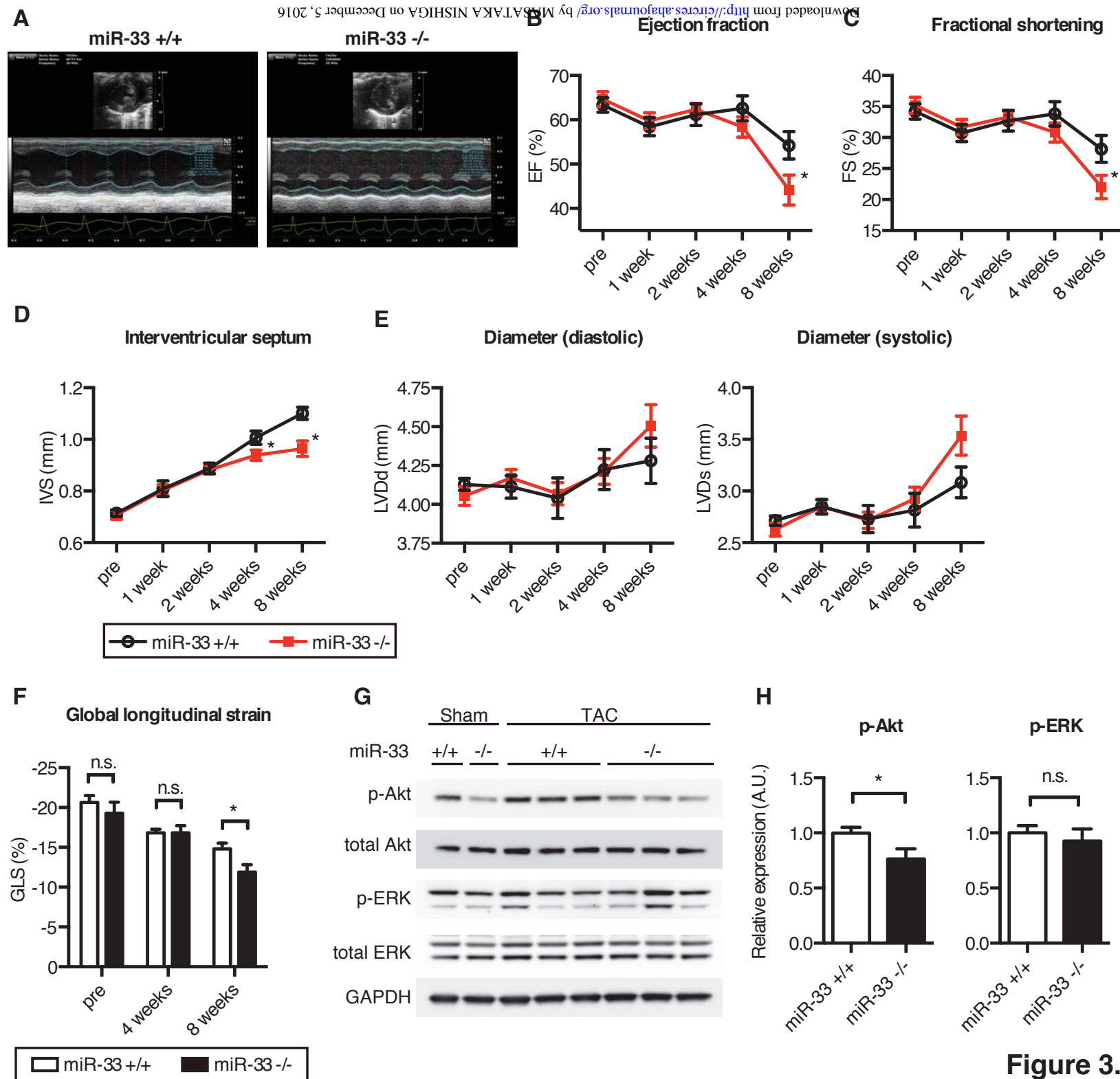


Figure 3.

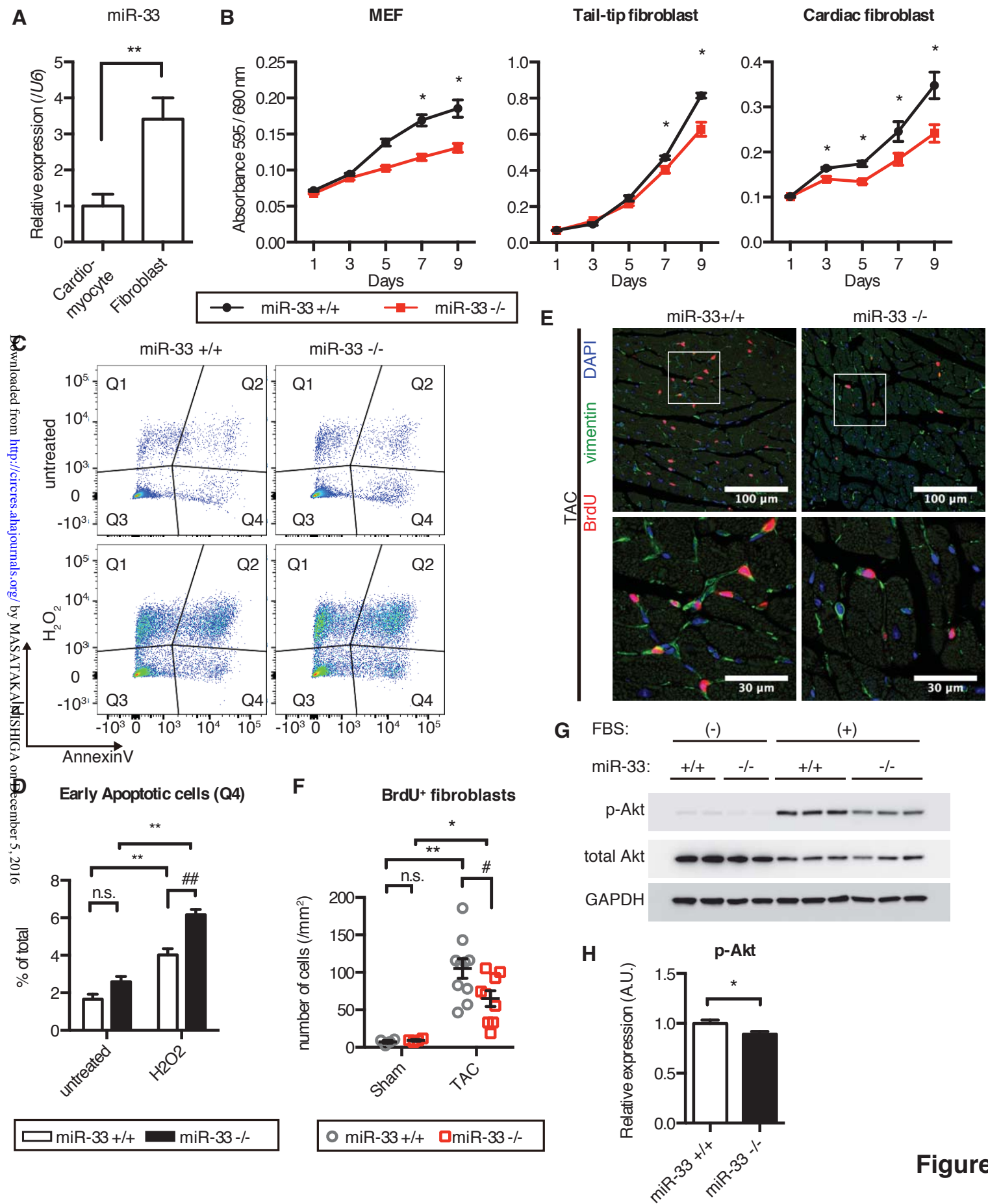


Figure 4.

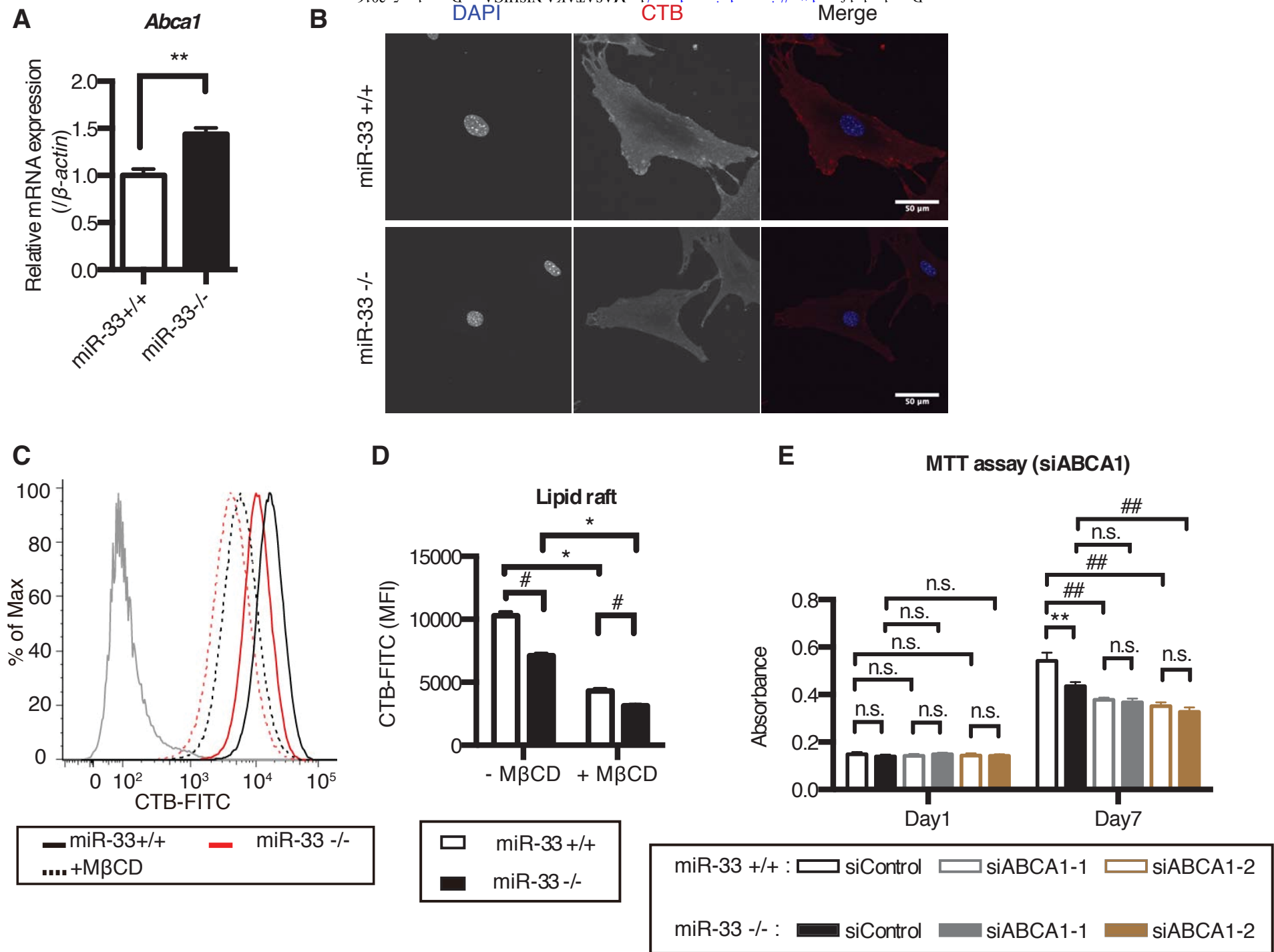


Figure 5.

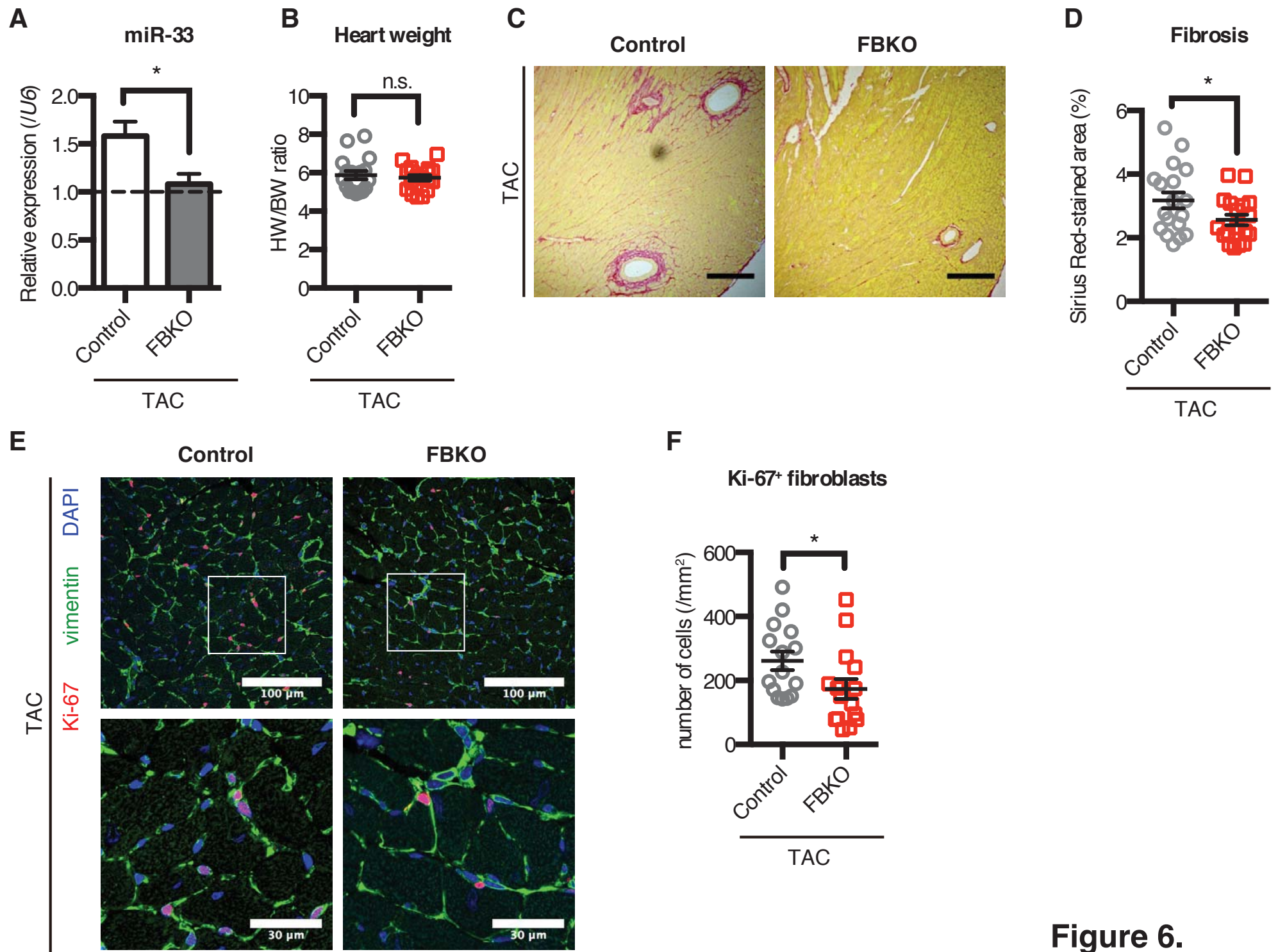


Figure 6.

MicroRNA-33 Controls Adaptive Fibrotic Response in the Remodeling Heart by Preserving Lipid Raft Cholesterol

Masataka Nishiga, Takahiro Horie, Yasuhide Kuwabara, Kazuya Nagao, Osamu Baba, Tetsushi Nakao, Tomohiro Nishino, Daihiko Hakuno, Yasuhiro Nakashima, Hitoo Nishi, Fumiko Nakazeki, Yuya Ide, Satoshi Koyama, Masahiro Kimura, Ritsuko Hanada, Tomoyuki Nakamura, Tsukasa Inada, Koji Hasegawa, Simon J Conway, Toru Kita, Takeshi Kimura and Koh Ono

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**MicroRNA-33 controls adaptive fibrotic response in the remodeling
heart by preserving lipid raft cholesterol**

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SUPPLEMENTAL MATERIAL

Detailed methods

Myocardial biopsy

We analyzed the expression of miR-33a/b in the tissues of left ventricle (LV) endomyocardial biopsies and examined the relationships between their expression levels and hemodynamic parameters in 33 patients who were diagnosed with dilated cardiomyopathy (DCM) at Osaka Red Cross Hospital.¹ The biopsies were performed to determine the pathogenesis of heart failure (HF) in accordance with the following institutional criteria: (i) new onset HF with clinical symptoms of dyspnea, chest pain, or palpitation, (ii) unexplained impairment of LV function and LV dilatation, (iii) no evidence of coronary artery disease or primary valvular disease. These criteria were in agreement with a guideline. Hemodynamic parameters were obtained by the catheterization. Final diagnoses were obtained on the basis of clinical history, laboratory examinations, electrocardiogram (ECG), echocardiography, other disease-specific tests such as computed tomography and nuclear imaging, biopsies from extracardiac tissues, as well as histological analysis of the biopsy samples. All patients provided written informed consent for the procedure and gene expression analyses. The Ethics Committee of Osaka Red Cross Hospital approved the study protocol.

Mice

MiR-33-deficient mice were generated as reported previously.² To match genetic background between WT and miR-33KO mice, a miR-33^{+/-} male mouse and a miR-33^{+/-} female mouse were

crossed to generate miR-33^{+/+} and miR-33^{-/-} mice, and the offspring mice were used for analyses. MiR-33-floxed mice were generated as described below. Periostin-Cre mice were generated by Dr. Conway and colleagues.^{3, 4} Mice were maintained in temperature-controlled rooms with a 14:10 h light:dark cycle in specific pathogen-free conditions at the Institute of Laboratory Animals of Kyoto University Graduate School of Medicine. This study was approved by the Kyoto University Ethics Review Board. The primer sequences for genotyping are listed in Online Table II. All the *in vivo* experiments were performed using male mice in a C57BL/6J background.

Generation of miR-33-floxed mice.

We generated miR-33-floxed mice by homologous recombination. First, a targeting vector was constructed by modifying bacterial artificial chromosome RP24-291F2 (BACPAC Resources Center) using defective prophage λ -Red recombination system.⁵⁻⁷ A loxP sequence was inserted to 40 bp upstream of the miR-33 coding region, and another loxP sequence was inserted to downstream of the miR-33 coding region. As a selection marker, a neomycin resistance cassette flanked by FRT was inserted into the site between the miR-33 coding region and the downstream loxP (Online Figure VIIIA). We electroporated the targeting vector into C57BL/6J mouse embryonic stem (ES) cells (CHEMICON, CMTI-2) using NucleofectorTM (Lonza) and a Mouse ES Cell Nucleofector[®] Kit (Lonza, VPH-1001). Positive clones were selected by incubating cells with 200 μ g/mL Geneticin[®] (Thermo Fisher Scientific, 10131-035) for 7 days, and homologous recombination was confirmed by Southern blotting (Online Figure VIIIC). Successfully recombined ES cells were injected into blastocysts from ICR mice supplied by

Unitech Inc, and chimeric mice were bred with C57BL/6J mice to generate F1 mice. The genotype of F1 mice was confirmed by PCR. The neomycin resistance cassette was removed from the mouse germ line by crossing with CAG-FLPe transgenic mice, which express FLPe recombinase under the control of the CAG promoter (Riken, RBRC01834). The offspring miR-33^{flox/+} mice without the CAG-FLPe allele were crossed with each other to generate miR-33^{flox/flox} mice. The genotype of miR-33^{flox/flox} mice was confirmed by PCR and Southern blotting (Online Figure VIIIB and VIID). To confirm Cre-mediated deletion of miR-33, we crossed miR-33^{flox/flox} mice with Ayu1-Cre mice, which express Cre-recombinase ubiquitously (B6;D2-Tg(Ayu1-Cre)8Imeg, a gift from Dr. Yamamura). We ensured that miR-33 was deleted at the genomic and transcript levels in the descendant Ayu1-Cre⁺miR-33^{flox/flox} (miR-33^{Δ/Δ}) mice (Online Figure IXA), and splicing of *Sreb2* mRNA was not altered (Online Figure IXB and IXC). To generate cardiac fibroblast (CF)-specific knockout mice, miR-33^{flox/flox} mice and Pn-Cre⁺miR-33^{flox/flox} mice were crossed, and miR-33^{flox/flox} littermates were used as control mice. Primer sequences for the probe for Southern blotting and genotyping are shown in Online Table II.

Pressure-overload model

Transverse aortic constriction (TAC) was performed as described previously.^{8,9} Briefly, 10- to 12-week-old mice were anesthetized with sodium pentobarbital (64.8 mg/kg) administered intraperitoneally and the proximal portion of the sternum was cut open to visualize the aorta. A 7-0 silk suture was placed around the aortic arch distal to the brachiocephalic artery. The suture was tightened firmly around a blunt 26-gauge needle placed adjacent to the aorta. The needle

was then removed, and the chest and overlying skin were closed. Sham-operated mice underwent the identical surgical procedure without ligation of aortic arch.

Primary neonatal rat cardiomyocytes and fibroblasts

Neonatal rat cardiomyocytes and CFs were isolated from 1-day old Sprague-Dawley rats, as described previously.^{10, 11} After enzymatic digestion with pancreatin (Sigma, P3292), cardiomyocytes and fibroblasts were purified by Percoll density gradient centrifugation (Sigma, P4937). We made two-layer density gradients consisting of red-colored 65% Percoll solution underneath transparent 45% Percoll solution in 15 mL tubes. The cell suspension was layered on top of the gradient and the tubes were centrifuged at 3,000 rpm for 30 min. The fraction of cardiomyocytes was harvested from the newly formed layer between the Percoll solutions, and the fraction of fibroblasts was harvested from the top of the transparent Percoll solution.

Culture of mouse fibroblasts

For isolation of adult CFs, hearts from 8-week-old mice were digested with collagenase/ dispase solution (Roche, 11097113001) and plated for 2 h.¹² Attached CFs were cultured in Dulbecco's modified Eagle's medium (DMEM, 1% glucose) (Nacalai Tesque) supplemented with 10% fetal bovine serum (FBS) and antibiotics (GibcoTM, 10378016) and non-essential amino acids (GibcoTM, 11140050). We used adult CFs at passage 3-4. For isolation of tail-tip fibroblasts (TTFs), the tails from 8-week-old mice were peeled, minced into 1 cm pieces, placed on gelatin-coated culture dishes, and incubated in DMEM (1% glucose) supplemented with 10% FBS and antibiotics for 5 days.¹³ Cells that migrated out of the graft pieces were transferred to

new plates (passage 1). We used TTFs at passage 4-5. Mouse embryonic fibroblasts (MEFs) were isolated from WT and miR-33KO embryos at E14.5 and were maintained in DMEM (4.5% glucose) supplemented with 10% FBS and antibiotics. We used MEFs at passage 5-6. In the experiment of Akt activation, fibroblasts on 6-well plates at a density of 2×10^5 cells/well were serum-starved for 2 h and stimulated with 3% FBS for 3 min. The fibroblasts were washed with chilled $1 \times$ phosphate-buffered saline (PBS) and then collected in chilled lysis buffer. In the experiment using LXR agonist, fibroblasts were cultured on 6-well plates at a density of 2×10^5 cells/well, and stimulated with 1 μ mol/L T0901317 (Cayman, 71810) for 24 h.

Culture of mouse macrophage

Peritoneal macrophages were obtained from the peritoneal cavity of WT and miR-33KO mice 4 days after intraperitoneal injection of 3 mL 3% thioglycolate. The cells were washed with RPMI1640 (Nacalai Tesque), spun at 1200 rpm for 3 min, and plated at a density of 1.0×10^6 cells/mL. Cells were washed 1 h later and incubated for 2 days, and then used for experiments.

Lentiviral transfection

Lentiviral stocks were produced in 293T packaging cells as described previously.^{2, 10, 11} Briefly, lentiviral vectors were transfected into 293T cells using Polyethylenimine Max (Polysciences, Inc.), and virus-containing medium was collected 24 h after transfection and filtered through a 0.45- μ m filter. One round of lentiviral infection was performed by replacing the medium with virus-containing medium containing 8 μ g/mL polybrene, followed by centrifugation at 1220 g for 30 min. For overexpression of miR-33, we used lentiviral expression vectors for miR-33 and

a negative control (miR-control) generated using a BLOCK-iT™ Pol II miR RNAi Expression Vector Kit (Thermo Fisher Scientific) in accordance with the manufacturer's protocol. The miR-control vector contained a hairpin structure just as for a regular premiRNA, but which was predicted not to target any known vertebrate gene (pcDNA6.2-GW/EmGFP-miR-neg control plasmid). For knockdown of *Abca1*, we used shRNA lentiviral vectors. The siRNA sequences were designed using siDirect version 2.0 (<http://sidirect2.rnai.jp/>), and then the designed shRNA oligonucleotides were inserted into lentiviral vectors. The siRNA target sequences were as follows: AAATGTACTGCGCGTGGAG for siControl, GAAGAATCTGACATTTCTGAAG for siABCA1-1, and GAAAGAAAGTTATGTATGAAG for siABCA1-2.

Transfection of microRNA mimics

For transfection of miR-33, fibroblasts were transfected with 1 nmol/L miR-33 mimic (mirVana™ miRNA mimic, P/N 4464066, ID: MC12410) or control mimic (P/N 4464058) using Lipofectamine® 2000 Transfection Reagent (Thermo Fisher Scientific) in accordance with the manufacturer's protocol. To analyze ABCA1 expression in Online Figure VIIB, CFs were transfected with 1nmol/L miR-33 mimic or control mimic, incubated for 24 h, and then treated with LXR agonist (1 µmol/L T0901317) for 24 h.

RNA extraction

To extract total RNA, tissue samples were homogenized using a polytron homogenizer in 1 mL TRI Reagent® (Sigma, T9424), and monolayer cells were lysed directly on culture dishes in 1 mL TRI Reagent®. Total RNA was purified in accordance with the manufacturer's protocol. The

quantity and quality of total RNA were measured by using a NanoDrop™2000 spectrophotometer.

Quantitative real-time PCR

To evaluate mRNA expression, single-strand cDNA was synthesized from 1 µg of total RNA by means of reverse transcriptase reaction using Verso™ cDNA Synthesis Kit (Thermo Fisher Scientific, AB1453A) in accordance with the manufacturer's protocol, and quantitative PCR was performed using a LightCycler®96 (Roche Diagnostics) with THUNDERBIRD® SYBR qPCR Mix (TOYOBO, QPS-201). Expression levels were normalized by the indicated housekeeping genes. The primer sequences are listed in Online Table III.

Quantitative real-time PCR for microRNAs

Expression levels of miR-33a and miR-33b were measured using TaqMan® MicroRNA Assays (Applied Biosystems) and StepOnePlus™ Real-Time PCR System (Applied Biosystems) in accordance with the manufacturer's protocol. Expression levels of miRs were normalized by *U6* small nuclear RNA and calculated by the $2^{-\Delta\Delta Ct}$ method.

Microarray and Gene Ontology analysis

To analyze gene expression comprehensively, we performed DNA microarray analysis. Five RNA samples from each group (WT sham, WT TAC, miR-33KO sham, and miR-33KO TAC) were pooled and analyzed using a DNA microarray (3D-Gene Mouse Oligo Chip 24k, TORAY). For microarray analysis of miRs, Mouse miRNA Oligo chip (TORAY) was used. After global

normalization, MA plots were made to visualize the normalized microarray data and to identify differentially expressed genes. Gene Ontology analyses were performed using data sets of differentially expressed genes with the clusterProfiler package in R/Bioconductor. Heatmaps were made using the gplots package in R/Bioconductor.

Western blotting

Western blotting was performed using standard procedures as described previously.¹⁴ *In vitro* cell lysates were collected using cell scrapers in chilled lysis buffer consisting of 100 mM Tris-HCl, pH 7.4, 75 mM NaCl and 1% Triton X-100 (Nacalai Tesque) supplemented with Complete Mini protease inhibitor cocktail (Roche, 11836153001), 0.5 mM NaF and 10 mM Na₃VO₄ just before use. *In vivo* tissue samples were homogenized using a polytron homogenizer and lysed in chilled lysis buffer. The protein concentration was determined using BCA protein assay kit (Bio-Rad, 5000006JA). All samples (10 µg of protein) were suspended in lysis buffer, fractionated using NuPAGE 4–12% Bis-Tris Mini gels (Thermo Fisher Scientific, NP0322BOX) and transferred to a Protran nitrocellulose transfer membrane (Whatman). The membrane was blocked using 1× PBS containing 5% non-fat milk for 30 min and incubated with the primary antibody (Online Table IV) overnight at 4 °C. After a washing step in PBS-0.05% Tween-20 (PBS-T), the membrane was incubated with the secondary antibody (Online Table IV) for 1 h at room temperature. The membrane was then washed with PBS-T and detected using Millipore IMMOBILON Western Chemiluminescent HRP Substrate (ECL) (Fisher Scientific Co., WBKLS0500) or Pierce Western Blotting Substrate (Thermo Fisher

Scientific, NCI3106), with an LAS-4000 Mini system (Fuji Film). For quantification of western blots, densitometric analyses were performed using Fiji software.¹⁵

Histology

After administration of an overdose of anesthetics, mice were perfused with 4% paraformaldehyde (PFA) before excising the heart, and the tissue samples were further fixed in 4%PFA at 4°C overnight. On the next day, the tissue samples were transferred to 70% ethanol for dehydration before embedding in paraffin. After the heart sections were deparaffinized, they were stained with hematoxylin and eosin, Masson's trichrome, and picrosirius red staining. Images were acquired using a microscope (BZ-9000, Keyence). We measured the fibrotic area of picrosirius-red staining images using Fiji.¹⁵ To analyze the perivascular fibrosis, the fibrotic areas around main coronary arteries in Masson's trichrome staining were measured using Fiji, and the areas were normalized using the corresponding vessel areas. For wheat germ agglutinin (WGA) staining, heart sections were incubated for 1 h at room temperature with FITC-labeled WGA (Sigma, L4895) to visualize myocyte membranes. Cardiomyocyte cross-sectional areas were measured using Fiji.¹⁵ More than 100 cells per heart were measured, and the average values were used for analysis.

Lipid raft staining

We used fluorescence-conjugated cholera toxin subunit B (CTB) for lipid raft labeling. To obtain images of lipid raft staining, fibroblasts were plated on chamber slides and stained with Vybrant® Alexa Fluor® 594 Lipid Raft Labeling Kit (Molecular Probes™, V34405). The slides

were observed using a confocal microscope. To quantify the signals from CTB-labeled lipid rafts on the cell surface, flow cytometric analyses were performed. Fibroblasts were plated on 10-cm culture dishes and cultured overnight. The cells were dissociated with trypsin EDTA, and stained with 1 µg/mL FITC-conjugated CTB (Sigma, C1655) at 4°C for 15 min, and then analyzed on FACS Aria II™ (Becton Dickinson). To make control samples with decreased lipid rafts, some samples were incubated with 5 mmol/L methyl-β-cyclodextrin (MβCD) for 1 h before labeling with CTB.

BrdU incorporation in vivo

In vivo bromodeoxyuridine (BrdU) incorporation was performed to analyze cell proliferation in the heart. Mice were subjected to TAC, and 7 days later 100 mg/kg BrdU (Sigma, B9285) was administrated intraperitoneally 2 h before sacrifice. Hearts were fixed as described above. After fixation, the tissues were transferred to 15% sucrose solution at 4°C for 4 h and to 30% sucrose solution at 4°C overnight, and then embedded in Tissue-Tek OCT compound (Sakura, Japan). BrdU staining was performed for 10-µm frozen sections. The sections were rinsed in PBS and autoclaved (110°C, 20 min) in 10 mmol/L sodium citrate buffer (pH 6) for heat-mediated antigen retrieval. The sections were blocked with 5% donkey serum/PBS at room temperature for 15 min, and then incubated with primary antibodies at 4°C overnight. An anti-BrdU antibody (Abcam, ab6326, 1:100 dilution) was used for BrdU detection. An anti-vimentin antibody (PROGEN, GP53, 1:100 dilution) was used for double staining with a fibroblast marker, vimentin. The slides were rinsed three times and then incubated with Alexa Fluor® 488- or 594-conjugated secondary antibodies (Online Table IV) with DAPI (1 µg/mL) at room

temperature for 1 h. The slides were washed and mounted in VECTASHIELD[®] Mounting Medium (Vector Laboratories, H-1000), and then observed under a Leica TCS SP8 confocal microscope with a 40× objective in accordance with the manufacturer's instructions. Slides without primary antibodies were used as negative controls. More than 10 images per heart were randomly acquired from LVs. The number of BrdU⁺ vimentin⁺ cells in each image was counted using Fiji,¹⁵ and the average value of the images from each sample was used for analysis.

Immunostaining

Immunostaining for Ki-67 or CD68 was performed using paraffin-embedded sections. After the heart sections were deparaffinized, they were rinsed in PBS and autoclaved for heat-mediated antigen retrieval in citrate buffer pH 6.0 for CD68 staining, and in EDTA buffer pH 8.0 for Ki-67 staining. The sections were blocked with 5% donkey serum/PBS at room temperature for 15 min, and then incubated with primary antibodies at 4°C overnight (Online Table IV). The slides were rinsed three times and then incubated with Alexa Fluor[®] 488- or 594-conjugated secondary antibodies (Online Table IV) with DAPI (1µg/mL) at room temperature for 1 h. The slides were washed and mounted in VECTASHIELD[®] Mounting Medium (Vector Laboratories, H-1000), and then observed using a Leica TCS SP8 confocal microscope with a 40× objective in accordance with the manufacturer's instructions. Slides without primary antibodies were used as negative controls. For quantification of Ki-67 positive cells, more than 10 images per heart were randomly acquired from LVs. The number of Ki-67⁺ vimentin⁺ cells in each image was counted using Fiji,¹⁵ and the average value of the images from each sample was used for analysis.

MTT assay

Proliferation of fibroblasts was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were plated on 96-well culture dishes at a density of 1,000 cells/well and incubated in 100 μ L medium containing 10% FBS. At the indicated time point, 10 μ L of MTT solution (5 mg/mL) was added. Cells were incubated at 37°C for 4 h and lysed by adding 100 μ L acidified isopropanol (40 mmol/L HCl). The absorbance at 595 nm was measured using a plate reader (ARVO X3, PerkinElmer). The reference absorbance at 690 nm was used to correct for non-specific background values.

Flow cytometric analysis of apoptotic cells

MEFs were plated on 10-cm culture dishes at a density of 3.0×10^5 cells/dish and cultured for 24 h in DMEM supplemented with 10% FBS. On the next day, the cells were stimulated with 1 mmol/L hydrogen peroxide (H_2O_2) for 4 h. The cells were dissociated with trypsin EDTA, and rinsed with PBS and stained using a Dead Cell Apoptosis Kit with Alexa[®] Fluor 488 annexin V and PI (Molecular Probes[™], V13241), and then analyzed on FACS Aria II[™] (Becton Dickinson) in accordance with the manufacturer's instructions. The flow cytometric data were analyzed using FlowJo version 10.1 (FLOWJO, LLC).

Southern blotting

Southern blotting was performed using a DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, 11585614910) in accordance with the manufacturer's protocol. Genomic DNA samples were purified and digested with *Bam*HI. Primer sequences used to amplify the probe are shown in Online Table II.

Echocardiography

To analyze the cardiac function of mice, we performed echocardiography (Vevo[®]2100, VISUALSONICS) at the indicated time points after TAC. Mice were kept under inhalation anesthesia with 2.0% isoflurane. Left ventricular wall thickness and diameters were measured in M mode of the parasternal short-axis view. To analyze speckle-tracking strain imaging, we used Vevo Strain (Vevo[®]2100). A parasternal long-axis view was used to measure global longitudinal strain (GLS) and longitudinal strain rate (LSR). Endocardium was traced, and GLS (negative value), LSR (negative value), and early diastolic LSR (positive value) were calculated in Vevo Strain in accordance with the manufacturer's protocol.

Statistical analysis

Measurements for clinical biopsy samples are presented as median $\pm$ interquartile range, and other measurements are presented as means $\pm$ standard error of the mean (SEM). To compare gene expression levels and the hemodynamic parameters in Figure 1A, linear-regression analysis was performed on R version 3.2.3 (The R Project). P values and r values are shown. For Gene Ontology analysis, the clusterProfiler package in R/Bioconductor was used for data sets with differentially expressed genes as described above. P values were adjusted by FDR

correction. For other statistical comparisons, unpaired Student's t-test (two groups, parametric), Mann–Whitney test (two groups, non-parametric), or one-way ANOVA with Sidak's post-hoc test (three or more groups) were used as indicated in the figure legends. A p value of <0.05 was considered as statistically significant. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc.).

Online Table I. Patient characteristics

	Total	Low-stage HF	High-stage HF	P value
Patients, n	33	17	11	
Age, y	55.8 ± 12.3	54.8 ± 14.2	58.4 ± 10.8	0.47
Female, n (%)	13 (39%)	8 (47%)	2 (18%)	0.23
Body Mass Index, kg/m ²	25.2 ± 4.2	24.3 ± 4.1	26.1 ± 4.1	0.28
Diabetic mellitus, n (%)	4 (12%)	1 (5.9%)	3 (27%)	0.27
Hypertension, n(%)	12 (36%)	7 (41%)	4 (36%)	1.0
Dyslipidemia, n (%)	7 (21%)	3 (18%)	2 (18%)	1.0
Hemodynamic parameters				
Cardiac Index, L/min/m ²	2.7 ± 1.0	3.2 ± 1.1	1.9 ± 0.2	0.0004***
mean PCWP, mmHg	11.1 ± 7.1	7.9 ± 2.7	16.0 ± 9.3	0.0022**
Ejection fraction, %	38.7 ± 15.6	41.4 ± 13.4	28.1 ± 9.8	0.015*
Serum lipid levels				
Total cholesterol, mg/dL	195.2 ± 57.0	207.6 ± 64.2	164.6 ± 25.8	0.052
Triglyceride, mg/dL	127.0 ± 71.1	138.0 ± 85.8	122.6 ± 46.1	0.59
HDL-C, mg/dL	47.1 ± 13.6	50.9 ± 12.6	40.5 ± 9.1	0.053
LDL-C, mg/dL	121.0 ± 42.7	128.2 ± 50.3	102.5 ± 17.0	0.13
Medication, n (%)				
Statin	5 (15%)	2 (12%)	1 (9.1%)	1.0
ACEI/ARB	18 (55%)	11 (65%)	6 (55%)	0.70
β blocker	20 (61%)	12 (71%)	7 (64%)	1.0
Furosemide	16 (48%)	4 (24%)	9 (82%)	0.0056**
Spironolactone	8 (24%)	3 (18%)	5 (45%)	0.20
Thiazide	1 (3.0%)	0 (0%)	1 (9.1%)	0.39

Data for continuous variables and categorical variables are presented as mean ± SD and number of patients (%), respectively. Five patients did not have the parameters for Forrester classification. P values for continuous variables and categorical variables were calculated by two-tailed unpaired Student's t-tests and two-tailed Fisher's exact tests, respectively.

*P<0.05, **P<0.01, ***P<0.001

Online Table II. Primer sequences for genotyping and Southern blotting probes

Genotype	Sequence	Product size (bp)
miR-33 ^{flox}	(F) TCTTCTCCCGAGCTCTCTTACTCTCA	465/327/223
	(R) TTATTCCCCACGATGGTTAGGTAGGC	(floxed/WT/ Δ)
miR-33 ^{floxNeo}	(F) GTCTGAGTAGGTGTCATTCTATTCTGGGGG	2445
	(R) GTACAATCTATGCCTCTCCCCTTACTGCTG	
Cre	(F) GAACCTGATGGACATGTTTCAGG	320
	(R) AGTGCGTTCTGAACGCTAGAGCCTGT	
Myogenin (internal control)	(F) TTACGTCCATCGTGGACAGC	250
	(R) TGGGCTGGGTGTTAGCCTTA	
probe (Southern blotting)	(F) AATGCAGTGAGCAGGTGGAGTTTG	865
	(R) ACTGCACTTGAGTTCAGACGCTAC	

F: Forward, R: Reverse

Online Table III. Primer sequences for quantitative real-time PCR

Species	Gene	Refseq		Sequence	Product size (bp)
Human	<i>SREBF2</i>	NM_004599	(F)	AGGAGAACATGGTGCTGA	318
			(R)	TAAAGGAGAGGCACAGGA	
	<i>I8S</i>	NR_003286	(F)	AGAAACGGCTACCACATCCA	158
			(R)	CCCTCCAATGGATCCTCGTT	
Mouse	<i>Srebf2</i>	NM_033218	(F)	GTGGAGCAGTCTCAACGTCA	150
			(R)	TGGTAGGTCTCACCCAGGAG	
	<i>Abcal</i>	NM_013454	(F)	AACAGTTTGTGGCCCTTTTG	157
			(R)	AGTTCCAGGCTGGGGTACTT	
	<i>Abcg1</i>	NM_009593	(F)	CTGCCTCACCTCACTGTTCA	80
			(R)	TCTCGTCTGCCTTCATCCTT	
	<i>Npc1</i>	NM_008720	(F)	ACTCTTGTGTGGGAGGGATG	209
			(R)	AGCAGTCCTGGCAGCTACAT	
	pri-miR-33		(F)	TGGCAGCTTCCTCTAAGTCC	155
			(R)	TGCAACTACAATGCACCACA	
	pre-miR-33	NR_029804	(F)	TGTGGTGCATTGTAGTTGCAT	67
			(R)	TGTTTCCACAGTGCATCACG	
	<i>Colla1</i>	NM_007742	(F)	GCCAAGAAGACATCCCTGAAG	139
			(R)	TCATTGCATTGCACGTCATC	
	<i>Postn</i>	NM_015784	(F)	AACCAAGGACCTGAAACACG	170
		NM_001198765	(R)	GTGTCAGGACACGGTCAATG	
		NM_001198766			
		XM_006501653			
	<i>Adgre1</i>	NM_010130	(F)	TCCAGAAGGCTCCCAAGGATA	200
	(F4/80)		(R)	GGGCACTTTTGTCTCACAGGTA	
	<i>IL-10</i>	NM_010548	(F)	AAATAAGAGCAAGGCAGTGGAG	81
			(R)	TCATTTCATGGCCTTGTAGACAC	
	<i>IL-6</i>	NM_031168	(F)	ACCACGGCCTTCCCTACTTC	200
			(R)	AGATTGTTTTCTGCAAGTGCATCA	
	<i>Tgfb1</i>	NM_011577	(F)	AGGGCTACCATGCCAATTCT	102
			(R)	CCGGGTGTGTTGGTTGTAGA	
	<i>Tgfb3</i>	NM_009368	(F)	GGAAGGCTGCACTCAGGAGA	101
			(R)	CGGCCAGTTCATTGTGCTC	
	<i>Nppb</i>	NM_008726	(F)	GCCAGTCTCCAGAGCAATTCA	112
			(R)	TGTTCTTTTGTGAGGCCTTGG	
	<i>Gapdh</i>	NM_001289726	(F)	AAATGGTGAAGGTCGGTGTG	86
		NM_008084	(R)	AATCTCCACTTTGCCACTGC	
	<i>β-actin</i>	NM_007393	(F)	AGATTACTGCTCTGGCTCCTA	221
			(R)	CAAAGAAAGGGTGTAAAACG	
	<i>I8S</i>	NR_003278	(F)	CGCGGTTCTATTTTGTGTTGGT	219

			(R)	AGTCGGCATCGTTTATGGTC	
Rat	<i>Nppb</i>	NM_031545	(F)	TTCCGGATCCAGGAGAGACTT	124
			(R)	CCTAAAACAACCTCAGCCCGT	
	<i>Colla1</i>	NM_053304	(F)	TCCTGACGCATGGCCAAGAA	145
			(R)	CATAGCACGCCATCGCACAC	
	<i>β-actin</i>	NM_031144	(F)	CCCATCTATGAGGGTTACGC	150
			(R)	TTTAATGTCACGCACGATTTC	

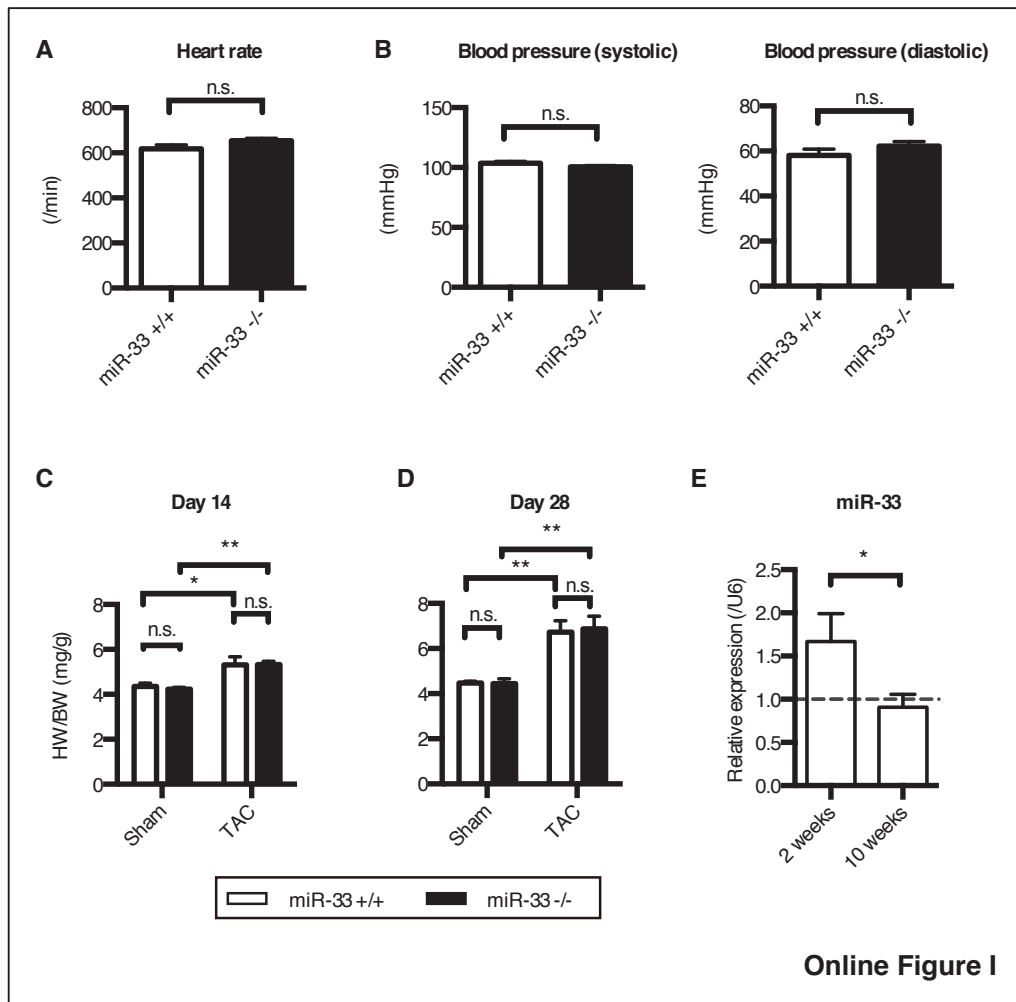
Online Table IV. Antibodies used for western blotting and immunostaining.

Methods	Antibodies	Product number	Dilution
Western blotting	anti-GAPDH	CST #2118	1:3000
	anti-ABCA1	Novus NB400-105	1:500
	anti-ABCG1	Novus NB400-132	1:500
	anti-NPC1	Novus NB400-148	1:1000
	anti-phospho-Akt (Ser 473)	CST #9271	1:1000
	anti-total Akt	CST #9272	1:1000
	anti- phospho-ERK	CST #9101	1:1000
	anti-total ERK	Santa Cruz sc-94	1:1000
	anti-CDK6	CST #3136	1:2000
	anti-Rabbit IgG, HRP-Linked	GE NA934V	1:2000
	anti-Mouse IgG, HRP-Linked	GE NA931V	1:2000
Immunostaining	anti-BrdU	Abcam ab6326	1:100
	anti-Ki67	Nichirei 418071	1:1
	anti-vimentin	PROGEN GP53	1:100
	anti-TroponinI	Abcam ab47003	1:200
	anti-CD68	BioRad MCA1957	1:200
	Alexa Fluor® 488 AffiniPure Donkey Anti-Guinea Pig IgG (H+L)	Jackson 706-545-148	1:200
	Donkey anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 488	ThermoFisher A21206	1:200
	Donkey anti-Rat IgG (H+L) Secondary Antibody, Alexa Fluor 594	ThermoFisher A21209	1:200
	Donkey F(ab') ₂ Anti-Rat IgG H&L (Alexa Fluor® 594) preadsorbed	Abcam ab150152	1:200

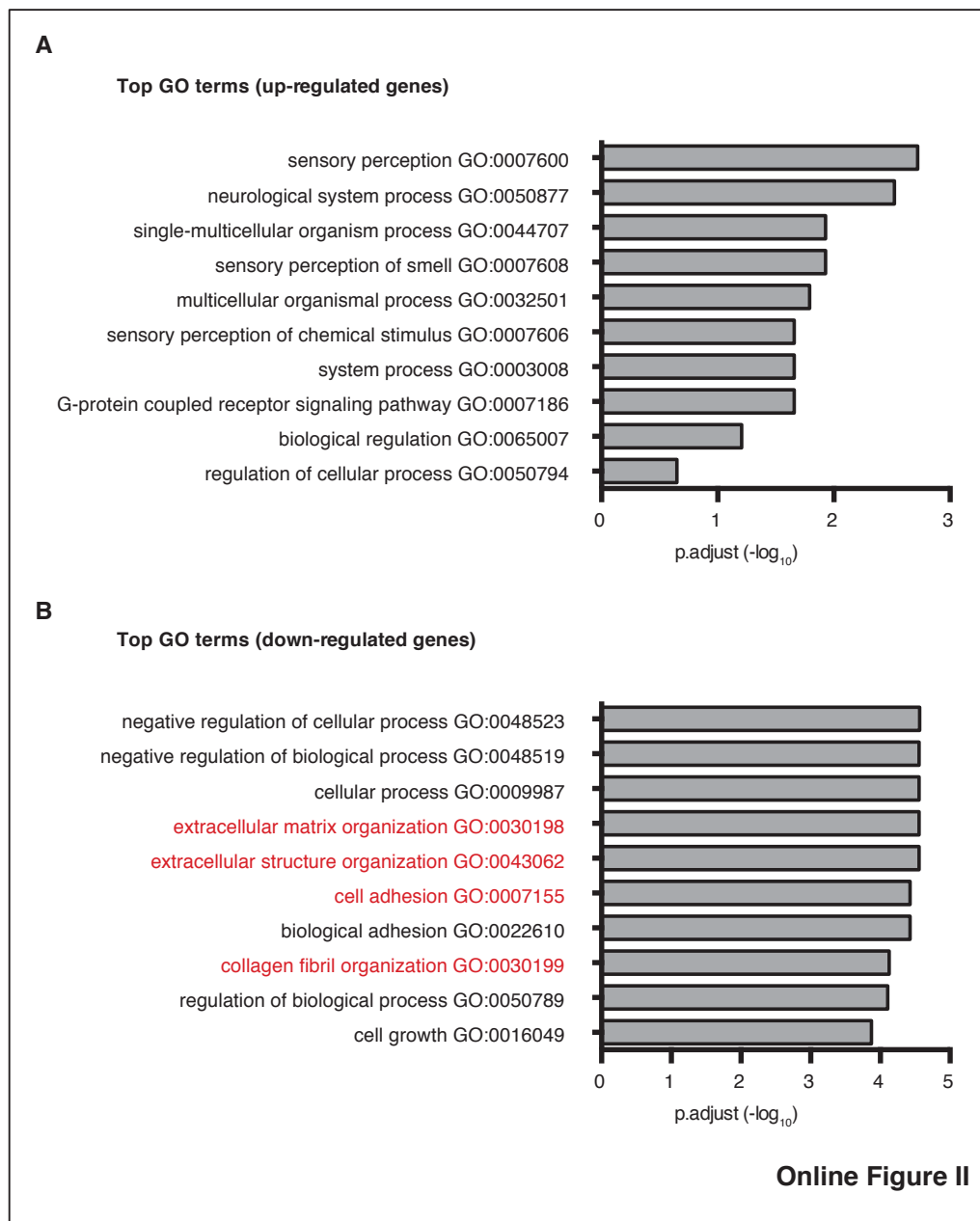
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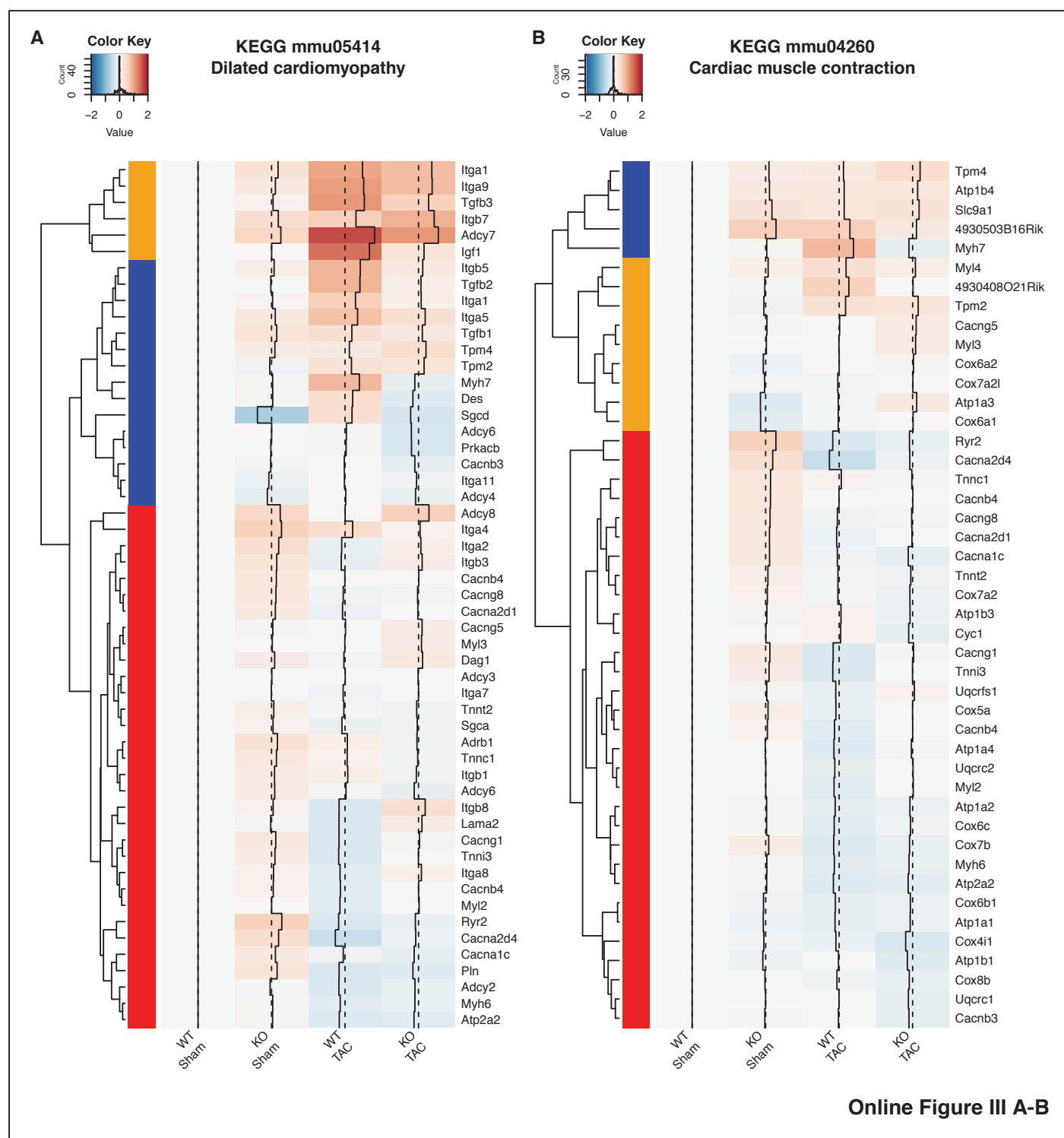
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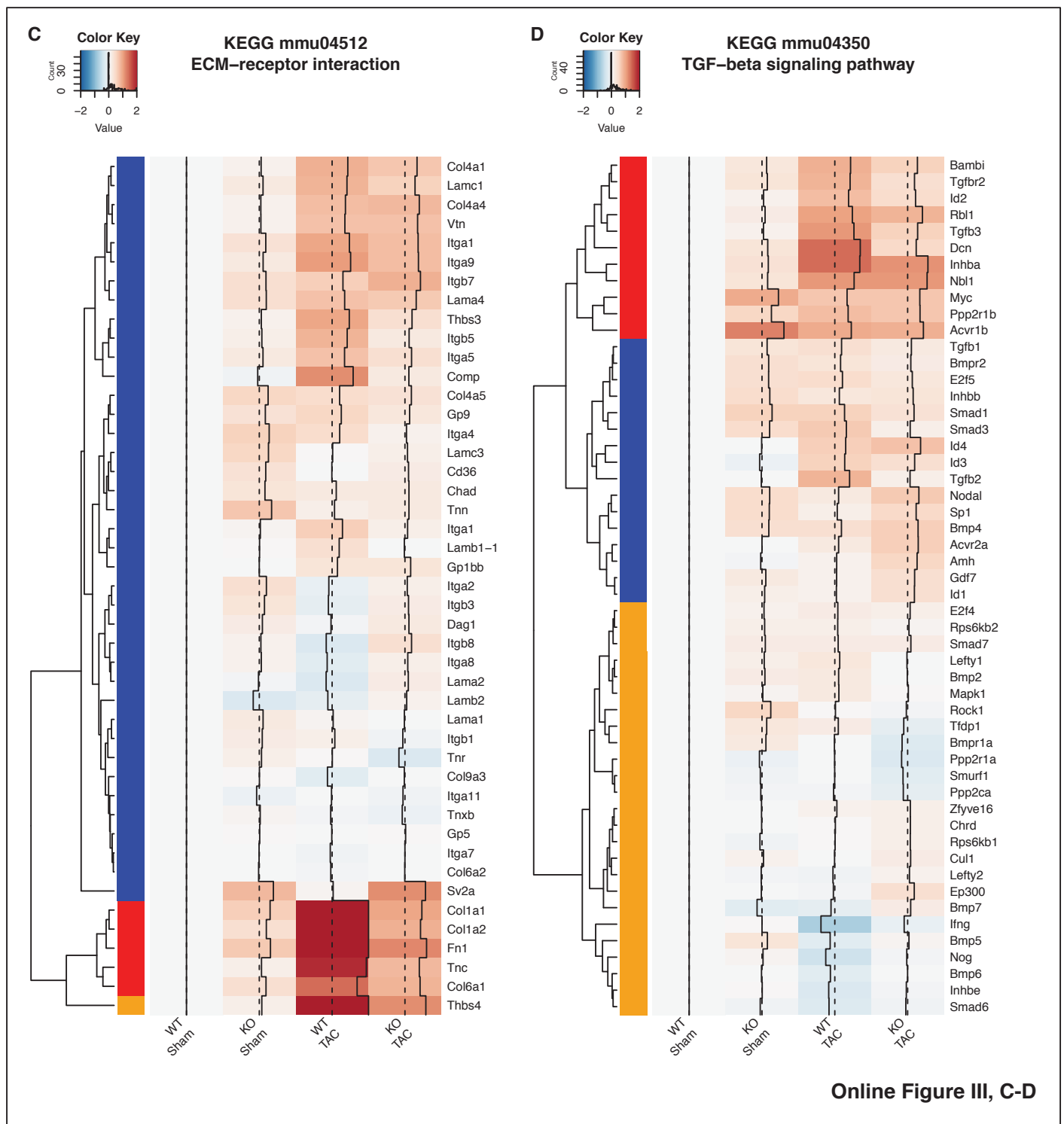
Online Figure I. Heart weight was increased similarly in WT and miR-33KO mice after TAC. **A** and **B**, Heart rate (**A**) and blood pressure (**B**) of WT and miR-33KO mice at the age of 10 weeks (n=5 and 7). **C** and **D**, Changes in heart weight in 2 weeks (**C**) or in 4 weeks (**D**) after TAC (n=5 each). Heart weight (mg) was normalized with body weight (g). *P<0.05, **P<0.01 vs sham, n.s. not significant vs WT, by ANOVA with Sidak correction. **E**, Expression levels of miR-33 in LVs in the early phase (2 weeks after TAC, n=6) and in the chronic phase (10 weeks after TAC, n=9). *P<0.05 by unpaired two-tailed Student's t-test. Data are presented as mean $\pm$ SEM.



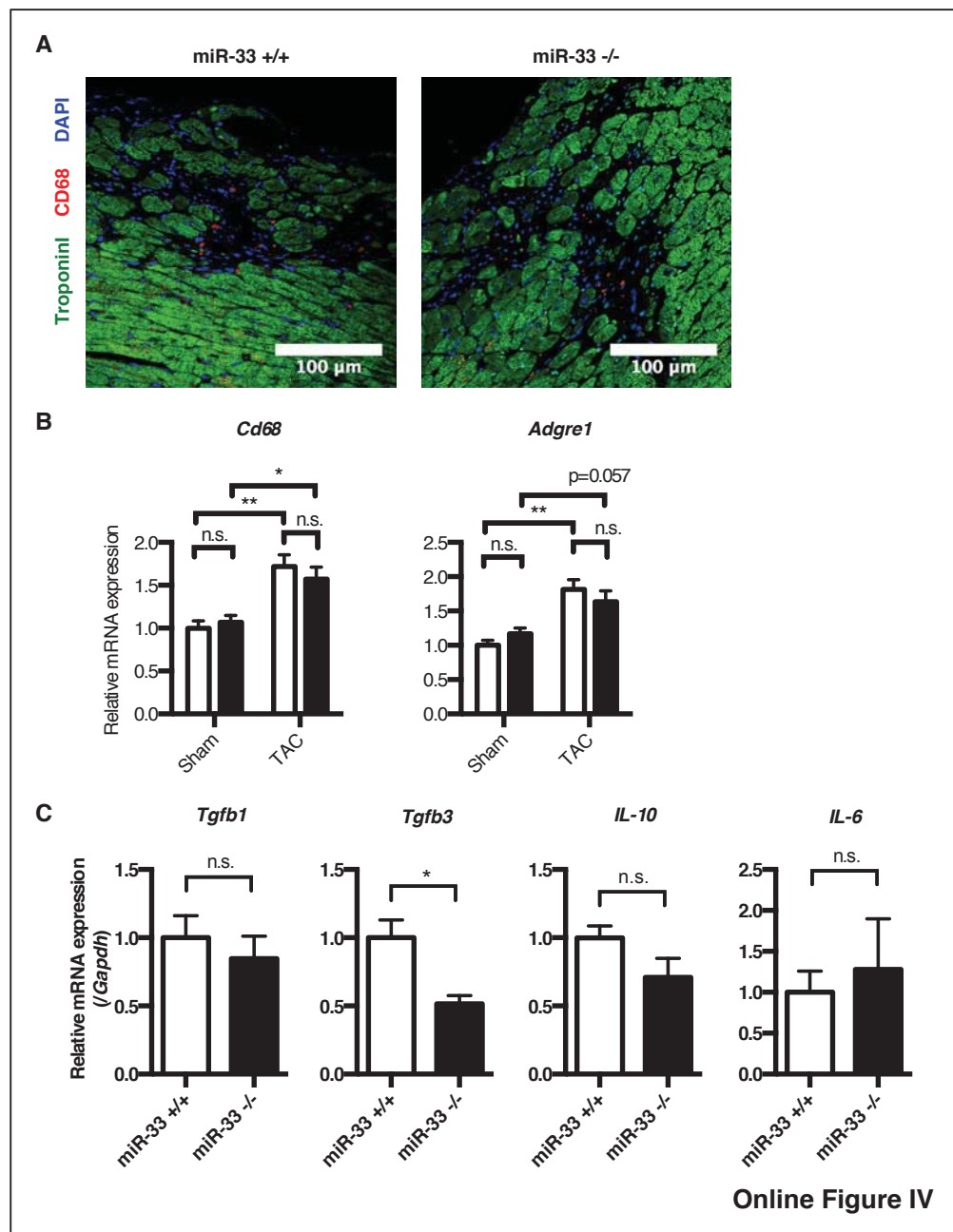
Online Figure II. Gene Ontology (GO) analysis showed enriched biological processes related to extracellular matrix and collagen. A and B, Top 10 GO terms (biological process) enriched in miR-33KO left ventricles. The microarray data detected 114 up-regulated genes (KO/WT fold change>2) and 148 down-regulated genes (KO/WT fold change<0.5) in miR-33KO hearts 2 weeks after TAC. The up-regulated genes (**A**) and the down-regulated genes (**B**) were categorized by GO analysis. Adjusted p values in each GO term were shown.



Online Figure III. Heatmap data showed modulated expression of fibrosis-related genes after TAC in miR-33KO mice. A, B, C, and D, Heatmap data of genes involved in cardiac remodeling. The sets of genes were picked up from Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The up-regulation of fibrosis-related genes induced by TAC was suppressed in miR-33KO mice (orange cluster in A, red and

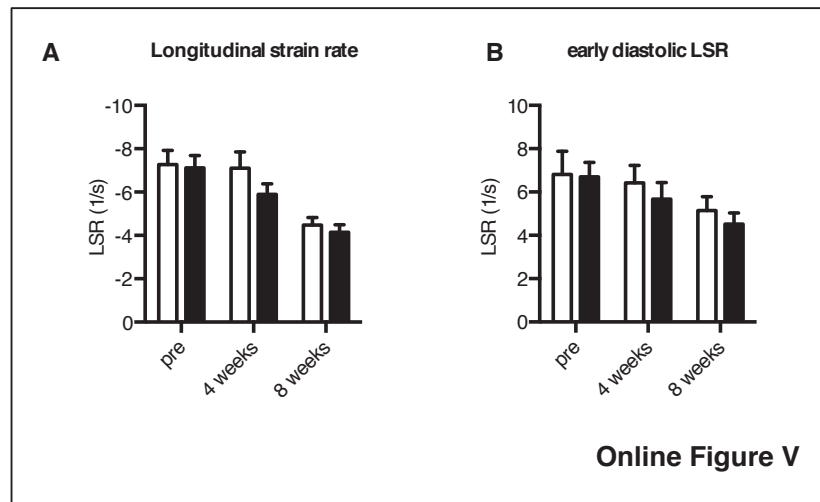


orange cluster in **C**, and red cluster in **D**), whereas the genes involved in cardiac muscle contraction showed only small changes (**B**).

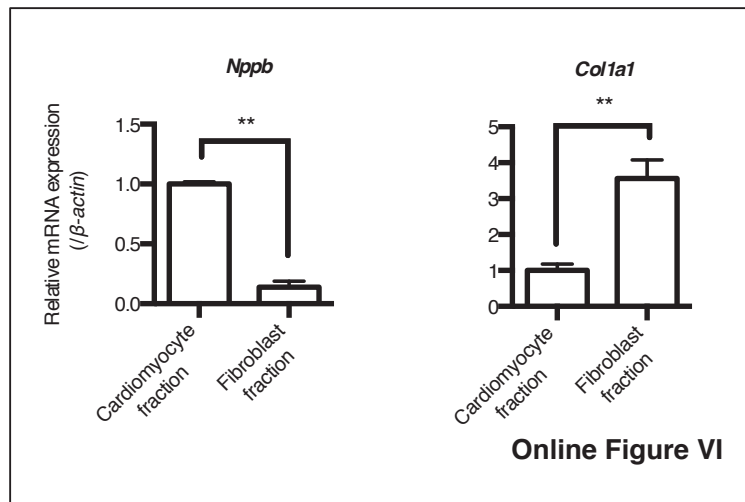


Online Figure IV. Deficiency of miR-33 did not affect macrophage infiltration induced by TAC *in vivo*. **A**, Immunostaining images of macrophage infiltration. CD68⁺ macrophages were seen mainly in the fibrotic area both in WT and miR-33KO mice at 2 weeks after TAC. **B**, Quantitative real-time PCR analysis of macrophage markers *Cd68* and *Adgre1* (known as F4/80) (n=7-10). *P<0.05, **P<0.01 vs sham, n.s. not significant vs WT, by ANOVA with Sidak correction. **C**, Gene expression of macrophages *in vitro*. Peritoneal macrophages from

WT and miR-33KO were analyzed by quantitative real-time PCR. * $P < 0.05$ by unpaired two-tailed Student's t-test ($n=4$). Data are presented as mean $\pm$ SEM.

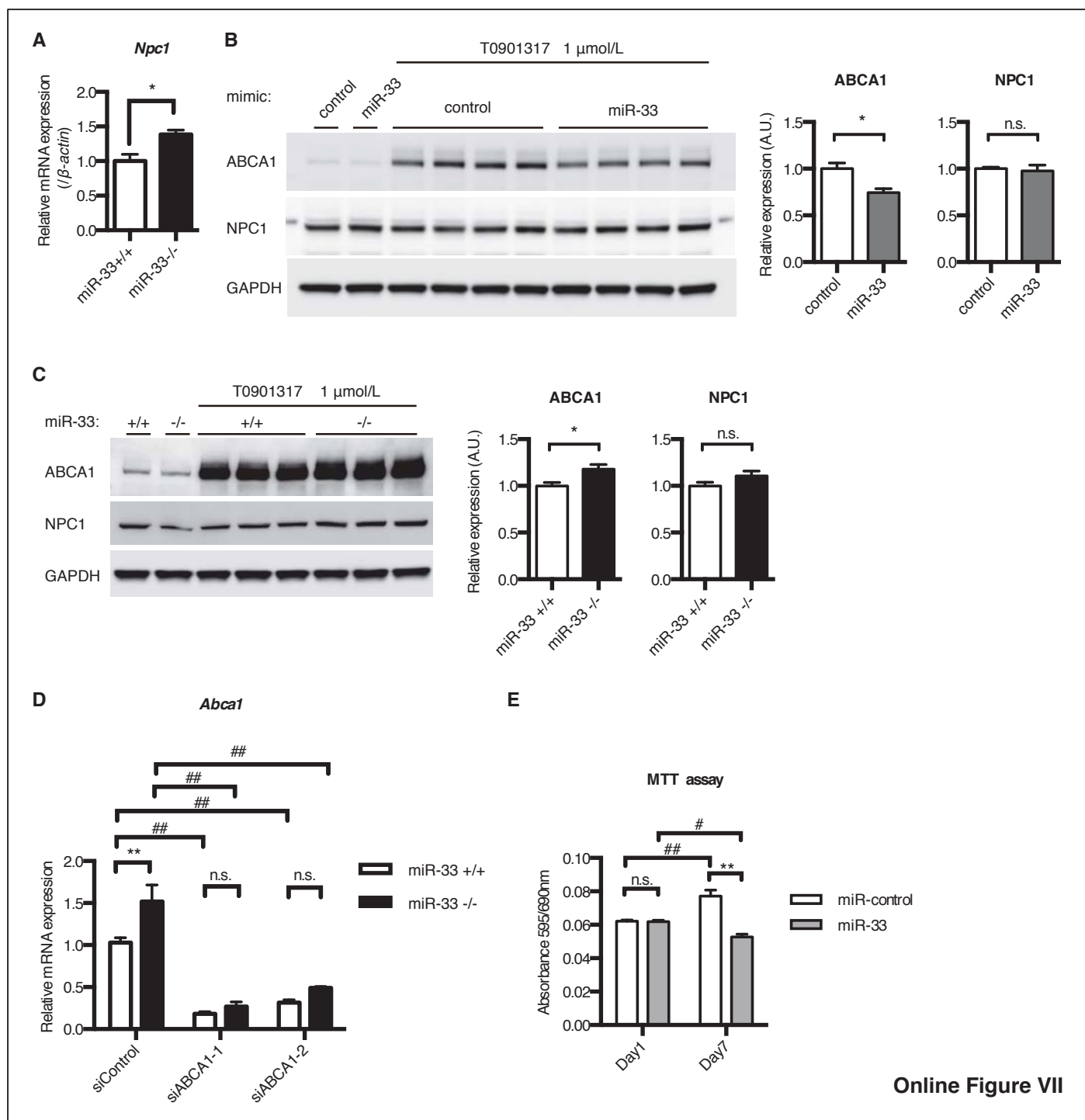


Online Figure V. Deficiency of miR-33 did not affect diastolic function. **A**, Longitudinal strain rate (LSR) analyzed using speckle-tracking strain imaging with Vevo2100. **B**, Early diastolic LSR (a parameter for diastolic function) analyzed using speckle-tracking imaging.



Online Figure VI. Isolated neonatal rat cardiomyocytes and cardiac fibroblasts were purified by Percoll gradient separation. Expression of *Nppb* and *Col1a1* in isolated cardiomyocyte and fibroblast fractions (n=5).

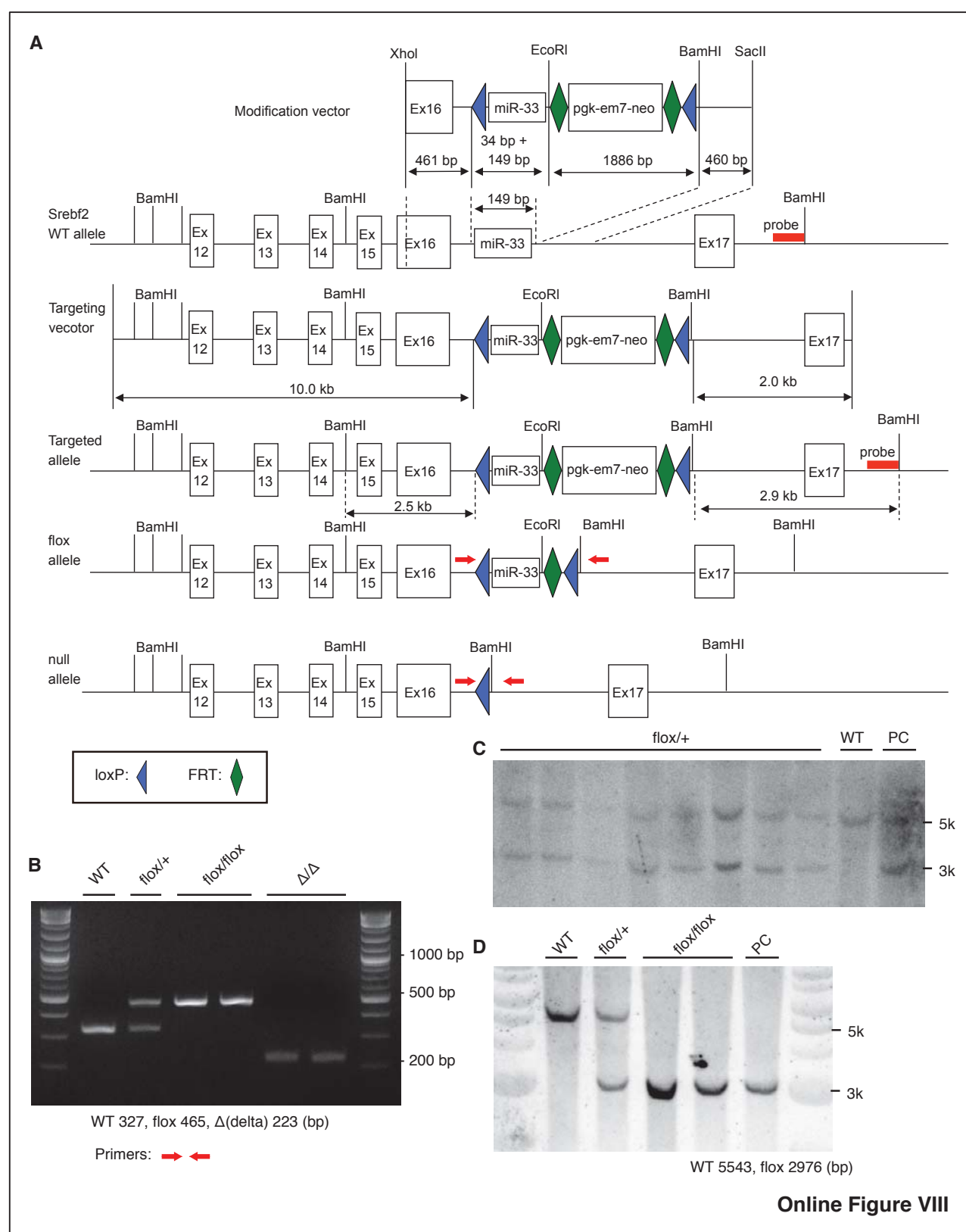
**P<0.01 by unpaired two-tailed Student's t-test. Data are presented as mean $\pm$ SEM.



Online Figure VII

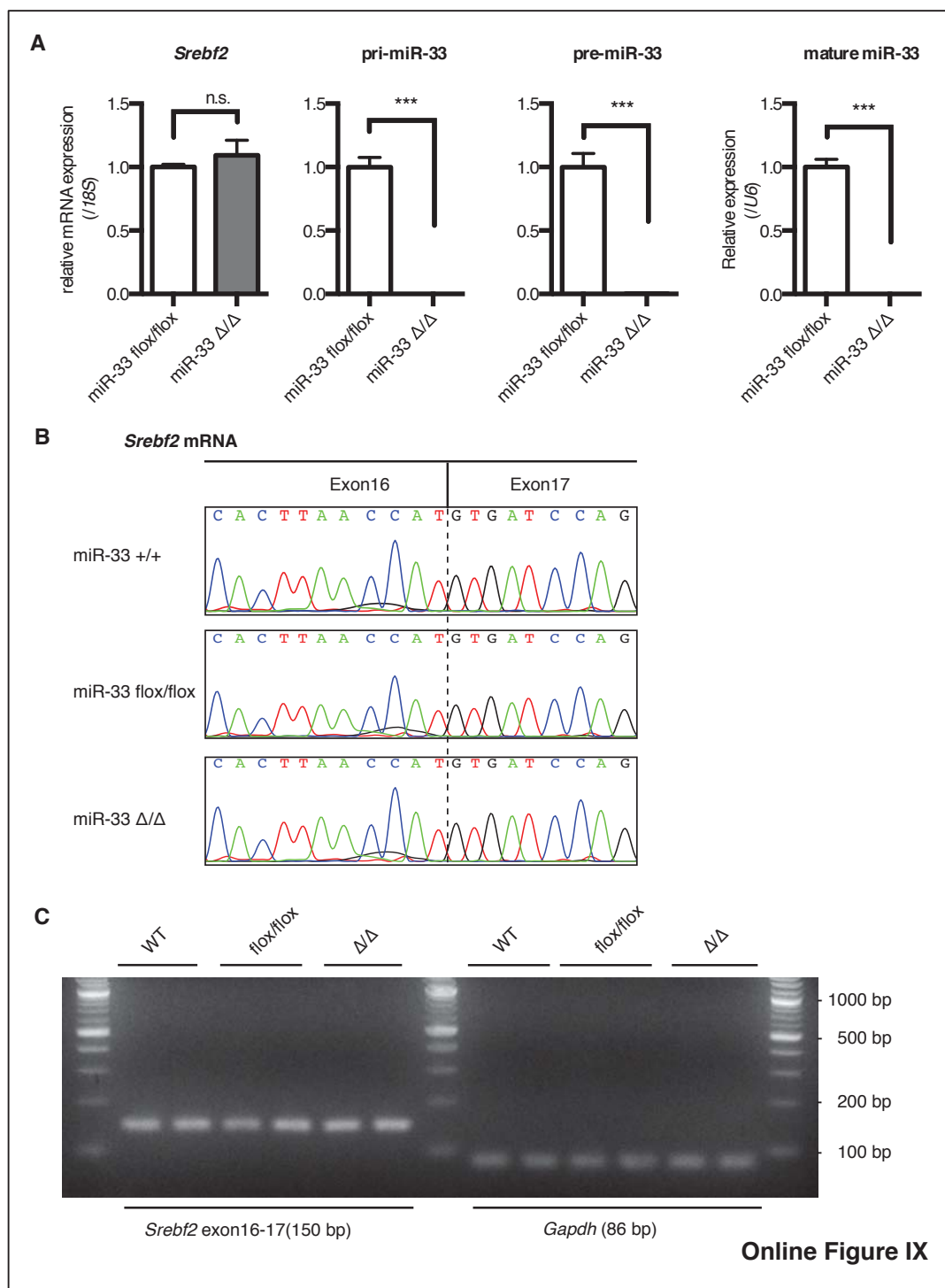
Online Figure VII. ABCA1 is regulated by miR-33. **A**, Expression levels of *Npc1* mRNA in cardiac fibroblasts (CFs). **B**, Western blotting analysis of ABCA1 and NPC1 in cardiac fibroblasts (CFs). CFs were transfected with control mimic or miR-33 mimic. T0901317 (LXR agonist) was used at 1 μ mol/L. GAPDH was

used as an internal control. *P<0.05 vs control, by unpaired two-tailed Student's t-test. **C**, Western blotting analysis of ABCA1 and NPC1 in WT and miR-33KO CFs. GAPDH was used as an internal control. *P<0.05 vs WT, by unpaired two-tailed Student's t-test. **D**, The efficiency of lentiviral knockdown of *Abca1*. The expression levels of *Abca1* in WT and miR-33KO MEFs with siABCA1 were analyzed by quantitative PCR (n=4). **P<0.01 vs WT, ##p<0.01 vs siControl by ANOVA with Sidak correction. **E**, Impaired proliferation of fibroblasts by miR-33 overexpression. CFs were transfected with a lentiviral miR-33 expression vector or a control miR expression vector (miR-control). MTT assay was performed 1 day and 7 days after transfection. **P<0.01 vs miR-control, ##p<0.01 vs Day 1, by ANOVA with Sidak correction. Data are presented as mean $\pm$ SEM.



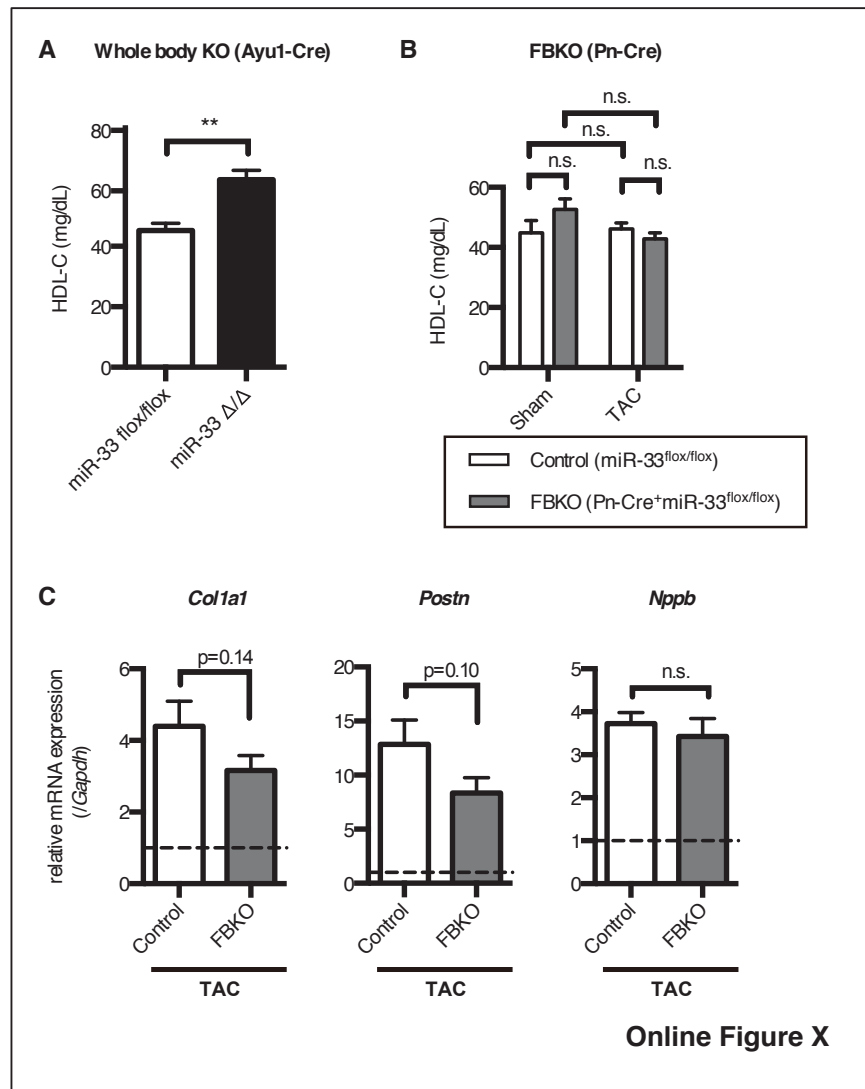
Online Figure VIII. Generation of miR-33-floxed mice. **A**, Schematic representation of the targeting vector and expected gene replacement at the miR-33 locus. white triangles, loxP sequences; white rhombuses, FRT

sequences. **B**, Genotyping PCR using tail genomes of indicated genotypes. In miR-33^{Δ/Δ} mice, miR-33 loci were deleted by crossing with Ayu1-Cre mice. **C** and **D**, Southern blotting using the genome of ES cells (**C**) and tail genome (**D**) of indicated genotypes.



Online Figure IX. Expression and splicing of *Srebf2* mRNA were not altered in miR-33-floxed or miR-33-deleted mice. A, Expression levels of *Srebf2* mRNA, precursors of miR-33, and mature miR-33 in the livers of 8-week-old mice (n=4). Expression levels of *Srebf2*, pri-miR-33, and pre-miR-33 were normalized by

18S ribosomal RNA. Expression levels of mature miR-33 were normalized by *U6*. P values by unpaired Student's t-test are shown. n.s. not significant. Data are presented as mean $\pm$ SEM. **B**, Sequencing alignment at the junction between exons 16 and 17 of *Srebf2* mRNA in the indicated genotypes. **C**, RT-PCR analysis of *Srebf2* in the indicated genotypes. There was no other bands except for that of the correct size.

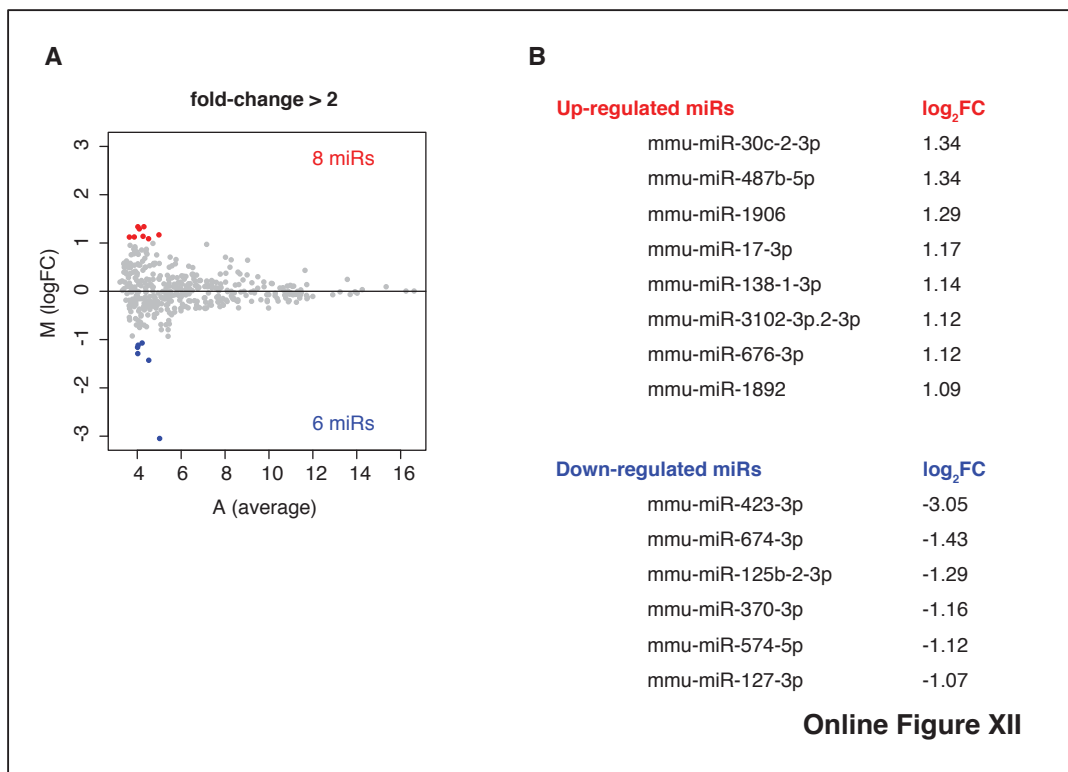


Online Figure X. HDL-C levels and gene expression levels in fibroblast-specific miR-33 KO (FBKO)

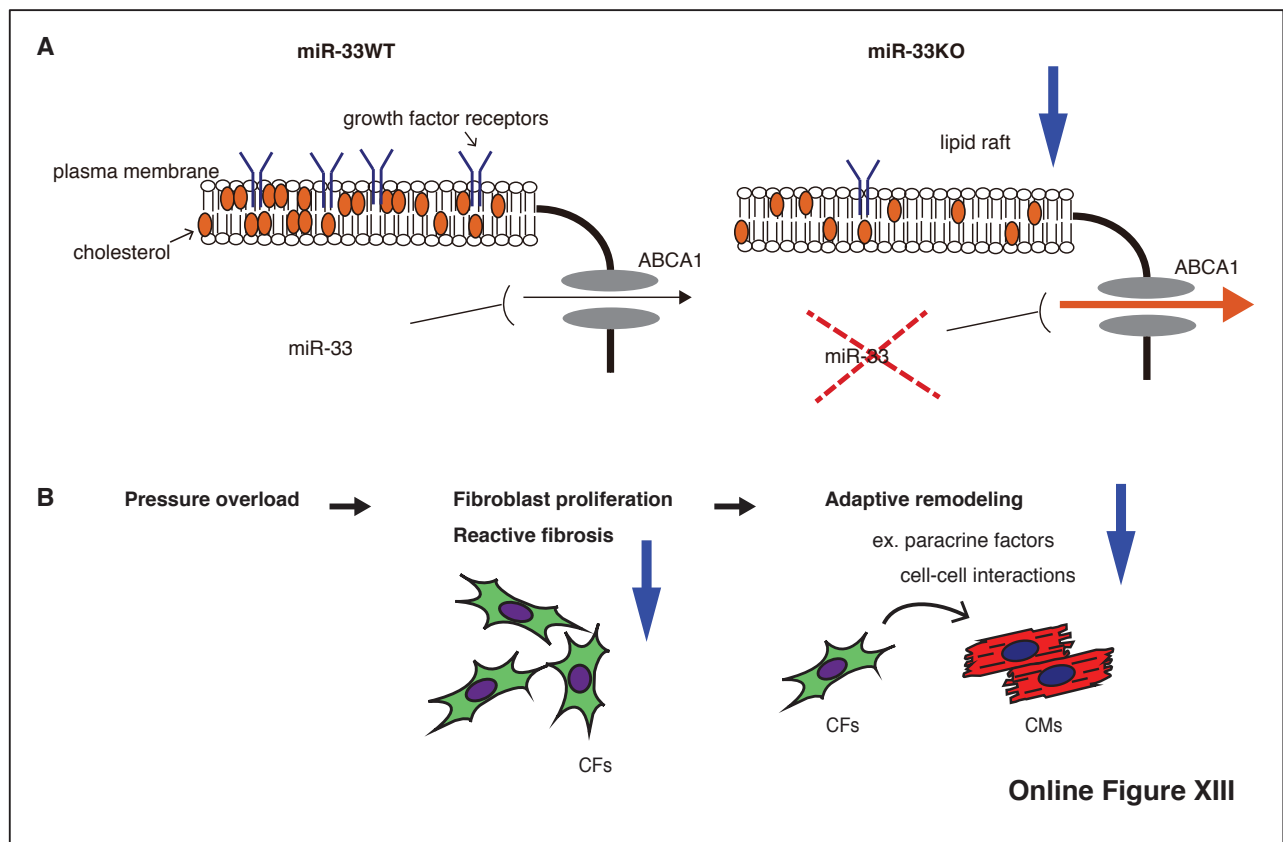
mice. **A** and **B**, HDL-C levels in miR-33^{Δ/Δ} (Ayu1-Cre⁺miR-33^{flx/flx}) mice (**A**) (n=4) and FBKO (Pn-Cre⁺miR-33^{flx/flx}) mice (**B**) (sham, n=6; TAC, n=18). **C**, Quantitative real-time PCR analysis in FBKO mice 2 weeks after TAC (n=18). Mean expression levels in five sham-operated miR-33^{flx/flx} mice were defined as 1.0. Expression levels were normalized by *Gapdh*. P values by unpaired Student's t-test (**A** and **C**) or by one-way ANOVA with Sidak correction (**B**) are shown. **P<0.01. Data are presented as mean ± SEM. Control, miR-33^{flx/flx} mice. FBKO, Pn-Cre⁺miR-33^{flx/flx} mice.



Online Figure XI. Western blotting analysis of cyclin-dependent kinase 6 (CDK6). CDK6 was not up-regulated in miR-33KO fibroblasts.



Online Figure XII. Deficiency of miR-33 had little impact on the expression of other miRs. A, Differentially expressed miRNAs in miR-33KO heart. Total RNA samples from WT and miR-33KO hearts were analyzed using a microRNA-microarray. Only small number of miRNAs were differentially expressed (fold change>2). **B,** The list of differentially expressed miRNAs shown in A.



Online Figure XIII. Mechanism model of lipid raft maintenance by miR-33. **A**, Cholesterol is enriched in microdomains on the plasma membrane called lipid rafts, which function as platforms that concentrate and segregate proteins such as growth factor receptors. In wild-type cells, expression levels of ABCA1 and ABCG1, which are involved in cholesterol efflux, are suppressed by miR-33 post-transcriptionally. Accordingly, lipid rafts on the plasma membrane are preserved. However, in miR-33KO cells, efflux of cholesterol is accelerated because of loss of inhibition of ABCA1 and ABCG1. Therefore, lipid raft cholesterol content is decreased. As a result, signal transduction from outside the cell is impaired in miR-33KO cells. **B**, In pressure overload model, proliferation of cardiac fibroblasts (CFs) is inhibited in miR-33KO mice because of the decreased lipid rafts as shown in A. Accordingly, reactive fibrosis, which is not a replacement of dead cardiomyocytes but an adaptive response to pathological stimuli, is impaired. As a result, adaptive remodeling such as producing protective paracrine factors is insufficient in miR-33KO mice.

MicroRNA-451 Exacerbates Lipotoxicity in Cardiac Myocytes and High-Fat Diet-Induced Cardiac Hypertrophy in Mice Through Suppression of the LKB1/AMPK Pathway

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Rationale: In some patients with type 2 diabetes mellitus (DM) without hypertension, cardiac hypertrophy and attenuated cardiac function are observed, and this insult is termed diabetic cardiomyopathy. To date, microRNA (miRNAs or miR) functions in diabetic cardiomyopathy remain to be elucidated.

Objective: To clarify the functions of miRNAs involved in diabetic cardiomyopathy caused by type 2 DM.

Methods and Results: C57BL/6 mice were fed a high-fat diet (HFD) for 20 weeks, which induced obesity and type 2 DM. miRNA microarray analyses and real-time polymerase chain reaction revealed that miR-451 levels were significantly increased in the type 2 DM mouse hearts. Because excess supply of saturated fatty acids is a cause of diabetic cardiomyopathy, we stimulated neonatal rat cardiac myocytes with palmitic acid and confirmed that miR-451 expression was increased in a dose- and time-dependent manner. Loss of miR-451 function ameliorated palmitate-induced lipotoxicity in neonatal rat cardiac myocytes. Calcium-binding protein 39 (Cab39) is a scaffold protein of liver kinase B1 (LKB1), an upstream kinase of AMP-activated protein kinase (AMPK). Cab39 was a direct target of miR-451 in neonatal rat cardiac myocytes and Cab39 overexpression rescued the lipotoxicity. To clarify miR-451 functions in vivo, we generated cardiomyocyte-specific miR-451 knockout mice. HFD-induced cardiac hypertrophy and contractile reserves were ameliorated in cardiomyocyte-specific miR-451 knockout mice compared with control mice. Protein levels of Cab39 and phosphorylated AMPK were increased and phosphorylated mammalian target of rapamycin (mTOR) was reduced in cardiomyocyte-specific miR-451 knockout mouse hearts compared with control mouse hearts.

Conclusions: Our results demonstrate that miR-451 is involved in diabetic cardiomyopathy through suppression of the LKB1/AMPK pathway. (*Circ Res.* 2015;116:279-288. DOI: 10.1161/CIRCRESAHA.116.304707.)

Key Words: AMP-activated protein kinase ■ cardiomegaly ■ microRNAs ■ type 2 diabetes mellitus

Obesity is a major burden worldwide, especially in developed countries. Obesity and comorbid type 2 diabetes mellitus (DM) induce structural and functional changes in the heart. Cardiac hypertrophy and decreased cardiac function are recognized in some patients with obesity and type 2 DM without hypertension, coronary artery disease, or valvular heart disease. This cardiac insult is termed diabetic cardiomyopathy.¹ Because cardiac hypertrophy and dysfunction result in cardiac death, uncovering the mechanisms is important research goal.

Several mechanisms that elicit diabetic cardiomyopathy have been proposed. Cytokines produced by the expanded adipose tissue, such as leptin and resistin, and triglyceride

accumulation² induce cardiac hypertrophy. Another proposed mechanism is excess supply of saturated fatty acids (FAs), such as palmitic acid. Unoxidized FAs in cardiac myocytes produce oxidative stress, mitochondrial dysfunction, and ceramide accumulation, resulting in lipotoxicity.^{3,4}

Editorial, see p 229

AMP-activated protein kinase (AMPK) is a major cellular sensor of energy availability. Liver kinase B1 (LKB1) and Ca²⁺/calmodulin-dependent protein kinase kinase β can act as major upstream kinases of AMPK in mammalian cells and phosphorylate AMPK at Thr¹⁷².⁵ LKB1 forms a heterotrimeric complex with ste20-related adaptor and calcium-binding

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Nonstandard Abbreviations and Acronyms

α MHC	α -myosin heavy chain
AMPK	AMP-activated protein kinase
Cab39	calcium-binding protein 39
DIO	diet-induced obesity
FA	fatty acid
FXR	farnesoid X receptor
HFD	high-fat diet
LKB1	liver kinase B1
MIF	macrophage inhibitory factor
mTOR	mammalian target of rapamycin
NC	normal chow
NRCMs	neonatal rat cardiac myocytes
PCR	polymerase chain reaction
UTR	untranslated region

protein 39 (Cab39, also known as MO25 α).⁶ Although several lines of evidence indicate that AMPK phosphorylation is attenuated in diet-induced obesity (DIO) mouse hearts,^{7,8} the mechanisms by which AMPK phosphorylation is decreased are not fully understood.

MicroRNAs (miRNAs or miRs) are a large class of small noncoding RNAs. miRNAs suppress the translation of a target mRNA depending on the complementarity between the 5' side seed sequence of a miRNA and the 3' untranslated region (3'UTR) of the target mRNA. Although several reports indicate that miRNAs are involved in diabetic cardiomyopathy in a type 1 DM model of streptozotocin-induced diabetic mice,^{9–11} miRNA functions in diabetic hearts induced especially by type 2 DM remain to be elucidated.

In the present study, we used DIO mice to identify differentially regulated miRNAs in hearts. One of these miRNAs, miR-451, was upregulated in the hearts, and palmitic acid stimulation increased miR-451 levels in neonatal rat cardiac myocytes (NRCMs). miR-451 directly targeted Cab39 in NRCMs, and loss of miR-451 function partly rescued lipotoxicity in vitro. Furthermore, we revealed that high-fat diet (HFD)-induced cardiac hypertrophy was ameliorated in cardiomyocyte-specific miR-451 knockout (miR-451 cKO) mice. Finally, we showed that AMPK phosphorylation was increased and mammalian target of rapamycin (mTOR) phosphorylation was decreased in the HFD-fed miR-451 cKO mice compared with the HFD-fed control mice. These data strongly suggest that cardiac-specific inhibition of miR-451 is a strategy for treating diabetic cardiomyopathy.

Methods

Detailed methods are provided in the Online Data Supplement.

Results

miRNA-451 Expression Is Significantly Increased in DIO Mouse Hearts

To clarify miRNA expression changes in DIO mouse hearts, we used HFD-fed C57BL/6 mice. This is a well-established

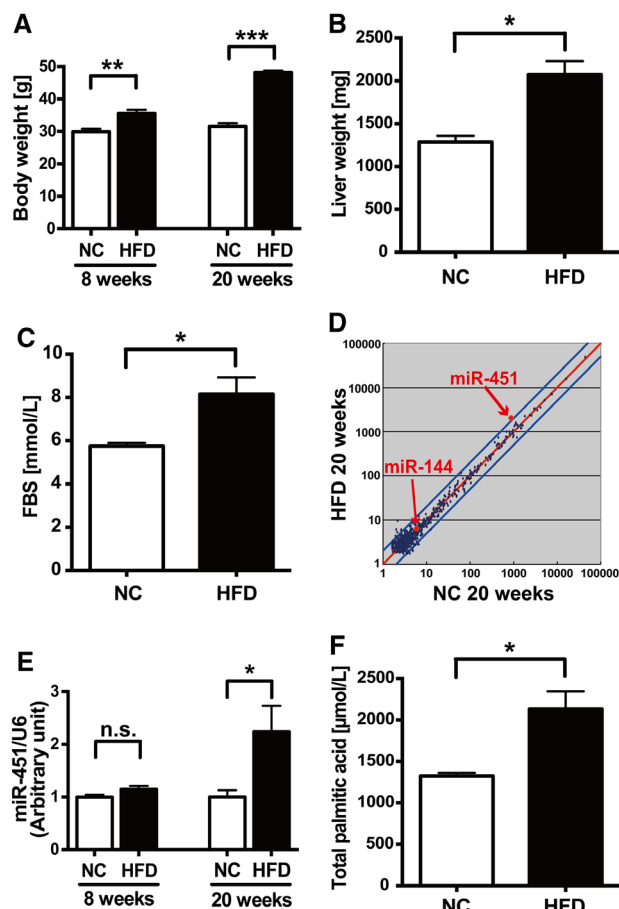


Figure 1. Cardiac microRNA (miR)-451 is upregulated in high-fat diet (HFD)-induced obese mice. **A**, Body weight changes in mice fed normal chow (NC) containing 10 kcal% fat or HFD containing 45 kcal% fat; 8 weeks: $n=4$; 20 weeks: $n=5$. **B** and **C**, Liver weights (**B**) and fasting blood sugar (FBS) concentrations (**C**) in mice fed NC or HFD for 20 weeks, $n=4$. **D**, miRNA expression profiling was performed using miRNA microarrays. The red diagonal line indicates the unchanged line. Two blue lines represent 2-fold changes. **E**, miR-451 expression levels in obese mice hearts fed NC or HFD for 8 or 20 weeks, $n=4$ to 5. **F**, Plasma concentrations of palmitic acid in NC- or HFD-fed mice, $n=5$. Data are presented as mean $\pm$ SEM. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

obesity and type 2 DM model,¹² and the C57BL/6 mice become obese depending on the duration of HFD feeding, as shown in Figure 1A. In line with our and other reports,^{8,12,13} liver weights approximately doubled, and fasting blood sugar concentrations increased significantly by 1.5-fold after feeding 45 kcal% fat-containing HFD for 20 weeks (Figure 1B and 1C). These data suggest that this mouse model had marked insulin resistance and fatty liver and mimicked obesity with type 2 DM in humans.

To evaluate miRNA expression changes in the heart, we performed microarray analyses of miRNAs extracted from the hearts of mice fed HFD for 8 and 20 weeks. We focused on miRNAs meeting the following 3 criteria. The miRNA was evidently expressed in the heart (miRNA spot intensity, >100), the expression levels increased depending on the duration of HFD feeding, and the levels were 2-fold higher than those in control mouse hearts. Only miR-451 met these criteria (Online

Figure I and Figure 1D). As shown in Figure 1E, miR-451 expression levels were validated using Taqman microRNA real-time polymerase chain reaction (PCR). These data suggest that only miR-451 levels were significantly increased in DIO mouse hearts. It is reported that miR-451 and miR-144 are transcribed as a bicistronic transcript to yield 2 mature miRNAs.^{14,15} Therefore, we also evaluated miR-144 levels in the microarray analysis. Interestingly, the spot intensity of miR-144 was extraordinarily lower than that of miR-451 (Online Figure I and Figure 1D). Because saturated FAs, such as palmitic acid, are one of the mediators of lipotoxicity,⁴ we measured the plasma concentrations of total palmitic acid in the normal chow (NC)-fed and HFD-fed mice. The plasma concentration of total palmitic acid was significantly higher in the HFD-fed mice compared with the NC-fed mice (Figure 1F). Because we did not remove lipoproteins from plasma, which contain considerable amounts of triglycerides, we evaluated palmitic acid incorporated within apoB-containing lipoproteins, such as very-low-density lipoprotein and low-density lipoprotein. After precipitation with a polyethyleneglycol solution, the palmitic acid levels in serum samples of NC-fed mouse were significantly reduced, as shown in Online Figure II.

miR-451 Expression Is Induced by Palmitic Acid Stimulation, and Elevated miR-451 Causes Cell Toxicity in NRCMs

miR-451 is expressed abundantly in blood cells, such as erythrocytes and macrophages.^{14–16} Thus, we sought to isolate cardiac myocytes and cardiac fibroblasts and determine whether miR-451 is expressed in these cells. Cardiac myocytes do not have cell surface-specific markers, and a report indicated that cardiac myocytes have higher mitochondrial content than non-cardiac myocytes and could be purified by mitochondria-labeling dyes.¹⁷ To mark cardiac fibroblasts, we used allophycocyanin-conjugated Thy-1.2 antibody. As shown in Online Figure IIIA, isolated cells from neonatal mouse ventricles were analyzed and sorted by fluorescent-activated cell sorting. In line with a previous study, the mRNA levels of brain natriuretic peptide were significantly higher in the populations with high Mitotracker Green fluorescent content and negative Thy-1.2 (Mito^{high}/Thy-1.2^{neg}) than in populations with low Mitotracker Green fluorescent content and positive Thy-1.2 (Mito^{low}/Thy-1.2^{posi}; Online Figure IIIB), indicating that the Mito^{high}/Thy-1.2^{neg} populations contain many cardiac myocytes.¹⁷ Whereas the mRNA levels of collagen type 1 α 1 were significantly higher in Mito^{low}/Thy-1.2^{posi} populations than in Mito^{high}/Thy-1.2^{neg} populations (Online Figure IIIC), indicating that cardiac fibroblasts comprise Mito^{low}/Thy-1.2^{posi} populations. miR-451 levels were significantly higher in cardiac myocytes than in cardiac fibroblasts (Online Figure IIID), which suggested that miR-451 was expressed in cardiac myocytes. In addition, we tried to compare the miR-451 levels in cardiac myocytes and erythrocytes. We collected peripheral blood from a neonatal mouse, and fluorescent-activated cell sorting sorted erythrocytes that were labeled with an anti-Ter119 antibody, a marker of erythrocytes (Online Figure IVA). Because the total RNA mass extracted from erythrocytes was extremely low, and the U6 level was also lower than that in cardiac myocytes, we did

not think that U6 was suitable for use as an internal control in erythrocytes. Thus, the miR-451 level per cell was determined from a standard curve for artificial miR-451, the total RNA mass obtained, and the numbers of cells collected by fluorescent-activated cell sorting. The miR-451 levels in cardiac myocytes were $\approx$ 40-fold higher than those in erythrocytes (Online Figure IVB). These data were consistent with those in a previous report.¹⁸ Thus, we attempted to investigate miR-451 functions in cardiac myocytes.

We next stimulated NRCMs with palmitate to determine whether this induced miR-451 expression in cardiac myocytes as both albumin-bound FAs and FAs incorporated within lipoproteins are used as fuel by cardiac myocytes,^{19,20} and the total palmitic acid concentration was significantly higher in the plasma of HFD-fed mice than in that of NC-fed mice (Figure 1F). As shown in Figure 2A and 2B, palmitate stimulation resulted in elevated miR-451 levels in a dose- and time-dependent manner. We also measured the expression levels of miRNAs expressed abundantly in muscle cells, including miR-133a, miR-208a, and miR-499, but we did not observe any significant increases in their levels (Figure 2B). In addition, we assessed the effects of oleic acid, an unsaturated FA, on miR-451 expression in NRCMs. Our data indicated that the total oleic acid level in plasma was approximately one half of the total palmitic acid level (data not shown). Thus, we stimulated NRCMs with oleic acid at 250 μ mol/L. Although miR-451 level was also upregulated by oleic acid, this increase was modest; and the miR-451 upregulation by oleic acid was significantly lower than that by palmitic acid at 250 μ mol/L (Online Figure VA).

We then overexpressed miR-451 in NRCMs using a lentiviral system to elucidate miR-451 functions. Although the miR-451 level was high in NRCMs infected with a miR-451 expression vector (Online Figure VI), cell survival was significantly reduced after miR-451 overexpression (Figure 2C). Palmitate stimulation caused miR-451 upregulation in NRCMs, and ectopic expression of miR-451 induced cell toxicity; therefore, we hypothesized that miR-451 knockdown would ameliorate palmitate-induced cell toxicity. miR-451 decoys were induced in NRCMs, and these NRCMs were stimulated with palmitate. We found that cell injury measured by the lactate dehydrogenase assay was partly rescued (Figure 2D). To clarify the induction of miR-451 and lipotoxicity in NRCMs at physiological ratios of palmitate to albumin, the albumin concentration was fixed at 550 μ mol/L and NRCMs were stimulated with various concentrations of palmitate. miR-451 levels were significantly upregulated at palmitate to albumin ratios of 1:2 and 1:1 (Figure 2E). Palmitate-induced lipotoxicity was observed at a ratio of 1:2, and the lipotoxicity was significantly ameliorated when miR-451 was knocked down (Figure 2F). These data imply that palmitate induces miR-451, and the elevation in miR-451 levels in part exacerbates lipotoxicity in NRCMs.

Cab39 Is a Direct Target of miR-451 in NRCMs and Mice

We next sought to identify the target of miR-451 in cardiac myocytes. To date, macrophage inhibitory factor (MIF),²¹ Cab39,²² 14-3-3 ζ ,¹⁵ CUG triplet repeat-binding protein 2,²³ and Ras-related C3 botulinum toxin substrate 1¹⁴ have been

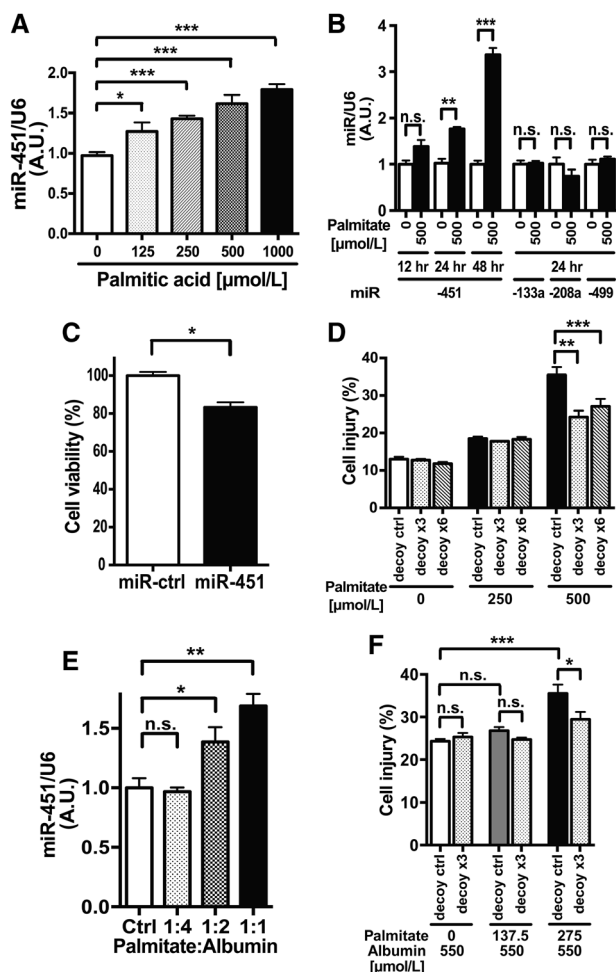


Figure 2. Lipotoxicity is partly because of microRNA (miR)-451, which is upregulated by palmitic acid stimulation in neonatal rat cardiomyocytes (NRCMs). **A**, miR-451 levels after palmitic acid stimulation for 24 hours in NRCMs, $n=4$ to 6. **B**, miR-451, miR-133a, miR-208a, and miR-499 levels in palmitate-stimulated NRCMs, $n=3$. **C**, Cell viability of NRCMs transfected with the miR-451 overexpression vector, $n=4$. **D**, Cell injury evaluation using lactate dehydrogenase (LDH) assays in miR-451 decoy-introduced NRCMs. These NRCMs were stimulated with palmitate for 24 hours, $n=3$ to 4. **E**, miR-451 levels in NRCMs stimulated for 48 hours with various palmitate to albumin ratios. Albumin concentration was fixed at 550 $\mu\text{mol/L}$, $n=6$ to 12. **F**, Cell injury was evaluated using LDH assays in miR-451 decoy-introduced NRCMs. These NRCMs were stimulated for 72 hours with the indicated palmitate and albumin concentrations, $n=4$. Data are presented as mean $\pm$ SEM. * $P<0.05$; ** $P<0.01$; *** $P<0.001$. AU indicates arbitrary unit.

identified as direct miR-451 targets. Of these bona fide targets, we focused on 2 proteins that have cell protective functions, MIF²⁴ and Cab39,^{5,22} because our data indicate that miR-451 knockdown ameliorates palmitate-induced lipotoxicity. To examine whether miR-451 can directly bind to the 3'UTRs of MIF and Cab39, wild-type (WT) or mutated (Mut) miR-451-binding sites of MIF or Cab39 were inserted into the 3'UTR of a luciferase plasmid, and we performed dual luciferase assays in 293T cells. As shown in Online Figure VIIA and Figure 3A, luciferase activities were significantly decreased when miR-451 was co-overexpressed with luciferase plasmids harboring WT MIF-3'UTR or Cab39-3'UTR. This decrease

was abolished when Mut MIF-3'UTR or Cab39-3'UTR luciferase plasmids were cotransfected with the miR-451 overexpression plasmid. Western blotting analysis revealed that miR-451 overexpression reduced MIF and Cab39 protein levels in NRCMs (Online Figure VIIB; Figure 3B). These data indicate that MIF and Cab39 are direct targets of miR-451 in NRCMs. We also evaluated MIF and Cab39 protein levels in vivo. Although we did not observe a reduction in the MIF protein level, the Cab39 protein level in the hearts was decreased in the 45 kcal% fat-containing HFD-fed mice compared with the NC-fed mice (Online Figure VIIC and Figure 3C). Thus, we focused on Cab39. To strengthen the finding that Cab39 is a target of miR-451 in NRCMs, we attempted to rescue miR-451-induced cell toxicity by Cab39 overexpression. As shown in Figure 3D, Cab39 overexpression rescued miR-451-induced cell toxicity. Furthermore, Cab39 overexpression ameliorated cell toxicity in palmitate-stimulated NRCMs (Figure 3E). Together, these data suggest that palmitic acid stimulation upregulates miR-451 expression, which reduces Cab39 levels, resulting in cell toxicity.

Cab39 is a scaffold protein of LKB1 that stabilizes the activity through the formation of a heterotrimeric complex with ste20-related adaptor in the cytoplasm.⁶ It is well established that LKB1 is an upstream kinase of AMPK. To verify the significance of the LKB1/AMPK pathway in NRCMs, we performed immunoblotting for AMPK. After palmitate stimulation, AMPK phosphorylation in NRCMs was significantly decreased (Figure 3F). To determine the specificity of palmitic acid on AMPK phosphorylation, we stimulated NRCMs with oleic acid. Suppression of AMPK phosphorylation was not observed even with oleic acid at 500 $\mu\text{mol/L}$ (Online Figure VB and VC). Furthermore, we evaluated Cab39 protein levels and AMPK phosphorylation in vivo. Western blotting analysis revealed that AMPK phosphorylation was reduced in the hearts of mice fed 45 kcal% fat-containing HFD for 20 weeks in association with the decrease of Cab39 (Figure 3C).

miR-451 cKO Mice Attenuated HFD-Induced Cardiac Hypertrophy

We required miR-451 knockout mice to reveal miR-451 functions in the HFD-fed mouse hearts. miR-451 is transcribed with miR-144 as a bicistronic transcript. O'Carroll and colleagues¹⁴ reported that miR-144 and miR-451 double knockout (miR-144/451^{-/-}) mice show ineffective erythropoiesis and mild anemia, and the phenotype of miR-451 single knockout mice was fairly distinguishable from that of the miR-144/451^{-/-} mice.¹⁵ We thought that anemia induced by miR-451 deletion could affect the phenotype in the hearts of the HFD-fed mice. Thus, we crossed the α -myosin heavy chain (α MHC)-Cre transgenic mice and floxed miR-451 mice to generate the α MHC-Cre;miR-451^{f/f} or miR-451 cKO mice. To eliminate the direct effect of Cre-recombinase in cardiac myocytes, α MHC-Cre;miR-451^{+/+} mice were used as the control mice. We attempted to validate the manipulation of the miR-451 locus in the genome using PCR analysis. As shown in Online Figure VIII, WT miR-451 and floxed miR-451 could be detected as bands of 393 and 580 nucleotides, respectively. When we performed PCR using DNA extracted from the miR-451 cKO mouse hearts as a template, we observed ablated

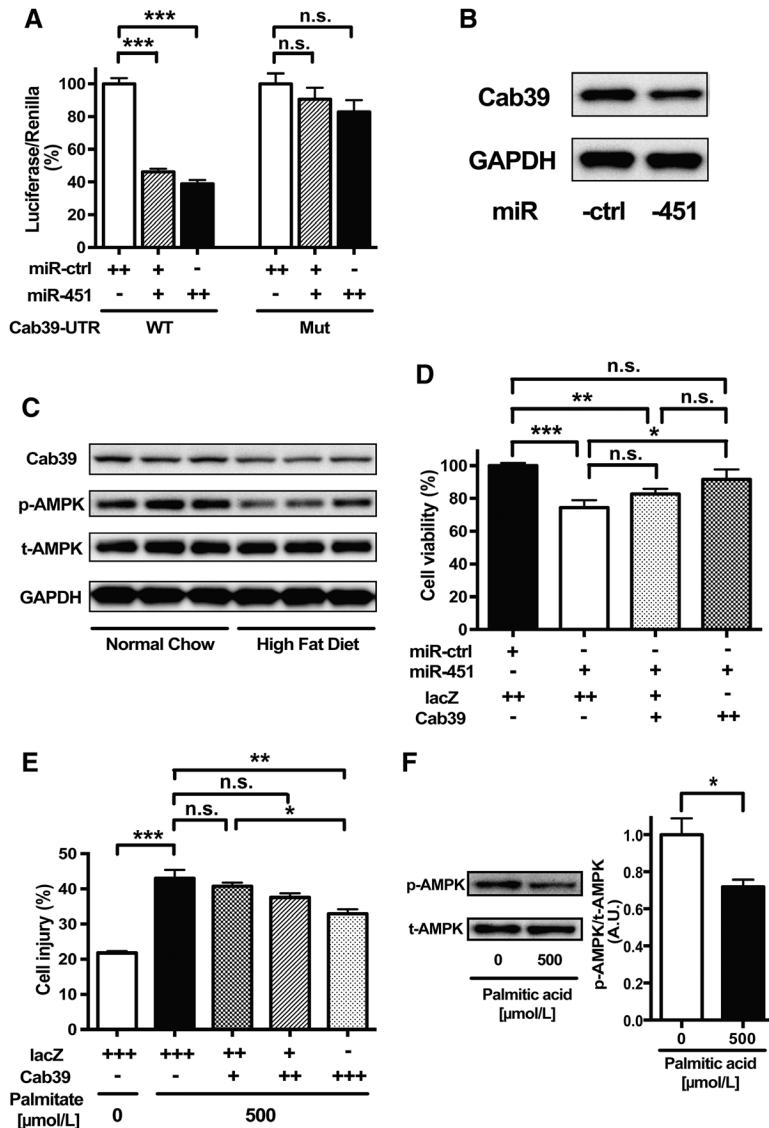


Figure 3. Calcium-binding protein 39 (Cab39) is a direct target of microRNA (miR)-451 in cardiomyocytes, and lipotoxicity is partially rescued by Cab39 overexpression.

A, 293T cells were transfected with an overexpression plasmid for miR-control (miR-ctrl) or miR-451 and a plasmid encoding luciferase wild-type (WT) Cab39 3' untranslated region (UTR) or mutated (Mut) Cab39 3'UTR lacking the miR-451-binding site. Luciferase activities were determined 24 hours later, $n=4$. **B**, Western blotting for Cab39 in neonatal rat cardiac myocytes (NRCMs) infected with the miR-ctrl or miR-451 vector. **C**, Western blotting of Cab39, phosphorylated AMP-activated protein kinase (p-AMPK), and total AMPK (t-AMPK) in the hearts of mice fed normal chow or 45 kcal% fat-containing high-fat diet. GAPDH: loading control. **D**, Cell viability in NRCMs infected with the miR-ctrl or miR-451 vectors and lacZ or Cab39 expression vectors, $n=3$ to 5. **E**, Cell injury ratio of Cab39-overexpressing NRCMs. NRCMs were stimulated with palmitate for 48 hours, $n=4$. **F**, **Left**, Representative images of Western blotting for p-AMPK and t-AMPK in NRCMs after palmitate stimulation. **Right**, Quantification of p-AMPK by densitometry analysis, $n=4$. Data are presented as mean $\pm$ SEM. * $P<0.05$; ** $P<0.01$; *** $P<0.001$. AU indicates arbitrary unit.

miR-451 as an ≈ 250 -nucleotide long band. A nonablated band was also observed after PCR analysis of heart DNA from the miR-451 cKO mice. We assumed the reason was that floxed miR-451 remained in non-cardiac myocytes.

The α MHC-Cre;miR-451^{fl/fl} and α MHC-Cre;miR-451^{+/+} mice started feeding on NC or 60 kcal% fat-containing HFD when they were 8 weeks old and were fed on their respective diets until the age of 28 weeks. No differences were found in body weights, liver weights, or fasting blood sugar concentrations between the NC- or HFD-fed control and miR-451 cKO mice (Figure 4A; Online Figure IXA and IXB). The miR-451 levels showed a clear declining trend in the NC-fed miR-451 cKO mice (Figure 4B), and the levels in the hearts were significantly increased in the HFD-fed control mice compared with the NC-fed control mice. As expected, this miR-451 up-regulation in hearts was not observed in the HFD-fed miR-451 cKO mice.

Because it was reported that GATA4 regulated miR-451 expression in cardiac myocytes,²⁵ we performed Western blotting for GATA4 to identify the mechanism by which miR-451 was upregulated in HFD-fed mouse hearts. As shown in Online

Figure XA and XB, protein levels of GATA4 were significantly increased in HFD-fed mouse hearts. This upregulated GATA4 may have induced miR-451 in HFD-fed mouse hearts.

In agreement with a previous report,¹³ the heart weight and the ratio of heart weight-to-tibial length were significantly increased in the HFD-fed control mice compared with the NC-fed control mice (Online Figure IXC; Figure 4C). The ratio of heart weight-to-tibial length was significantly decreased in the HFD-fed miR-451 cKO mice compared with in the HFD-fed control mice (Figure 4C). Transthoracic echocardiography also revealed that left ventricular wall thickness was significantly reduced in the HFD-fed miR-451 cKO mice compared with the HFD-fed control mice (Figure 4D; Online Figure IXD), although cardiac functions were not different between the HFD-fed control and miR-451 cKO mice at steady state (data not shown). Representative lectin staining of cross-sections of ventricles is shown in Figure 4E. Quantification of lectin staining revealed that HFD-induced cardiac hypertrophy was noticeably diminished in the miR-451 cKO mice compared with the control mice (Figure 4F). Because miR-451 overexpression caused cell toxicity in NRCMs, we

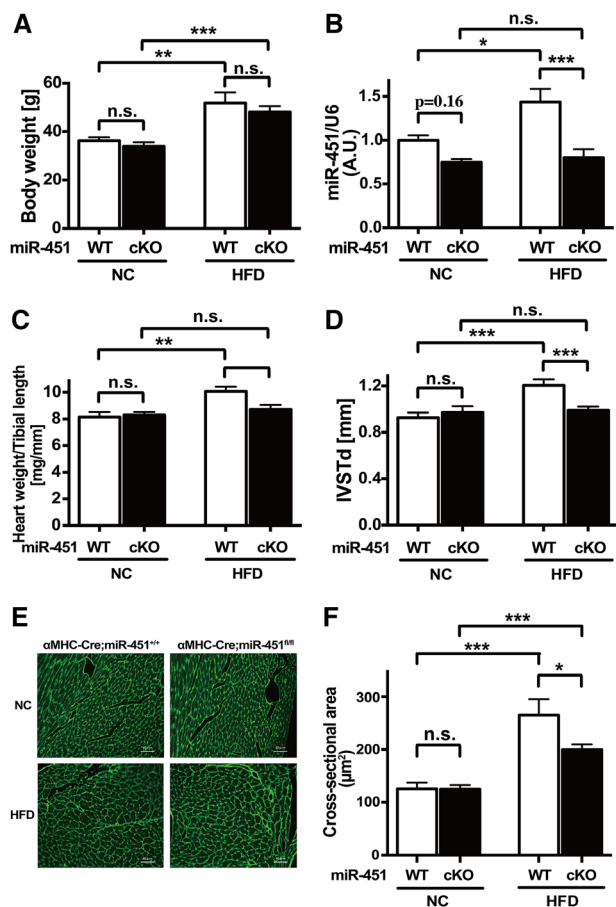


Figure 4. High-fat diet (HFD)-induced cardiac hypertrophy is ameliorated in cardiomyocyte-specific microRNA (miR)-451 knockout (miR-451 cKO) mice. Wild-type (WT) and cKO indicate α MHC-Cre;miR-451^{+/+} and α MHC-Cre;miR-451^{fl/fl}, respectively. **A**, Body weights of mice fed 10 kcal% fat-containing normal chow (NC) or 60 kcal% fat-containing HFD for 20 weeks, n=10 to 18. **B to D**, miR-451 levels in hearts (B, n=6–13), heart weight normalized using tibial length (C, n=10–14), and interventricular septum thickness at diastolic phase (IVSTd; D, n=6–9) of indicated NC- or HFD-fed mice. **E**, Representative images of lectin staining. White bar indicates 40 μ m. **F**, Quantification of cardiomyocyte cross-sectional area in NC- or HFD-fed control and miR-451 cKO mice, n=3 to 4. Data are presented as mean $\pm$ SEM. * P <0.05; ** P <0.01; *** P <0.001. AU indicates arbitrary unit.

performed Western blotting for cleaved caspase-3 and single strand DNA staining to evaluate cell toxicity in vivo. We did not detect cleaved caspase-3 (Online Figure XIA) and there was no difference in single strand DNA-positive cardiac myocytes (Online Figure XIB–XIE). To evaluate fibrosis, we also performed picrosirius red staining and quantitative reverse transcription PCR. However, we did not observe a significant difference in fibrosis area and collagen type 1 α 1 mRNA levels between the HFD-fed miR-451 cKO and control mice (Online Figure XII). These data suggest that HFD-induced cardiac hypertrophy is ameliorated in the miR-451 cKO mice.

AMPK Phosphorylation in the Heart Is Strengthened in HFD-Fed miR-451 cKO Mice

Western blotting analysis was performed to determine the signaling pathways activated in the control and miR-451 cKO mouse hearts. As expected, Cab39 protein levels in the hearts

were significantly increased in the HFD-fed miR-451 cKO mice compared with the HFD-fed control mice (Figure 5A). AMPK phosphorylation at Thr¹⁷², a downstream target of the Cab39/LKB1 complex, was also substantially enhanced in the hearts of the HFD-fed miR-451 cKO mice compared with the HFD-fed control mice (Figure 5B). Next, we evaluated the phosphorylation of mTOR, a regulator of protein synthesis,²⁶ and S6 ribosomal protein, which is a substrate of S6 kinase—a downstream target of mTOR. mTOR phosphorylation and S6 phosphorylation were significantly suppressed in the HFD-fed miR-451 cKO mice compared with the HFD-fed control mice (Figure 5C and 5D). Because AMPK suppresses mTOR signaling,²⁶ these data suggested that HFD-induced cardiac hypertrophy was attenuated, at least in part, via the

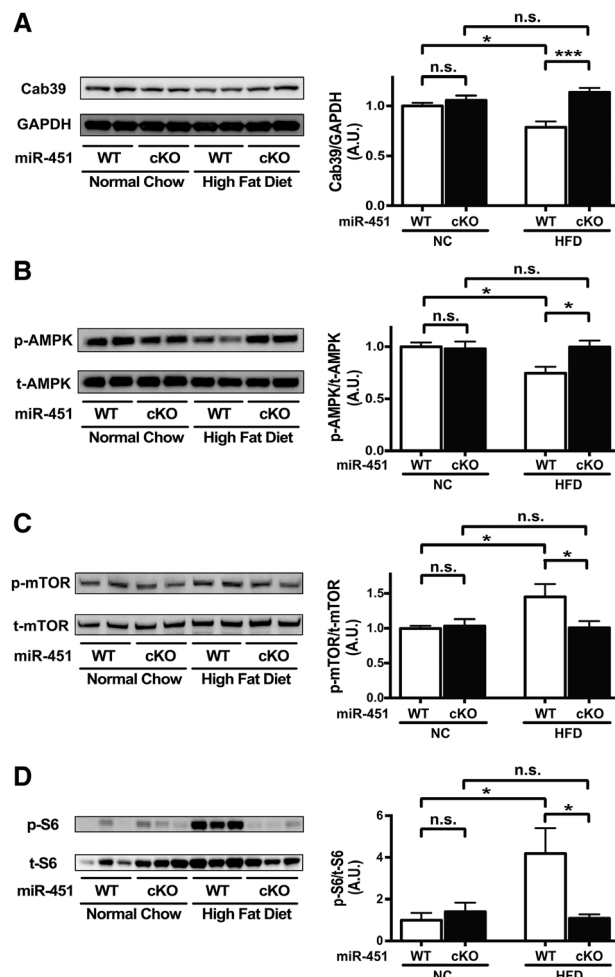


Figure 5. Phosphorylation of AMP-activated protein kinase (AMPK) in hearts is restored in high-fat diet (HFD)-fed microRNA (miR)-451 cKO (cardiomyocyte-specific knockout) mice. Western blotting analyses and the densitometric analyses of normal chow (NC)-fed or HFD-fed control (wild-type [WT]) and cardiomyocyte-specific miR-451 knockout (cKO) mice. **Left**, Representative images of Western blotting. **Right**, Quantifications by densitometric analysis. n=10 to 12. **A**, Calcium-binding protein 39 (Cab39). GAPDH was used as a loading control. **B**, Phosphorylated-AMPK (p-AMPK) and total-AMPK (t-AMPK). **C**, p-mammalian target of rapamycin (p-mTOR) and t-mTOR. **D**, p-S6 ribosomal protein (p-S6) and t-S6. n=6. Data are presented as mean $\pm$ SEM. * P <0.05; *** P <0.001. AU indicates arbitrary unit.

upregulation of the LKB1/AMPK signaling pathway in the miR-451 cKO mice.

Accumulation of Lipid Intermediates and Reactive Oxygen Species Production

Previous reports have shown that several lipid intermediates, such as triglyceride and ceramide, may be cell toxic for cardiac myocytes.^{8,27} Thus, we evaluated the accumulation of neutral lipid using BODIPY 505/515 staining. We detected small green droplets in cardiac myocytes. However, we did not observe any differences in the accumulation between HFD-fed control mouse hearts and HFD-fed cKO mouse hearts (Online Figure XIII A–XIII D). We next performed immunohistochemistry for ceramide⁸ and quantified ceramide content in the hearts. Long-term HFD feeding resulted in ceramide accumulation in control mouse hearts, whereas this accumulation was reduced in miR-451 cKO mouse hearts (Figure 6A–6C). Furthermore, we attempted to analyze the levels of reactive oxygen species (ROS). It has been reported that ROS peroxidize

ω -6 unsaturated FAs and generate 4-hydroxy-2-nonenal, an aldehyde. The 4-hydroxy-2-nonenal binds to proteins and composes 4-hydroxy-2-nonenal adducts.²⁸ Interestingly, Western blotting for 4-hydroxy-2-nonenal adducts revealed that ROS levels were higher in HFD-fed miR-451 cKO mouse hearts than in HFD-fed control mouse hearts (Figure 6D). We also performed dihydroethidium staining. As shown in Figure 6E and 6F, dihydroethidium fluorescence intensity was significantly increased in HFD-fed miR-451 cKO mice compared with HFD-fed control mice. These data suggest that ROS levels are significantly increased in HFD-fed miR-451 cKO mouse hearts compared with HFD-fed control mouse hearts.

Functional Changes in HFD-Fed miR-451 cKO Mouse Hearts

Finally, we sought to reveal the functional changes in the hearts. Cardiac stress was induced by dobutamine infusion and was monitored by cardiac catheterization. We found that the contractile reserve was reduced in HFD-fed control mouse

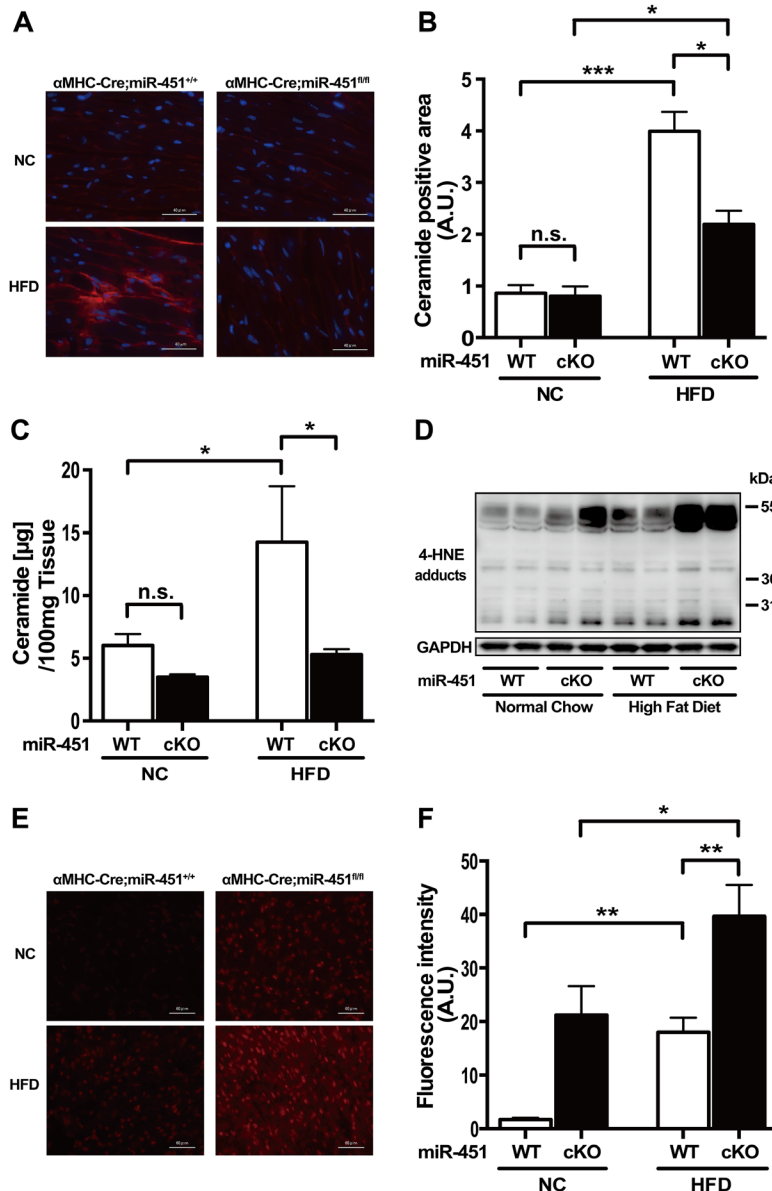


Figure 6. Accumulation of ceramides and reactive oxygen species levels. **A**, Representative images of ceramide staining. White bars indicate 40 μ m. **B**, Quantitative results for ceramide positive area. $n=5$ to 7. **C**, Ceramide content in the hearts. $n=3$ to 5. **D**, Western blotting for 4-hydroxy-2-nonenal (4-HNE) adducts in normal chow (NC)-fed or high-fat diet (HFD)-fed control (wild-type [WT]) and cardiomyocyte-specific miR-451 knockout (cKO) mouse hearts. Numbers indicate standard molecular weights. GAPDH was used as a loading control. **E**, Representative images of dihydroethidium (DHE) staining. White bars indicate 60 μ m. **F**, Quantitative results for DHE fluorescence intensity. $n=5$ to 7 for each group. Data are presented as mean $\pm$ SEM. * $P<0.05$; ** $P<0.01$; *** $P<0.001$. AU indicates arbitrary units.

heart compared with NC-fed control mouse heart, in line with a previous report¹³ (Figure 7A), although no difference in diastolic reserve was observed (Figure 7B). The reduced contractile reserve was ameliorated, at least in part, in the HFD-fed miR-451 cKO mouse hearts, as shown in Figure 7A.

Discussion

Our data suggest that miR-451 is involved in diabetic cardiomyopathy. Only miR-451 was upregulated in our type 2 DM model mice, and this was dependent on the duration of HFD feeding. Lu et al²⁹ found that miR-223 is differentially regulated in the cardiac tissue of patients with type 2 DM. To date, most researchers have used streptozotocin-induced diabetic mice to investigate miRNA involvement in diabetic cardiomyopathy.^{9–11} Although Shen et al¹⁰ used real-time PCR to validate 16 miRNAs identified by microarray analysis, the number of dysregulated miRNAs was small in DIO mouse hearts, a type 2 DM model. We considered that this was the case for 2 reasons. First, changes in miRNA expression levels associated with diabetic cardiomyopathy are dependent on the type of diabetes mellitus. Secretion defects of insulin and insulin resistance may invoke different miRNA changes in the heart. Second, the duration of HFD feeding may have been relatively short. To evaluate these possibilities, C57BL/6 mice fed HFD for a longer period or genetically manipulated mice, such as ob/ob or db/db mice, may be needed.

Because miR-144 and miR-451 are transcribed as a bicistronic transcript,^{14,15} it was surprising that miR-144 expression was unchanged in DIO mouse hearts. Considering our microarray results, miR-451 levels in the hearts were noticeably higher than miR-144 levels. We hypothesize that miR-451 may be more stable than miR-144 and miR-144 may be more fragile than miR-451, at least in hearts. It was reported that miR-144/451 double knockout mice showed insufficient maturation

of erythrocytes, resulting in mild anemia, and the phenotypes of the miR-144/451 double knockout mice and miR-451 single knockout mice were almost indistinguishable.¹⁴ These results suggest that miR-451 is functionally dominant compared with miR-144, at least in bone marrow. Furthermore, it was shown that miR-451 biogenesis was Dicer independent and required Argonaute2 catalytic activity.¹⁶ Taken together, the evidence indicates that miR-451 plays more important roles in the heart than miR-144, and post-transcriptional regulation of miR-451 expression may be altered in diabetic hearts.

Our data indicate that palmitic acid induced miR-451 upregulation in NRCMs. How is miR-451 expression regulated? Many reports showed that palmitic acid enhances nuclear factor κ B signaling^{3,30}; therefore, 1 possible mechanism is that activation of inflammation signaling, including nuclear factor κ B, induces miR-451. Others indicated that the miR-144/451 cluster was directly regulated by GATA4.²⁵ Numerous reports suggest that GATA4 plays a pivotal role in cardiac hypertrophy *in vitro* and *in vivo*; therefore, GATA4 may regulate miR-451 levels in HFD-induced hypertrophic hearts. Indeed, we observed the upregulation of GATA4 in HFD-fed mouse hearts. Recent reports demonstrated that activation of farnesoid X receptor (FXR) increased miR-144 and miR-451 levels in the liver.³¹ The nuclear receptor FXR regulates genes involved in the synthesis, secretion, and resorption of bile acids. Although abundant FXR expression is observed in the liver, intestine, kidney, and adrenal gland, bile acids are endogenous ligands for FXR, and some reports suggest that bile acid levels in serum are increased in obese or type 2 DM mice and humans.^{32,33} Hence, another possible mechanism is that bile acid-activated FXR induces miR-451 expression. Further studies are required to elucidate the transcription factors that regulate miR-451 expression.

Clinical data indicated that obesity and type 2 DM are linked to cardiac hypertrophy independently of blood pressure.³⁴ The present study and others show that long-term feeding of HFD to C57BL/6 mice results in cardiac hypertrophy.^{13,35} The phenotypes of HFD-induced obesity mouse hearts were similar to those of cardiomyocyte-specific LKB1 knockout (LKB1 cKO) mouse hearts. Ikeda et al reported that the ventricular weight-to-body weight ratio was significantly increased in the LKB1 cKO mice at 12 weeks of age compared with the control mice and fibrosis markers in cardiac ventricles were not different between the LKB1 cKO mice and control mice.³⁶ Our data strongly support that HFD-induced cardiac hypertrophy is partly because of decreased Cab39 levels through miR-451 upregulation. Collectively, the evidence indicates that miR-451 downregulation is a therapeutic strategy to attenuate HFD-induced cardiac hypertrophy.

Our data show that miR-451 overexpression resulted in cell toxicity, and miR-451 knockdown attenuated palmitate-induced lipotoxicity. Thus, to clarify the direct targets of miR-451, we focused on MIF and Cab39, which have cell protective functions.^{5,22,24} Our results show that the LKB1/AMPK signaling pathway plays a protective role in HFD-induced cardiac hypertrophy. These data are consistent with those of another report, which indicated that AMPK deficiency exacerbates obesity-induced cardiac hypertrophy.³⁵ Also, it is reported that miR-451 levels are significantly increased in the

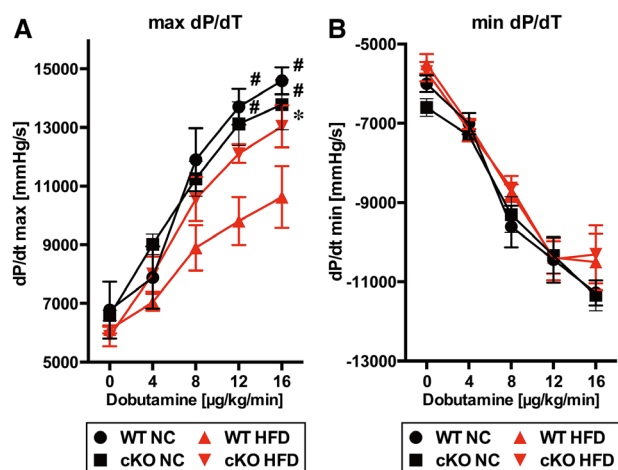


Figure 7. Evaluation of cardiac functional reserves. Cardiac stress was induced by continuous injection of dobutamine and cardiac functional reserves were monitored by cardiac catheterization. **A**, Changes in maximum (max) dP/dT. **B**, Changes in minimum (min) dP/dT. $n=5$ to 7 for each group. Data are presented as mean $\pm$ SEM. # $P<0.01$ vs high-fat diet (HFD)-fed control mice (wild-type [WT]) at the same dobutamine dose; * $P<0.05$ vs HFD-fed control mice at the same dobutamine dose. cKO indicates cardiomyocyte-specific miR-451 knockout mice; and NC, normal chow.

hearts of a hypertrophic cardiomyopathy mouse model and are involved in Cab39 expression in C2C12 cells.¹⁸ However, other unrecognized direct targets of miR-451 may be involved in diabetic cardiomyopathy because growing evidence suggests that miRNAs have large numbers of direct targets.

We used 45 kcal% fat-containing HFD-fed animals to screen dysregulated miRNAs in DIO mouse hearts. C57BL/6 mice were fed 45 kcal% fat-containing HFD for 20 weeks, and we identified miR-451 levels as being markedly increased by ≈ 2 -fold. Because palmitic acid stimulation increased miR-451 levels in a dose-dependent manner in NRCMs, miR-451 cKO mice were fed 60 kcal% fat-containing HFD for increasing the FA supply to the heart. However, the miR-451 levels increased by ≈ 1.5 -fold, and we did not observe further miR-451 upregulation in the 60 kcal% fat-containing HFD-fed mice compared with the 45 kcal% fat-containing HFD-fed mice. Compensatory mechanisms may inhibit the additional increase in miR-451 expression in vivo.

Cab39 also stabilizes other STE20 family kinases, ste20-related proline alanine-rich kinase and oxidative stress-responsive kinase, in a manner similar to ste20-related adaptor. These kinases function mainly in the kidney, but these kinases or other Cab39-binding proteins may attenuate diabetic cardiomyopathy. LKB1 also targets members of an AMPK-related superfamily.³⁷ To date, the functions of these peptides in the hearts remain unknown. Because it is possible that these peptides are also involved in HFD-induced cardiac hypertrophy, further investigations are needed to clarify the exact mechanisms.

In the present study, we demonstrated that ROS levels were higher in HFD-fed miR-451 cKO mouse hearts than in HFD-fed control mouse hearts. Many reports have shown that ROS levels are increased in type 2 diabetes mellitus, and that these ROS are harmful. Under these conditions, AMPK phosphorylation is usually suppressed. However, based on our data, the AMPK-induced ROS or ROS in AMPK-suppressed situations may be protective. This concept is consistent with the results in a previous report,³⁸ which shows that increased mitochondrial function and superoxide production increased by AMPK activation are beneficial in diabetic kidney disease. In addition, it has been reported that some types of ROS may play protective role and suppress inflammation in autoimmune disease.³⁹ Thus, the relationship between ROS and AMPK warrants further investigation.

LKB1 is recognized as a tumor suppressor gene, and loss of LKB1 function causes Peutz-Jeghers syndrome. Patients predisposed to this syndrome develop early-onset polyposis throughout the entire gastrointestinal tract and are at increased risk for developing cancer at a relatively young age. It was also reported that obesity increases the risk of cancer as well as cardiovascular disease. Hence, we speculate that miR-451 expression is induced in the gastrointestinal epithelium in obese patients. If so, attenuated LKB1 activity could increase the risk of gastrointestinal cancer. This hypothesis is fascinating because miR-451-targeted therapy may be able to prevent gastrointestinal cancer. Indeed, it has been proposed that increased miR-451 levels increase cell proliferation.⁴⁰

In conclusion, miR-451 levels were markedly increased in palmitate-stimulated NRCMs and DIO mouse hearts. We found that Cab39 was a direct target of miR-451 in the heart. miR-451 knockdown partly rescued lipotoxicity in vitro, and HFD-induced cardiac hypertrophy was ameliorated in miR-451 cKO mice through the LKB1/AMPK pathway. Thus, cardiac-specific inhibition of miR-451 is a promising strategy for treating diabetic cardiomyopathy.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- In both humans and mice, obesity and comorbid type 2 diabetes mellitus are associated with cardiomyopathy, characterized by the presence of cardiac hypertrophy and decreased cardiac function.
- Diabetic cardiomyopathy is associated with lipotoxicity, which results from excess supply of saturated fatty acids, such as palmitic acid.
- Calcium-binding protein 39 (Cab39) is a scaffold protein of liver kinase B1 (LKB1), a kinase upstream of AMP-activated protein kinase (AMPK), and stabilizes the LKB1.

What New Information Does This Article Contribute?

- MicroRNA (miR)-451 is upregulated in cardiac myocytes stimulated with palmitic acid and in hearts of mouse model of high-fat diet–induced obesity.
- miR-451 knockdown in cardiac myocytes rescues palmitate-induced lipotoxicity via the LKB1/AMPK signaling pathway.
- Cardiac myocyte–specific miR-451 knockout in mice ameliorates diabetic cardiomyopathy caused by obesity and type 2 diabetes mellitus.

Increasing evidence suggests that microRNAs are involved in cardiovascular diseases. However, the role of microRNAs in the

diabetic cardiomyopathy induced by obesity and type 2 diabetes mellitus is not fully understood. Using a mouse model of diet-induced obesity, we identified that miR-451 was significantly upregulated in the heart. miR-451 was induced in cardiac myocytes stimulated with palmitic acid, and Cab39 was a direct target of miR-451 in cardiac myocytes. Palmitate-induced lipotoxicity was rescued by miR-451 knockdown in cardiac myocytes. Furthermore, we generated cardiac myocyte–specific miR-451 knockout mice and found that high-fat diet (HFD)–induced cardiac hypertrophy was attenuated in these cardiac myocyte–specific miR-451 knockout mice through the LKB1/AMPK signaling pathway. Toxic lipid accumulation, which was present in HFD-fed control mouse hearts, decreased in HFD-fed cardiac myocyte–specific knockout mouse hearts. Finally, we found that the contractile reserve was ameliorated in HFD-fed cardiac myocyte–specific miR-451 knockout mouse hearts as compared with that in HFD-fed control mouse hearts. These findings indicate that miR-451 is involved in diabetic cardiomyopathy, and that miR-451 could be a potential therapeutic target for cardiac dysfunction associated with obesity and type 2 diabetes mellitus.

MicroRNA-451 Exacerbates Lipotoxicity in Cardiac Myocytes and High-Fat Diet-Induced Cardiac Hypertrophy in Mice Through Suppression of the LKB1/AMPK Pathway

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Supplemental Material

Detailed Methods

Mice and diets

To examine miRNA expression changes in DIO mouse hearts, C57BL/6 male mice (Charles River Laboratories) were used. Floxed miR-451 mice¹ and α myosin heavy chain promoter-driven Cre-recombinase (α MHC-Cre) transgenic mice were kind gifts of Dónal O'Carroll¹ and Kinya Otsu, respectively. The floxed miR-451 mice and α MHC-Cre transgenic mice were backcrossed to C57BL/6 mice for at least 10 generations. The primer sequences for genotyping are listed in Online Table I. Specific pathogen-free animals were maintained in the animal laboratories of Kyoto University Graduate School of Medicine. This investigation was approved by the Kyoto University Ethics Review Board. Normal chow (NC), containing 10 kcal% fat (F-2), was purchased from Funabashi Farm Company. In the present study, two types of HFD were used. D12451 (45 kcal% fat) and D12492 (60 kcal% fat) were purchased from Research Diets, Inc. All mice were grown by NC feeding until they were 8 weeks old, after which they were fed NC or HFD for additional 8 or 20 weeks. Sixteen-hour fasted mice were euthanized, and blood was collected from the inferior vena cava in a heparinized syringe to measure the fasting glucose concentration. The hearts and livers were then excised. The organs were washed immediately in cold phosphate-buffered saline (PBS) and weighed. Cut hearts were frozen in liquid nitrogen and stored at -80°C until analysis. To evaluate plasma concentrations of palmitic acid, we collected blood as described above from mice fasted for 6–8 h. Collected blood was centrifuged at $3300 \times g$ for 10 min, and plasma was stored at -80°C .

When measuring palmitic acid levels in apoB precipitated serum, blood was collected with a syringe and then transferred to a tube containing a serum separating gel (Terumo). This tube was centrifuged at $1200 \times g$ for 10 min. Then polyethyleneglycol (PEG) was added to a serum aliquot to deplete apoB-containing lipoproteins². To measure tibial length, the lower legs were severed and rendered in 1 mol/L NaOH at 37 °C until the tibial bones were completely visible.

RNA extraction

To extract total RNA, organs were homogenized in 1 mL TRIzol[®] reagent (Invitrogen) using a homogenizer. Total RNA from cells was also isolated using 1 mL TRIzol[®] reagent. The pellet of total RNA was dissolved in diethylpyrocarbonate water. The quantity and quality of total RNA were determined using a spectrophotometer (GeneQuant pro, GE Healthcare).

Microarray analysis and quantitative real-time PCR

Each 500 ng of total RNA extracted from 4–6 mice was mixed and analyzed using an miRNA microarray analysis system (3D-Gene[™], Toray). miRNAs were quantified using TaqMan[®] MicroRNA Assays (Applied Biosystems) and a 7900HT Fast Real-Time PCR System (Applied Biosystems) in accordance with the manufacturer's instructions. miRNA levels were normalized by U6 small nuclear RNA and calculated by the $2^{-\Delta\Delta C_t}$ method³. To determine the miR-451 levels in erythrocytes, we prepared a standard curve using artificial miR-451 and calculated the miR-451 level in 100 ng of total RNA. Then the miR-451 level per cell was determined based on the total RNA mass obtained and the number of cells harvested by FACS sorting. To evaluate mRNA levels, complementary DNA was synthesized using the Transcriptor First

Strand cDNA Synthesis Kit (Roche) in accordance with the manufacturer's instructions, and PCR was performed in a 7900HT Fast Real-Time PCR System (Applied Biosystems) with SYBR Green PCR master mix (Applied Biosystems), normalized to β -actin. The primer sequences are listed in Online Table I. Quantification was performed by the $2^{-\Delta\Delta C_t}$ method.

Primary neonatal rat ventricular cardiomyocytes culture

Neonatal rat ventricular cardiomyocytes were isolated from 1-day-old Sprague-Dawley rats, as described previously⁴. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and plated in multi-well™ Primaria™ 6- or 24-well plates (Becton Dickinson) at 37 °C in a 5% CO₂ incubator. Lentivirus infection was induced 48 h after plating the cells.

Purification of neonatal mouse cardiomyocytes, cardiac fibroblasts, and erythrocytes

Mouse ventricles were digested in the same manner as described above with slight modifications. In brief, ventricles were isolated from 1-day-old C57BL/6 mice and dispersed in digestion buffer containing 1.3 mg/mL pancreatin (Sigma, P3292) and 0.45 mg/mL collagenase type II (Gibco, 17101-015). Collected cells were washed in a 3:1 mixture of DMEM and medium 199 (Gibco) supplemented with 10% horse serum (Gibco), 10% FBS (Sigma, 172012), 100 units/mL penicillin (Gibco), 100 μ g/mL streptomycin (Gibco), and 292 μ g/mL L-glutamine (Gibco). The cells were centrifuged at 280 $\times$ g for 5 min, mixed in serum-free DMEM, and stained for fluorescence-activated cell sorting (FACS) analyses.

As described previously⁵ and according to the manufacturer's instructions, cells were stained

in 200 nM MitoTracker[®] Green FM (Invitrogen, M7514) solution for 30 min at 37 °C, followed by blocking for Fcγ receptors using anti-mouse CD16/CD32 antibody (BD Pharmingen, 553142). To isolate cardiac fibroblasts, we used anti-mouse Thy-1.2 antibody conjugated with allophycocyanin (APC) (eBioscience, 17-0902-81). Rat IgG2a κ conjugated with APC (eBioscience, 17-4321-81) was used as an isotype control immunoglobulin. All dispersed cells were dissociated with cold PBS supplemented with 2% FBS and then analyzed on a FACS system (BD FACSAria[™] II, Becton Dickinson) using 515–545 and 650–670 nm bandpass filters to detect MitoTracker[®] Green and APC, respectively. Before sorting, pre-gating for eliminating doublet fractions, in which one droplet contains more than two cells, was performed in accordance with the manufacturer's instructions.

To collect erythrocytes, peripheral blood was obtained from the jugular vein of a neonatal mouse using a heparinized syringe. After blocking, erythrocytes were labeled with an anti-mouse Ter-119 antibody conjugated with FITC (eBioscience, 11-5921-81), and sorted by FACS.

Fatty acid stimulation

Palmitic acid (Sigma, P5585) was dissolved in ethanol, and a 500 mmol/L stock solution was stored at –20 °C. This stock solution was combined with 5 mmol/L of fatty acid-free bovine serum albumin (BSA) (Wako, 013-15143) at a molecular ratio of 10:1 (fatty acid:albumin) in serum-free medium^{6,7}. The indicated concentrations of palmitic acid were prepared in serum-free medium. To stimulate NRCMs with various concentrations of palmitic acid in a physiological concentration of albumin, 275 mmol/L of palmitic acid dissolved in ethanol was

added to serum-free medium containing 550 $\mu\text{mol/L}$ of fatty acid-free BSA. Two days after infecting cardiomyocytes with lentivirus, cardiomyocytes were stimulated with palmitic acid. An oleic acid (Sigma, O1008) solution was prepared using a method similar to that for making the palmitic acid solution.

Cell viability and cell injury evaluation

Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) assay. The cells were labeled with MTT at a final concentration of 0.5 mg/mL for at least 6 h at 37 °C. We measured the absorbance at 595 nm using an Elx800 microplate reader (BioTek Instruments, Inc.). The cell injury ratio was determined using the lactate dehydrogenase (LDH) Cytotoxicity Assay KitTM (Cayman Chemical Company) in accordance with the manufacturer's instructions.

Plasmids

An expression vector of miR-451 was generated using the BLOCK-iTTM Pol II miR RNAi Expression Vector Kit (Invitrogen) in accordance with the manufacturer's instructions. We generated two constructs for miR-451 knockdown, which were named "decoys." In decoy $\times 3$ and $\times 6$ constructs, three or six complementary tandem sequences for miR-451 were inserted in the 3' UTR of the pMIR-REPORTTM vector (Invitrogen), respectively, as described previously^{3, 8}. The unmodified pMIR-REPORTTM vector was used as the control vector. The pRL-TKTM *Renilla reniformis* luciferase (RL) plasmid was purchased from Promega. For dual luciferase assays, a macrophage inhibitory factor (MIF) or Cab39 3' UTR was also inserted in

the 3' UTR of the pMIR-REPORT™ vector. Mutated (Mut) 3' UTR constructs were created using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) in accordance with the manufacturer's instructions. For Cab39 overexpression, the subcloned Cab39-coding sequence was inserted into the multicloning site in the lentivirus vector. The primer sequences used are described in Online Table II. The constructs transfected into NRCMs were transferred to the lentivirus vector because transfection efficacy using this vector is very high^{4,8}.

Lentivirus production and DNA transduction

Lentiviral stocks were produced in 293T cells in accordance with the manufacturer's instructions (Invitrogen), as described previously^{4,8}. In brief, virus-containing medium was collected for 48 h after transfection and filtered through a 0.45- μ m filter. One round of lentiviral infection was performed by replacing the medium with virus-containing medium that contained 8 μ g/mL polybrene, followed by centrifugation at 1220 $\times g$ for 30 min.

Dual luciferase reporter assay

The dual luciferase assay was performed as described previously³. In total, 0.02 μ g firefly luciferase (FL) reporter plasmid, 0.25 μ g miR expression vector, and 0.02 μ g pRL-TK™ RL plasmid for normalizing transfection efficiency were transfected into 293T cells. After 2 days of incubation, both luciferase activities were measured using a dual luciferase reporter assay system (Toyo Ink).

Western blotting analysis

Immunoblotting analysis was performed using standard procedures as described previously⁸. Cultured cells were homogenized in lysis buffer consisting of 100 mM Tris-HCl (pH 7.4), 75 mM NaCl, and 1% TritonTM X-100 (Nacalai Tesque). The buffer was supplemented with Complete MiniTM protease inhibitor (Roche), aprotinin (Sigma), 50 mM NaF, and 1 mM Na₃VO₄ just prior to use. Protein concentrations were determined using the bicinchoninic acid protein assay kit (Bio-Rad). A total of 20 µg protein was fractionated using NuPAGETM 4–12% Bis-Tris (Invitrogen) gels and transferred to a ProtranTM nitrocellulose transfer membrane (Whatman). The membrane was blocked using 1× PBS containing 5% non-fat milk for 1 h and incubated with the primary antibody overnight at 4 °C. After being washed in 0.05% T-PBS (1× PBS and 0.05% Tween-20), the membrane was incubated with the secondary antibody for 1 h at 4 °C. After the membrane was washed again in 0.05% T-PBS, the immune complexes were detected using the ECL-PlusTM chemiluminescent detection reagent (Amersham Biosciences). The following primary antibodies were used: anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling, 14C10), 1:3000; anti-rat MIF (Torrey Pines Biolabs, Inc., TP234), 1:2000; anti-MO25α/Cab39 (Cell Signaling, C49D8), 1:1000; anti-phospho-AMPKα (Cell Signaling, Thr172, 2531), 1:500; anti-total AMPKα (Cell Signaling, 2532), 1:1000; anti-phospho-mTOR (Cell Signaling, Ser2448), 1:1000; anti-total mTOR (Cell Signaling, 7C10), 1:1000; anti-phospho-S6 ribosomal protein (Cell Signaling, D57.2.2.E), 1:1000; anti-total S6 ribosomal protein (Cell Signaling, 5G10), 1:1000; anti-cleaved caspase-3 (Cell Signaling, 9661), anti-GATA4 (Santa Cruz, sc-9053), 1:500; anti-4-hydroxy-2-nonenal (4-HNE) (Nikken Seil Co., Ltd, clone HNEJ-2, MHN-020P), 2 µg/mL. Anti-rabbit IgG (GE Healthcare) and anti-mouse IgG (GE Healthcare) were used as

secondary antibodies each at a dilution of 1:2000. Immunoblots were detected using LAS-3000 (Fujifilm). For quantification of western blotting, densitometric analyses were performed using ImageJ64 software (NIH).

Transthoracic echocardiography

Mice were anesthetized with 300 mg/kg of 2,2,2-tribromoethanol (Sigma, T48402), and heart rates were kept at 500–600 beats/min. Transthoracic echocardiography was performed using the SONOS 4500 Ultrasound Imaging System (Philips). Left ventricular wall thickness was measured in the parasternal short-axis view.

Lectin staining, cross-sectional area measurement, and other histological analyses using paraffin embedded hearts

We performed perfusion-fixed mice with 4% paraformaldehyde before excising the heart, which was further fixed in 4% paraformaldehyde at 4 °C overnight. The next day, the tissue was transferred to 70% ethanol for dehydration until paraffin embedding. Paraffin-embedded ventricular short-axis sections were stained with FITC-conjugated lectin (Sigma, L4895). Single strand DNA staining was performed using an anti-single strand DNA antibody (IBL, 18731). Picrosirius red staining was also performed using paraffin embedded section. Images were acquired with BZ-9000 (Keyence). Cardiomyocyte cross-sectional area measurements were made using BZ-H1M analyzing software (Keyence). Approximately 100 cells were measured per heart at $\times 400$ magnification, and the averages were used for analysis⁹.

Ceramide, neutral lipid, and DHE staining

Hearts were fixed as described above. After fixation, the specimens were incubated in 15% sucrose/PBS at 4°C for 2-3 h and in 30% sucrose/PBS at 4 °C overnight. The next day, the tissue samples were embedded in Tissue-Tek OCT (Sakura, Japan) compound. Ceramide staining was performed for 8µm frozen section. A heart section was washed with 0.05% T-PBS, and then blocked with 5% donkey serum in PBS at room temperature for 15 min. An anti-ceramide antibody (Enzo Life Sciences, Inc. MID 15B4) was used for ceramide detection¹⁰. After washing three times with 0.05% T-PBS, Alexa Fluor 594 donkey anti-mouse IgG antibody (Life technologies, A-21203) was used as a secondary antibody. For neutral lipid staining, heart sections were incubated in PBS containing 1 µmol/L of BODIPY 505/515 (Life Technologies, D-3921) dye at room temperature for 1 h. For dihydroethidium (DHE; Life Technologies, D-1168) staining, harvested hearts were washed in cold PBS, and then directly embedded in Tissue-Tek OCT (Sakura, Japan) compound. Frozen sections of 10 µm were incubated in PBS containing 10 µmol/L of DHE at room temperature for 1 h. After washing in PBS, sealed sections were observed using BZ-9000 (Keyence). Five to ten images were randomly acquired using the same exposure time for each mouse, after which fluorescence intensities were determined using ImageJ64 software (NIH).

Quantification of ceramide in hearts

Total lipids were extracted from the hearts by Folch method. Ceramide content was measured using high-performance liquid chromatography/evaporative light-scattering detection

(HPLC-ELSD)¹¹. A standard curve was determined using commercially available ceramide (Takasago International Corporation, Ceramide TIC-001).

Cardiac catheterization in mice

We analyzed cardiac functional reserve by cardiac catheterization of mice that were continuously administered dobutamine via the jugular vein^{12, 13}. Briefly, a mouse was anesthetized with isoflurane, intubated, connected to a rodent ventilator (Harvard Apparatus, Inspira ASVv), and maintained on 1.5% isoflurane. The mouse was then placed on a heat pad to maintain its body temperature and electrocardiography was monitored during this procedure. Ventilation was by positive-pressure respiration. The settings were determined according to the following formulas: tidal volume (ml) = $6.2 \times M^{1.01}$; respiration rate (min^{-1}) = $53.3 \times M^{-0.26}$; M = body weight (kg)¹⁴. Both vagal nerves were cut, and a 1.0 French mouse pressure catheter (Millar, SPR-1000) was inserted into the right carotid artery via a small incision. The catheter tip was manipulated across the aortic valve into the left ventricle. To infuse dobutamine, the left jugular vein was cannulated with a stretched polythene tube. After stabilizing hemodynamic conditions, dobutamine was administered intravenously with an infusion pump (KD Scientific, KDS100). The dobutamine dose was increased from 4 $\mu\text{g/kg/min}$ to 16 $\mu\text{g/kg/min}$ every 2 min¹². Left ventricular pressure signals obtained from 10 to 20 beats were averaged, and then analyzed using PowerLab software (ADInstruments, LabChart 5).

Statistical analysis

Measurements are presented as means $\pm$ SEM (standard error of the mean). For statistical

comparisons, Mann–Whitney U test (two groups) and one-way ANOVA (three or more groups) with Tukey's post-hoc test were used as appropriate. For statistical comparisons only in Online Figure II, unpaired t-test was used. To compare cardiac functional reserves, two-way ANOVA with Tukey's post-hoc test was used. A probability value of <0.05 was considered to indicate statistical significance. Statistical analyses were performed using GraphPad Prism 6 statistical packages (GraphPad Software, Inc.).

Online Table I. Primer sequences for genotyping miR-451^{fl/fl} mice and α MHC-Cre

transgenic mice and quantitative RT-PCR

Genotype	Forward (5' –3')	Reverse (5' –3')
miR-451 ^{fl/fl}	GGCTGGGATATCATCATATACT	AGTTAAATGACAAGTGCTCTTA
α MHC-Cre	GAACACACCTGGAAGATGCTCCT	CTGATTCTGGCAATTCGGCAAT
Target gene	Forward (5' –3')	Reverse (5' –3')
BNP	GCCAGTCTCCAGAGCAATTCA	TGTTCTTTTGTGAGGCCTTGG
Col1A1	GCCAAGAAGACATCCCTGAAG	TCATTGCATTGCACGTCATC
β -actin	AGATTACTGCTCTGGCTCCTA	CAAAGAAAGGGTGTAAAACG

α MHC-Cre: α myosin heavy chain promoter-driven Cre-recombinase; BNP: brain natriuretic

peptide; Col1A1: collagen type 1 α 1.

Online Table II. Primer sequences for subcloning mmu-Cab39

Direction	Sequence (5' –3')
Forward	GCTCTAGACACCATGCCGTTCCCATTTGGCAAGT
Reverse	CCGCTCGAGCTAGGCTTCCTGCTGGGCGGCTCTCTT

Cab39: calcium-binding protein 39. These sequences include restriction enzyme sites.

Supplemental References

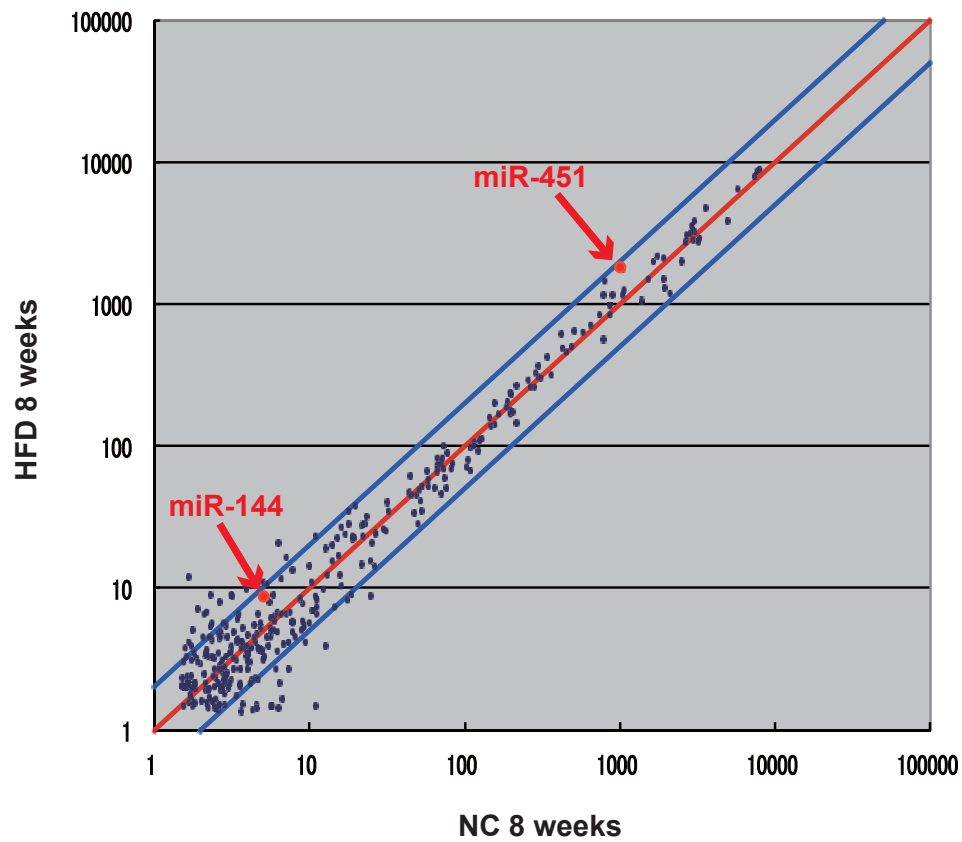
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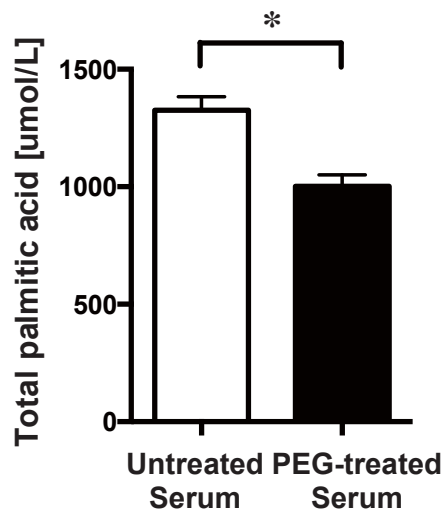
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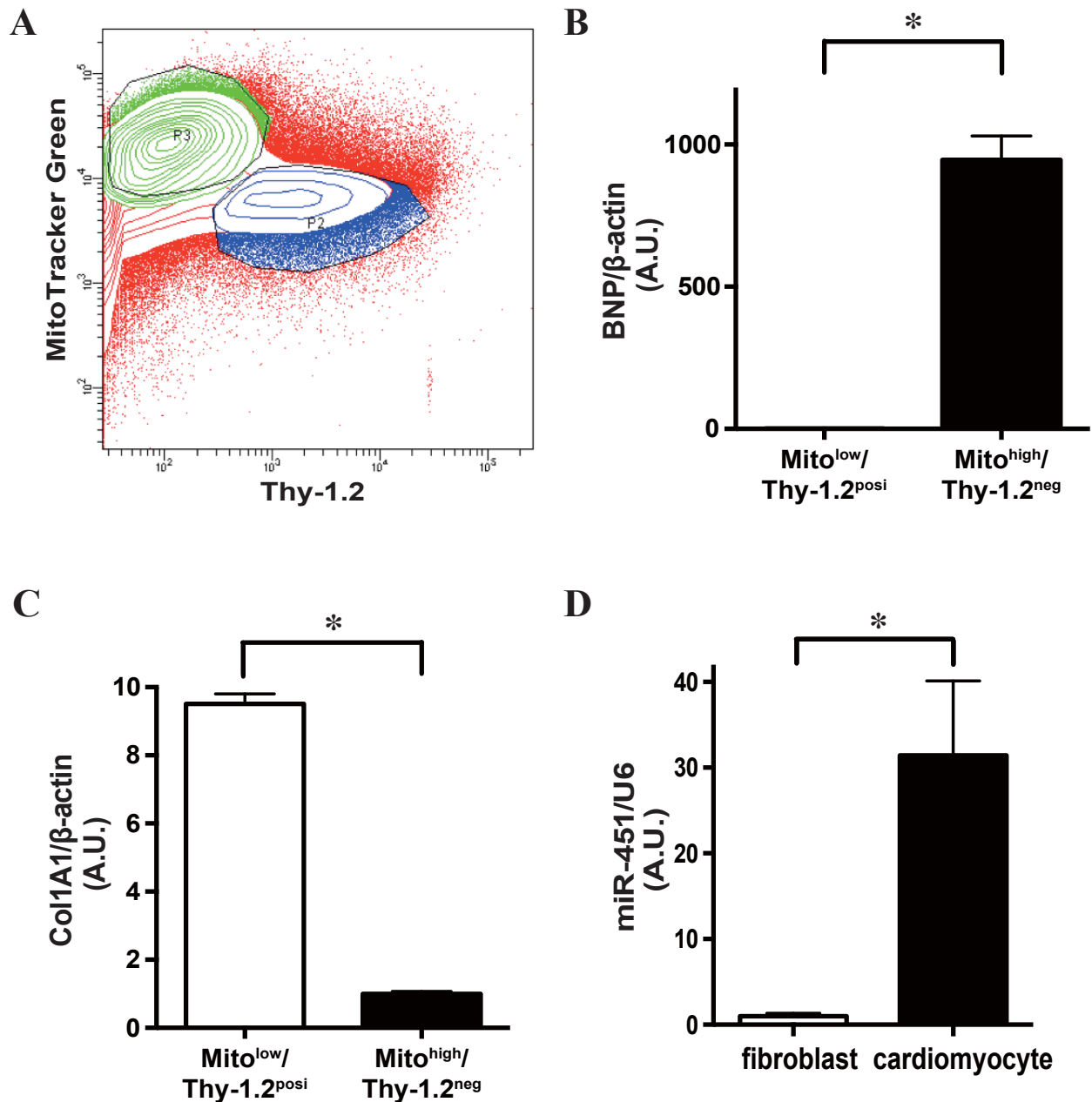


Online Figure I. Expression changes in miRNAs in the hearts of mice fed normal chow (NC) or high-fat diet (HFD) for 8 weeks.

miRNA expression profiling was performed using a miRNA microarray. The red diagonal line indicates the unchanged line. Two blue lines represent 2-fold changes.



Online Figure II. Total palmitic acid levels in untreated and in PEG-treated serum. Apo-B containing lipoproteins were precipitated by PEG, after which palmitic acid levels were measured. PEG: polyethyleneglycol, n=3. Data are presented as mean±SEM. *, $p < 0.05$.



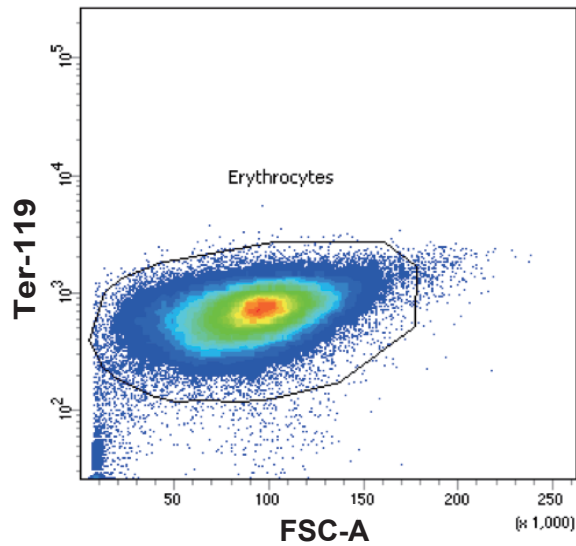
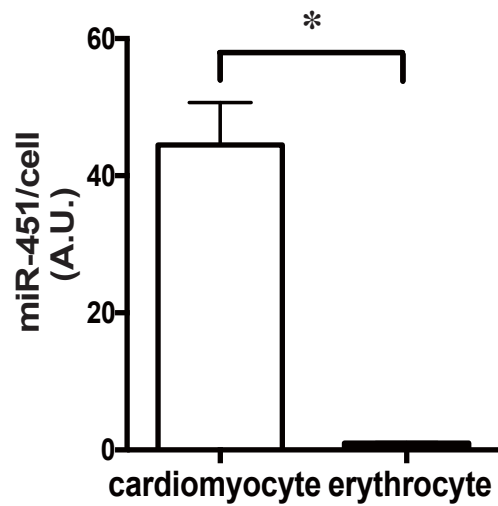
Online Figure III. miR-451 levels in cardiomyocytes and cardiac fibroblasts.

A, Isolating cardiomyocytes and cardiac fibroblasts from neonatal mouse hearts.

Fluorescence-activated cell sorting (FACS) analysis of neonatal mouse ventricle-derived cells stained with Mitotracker Green dye and APC-conjugated Thy-1.2 antibody. Blue (P2) and green (P3) populations indicate the Mitotracker^{low}/Thy-1.2^{posi} and Mitotracker^{high}/Thy-1.2^{neg} populations, respectively. B and C, mRNA levels of brain natriuretic peptide (BNP) and collagen type 1 α 1 (Col1A1) in FACS-sorted cell populations. n=4. D, miR-451 levels normalized to U6 levels in cardiomyocytes and cardiac fibroblasts, n=4. A.U.: arbitrary units.

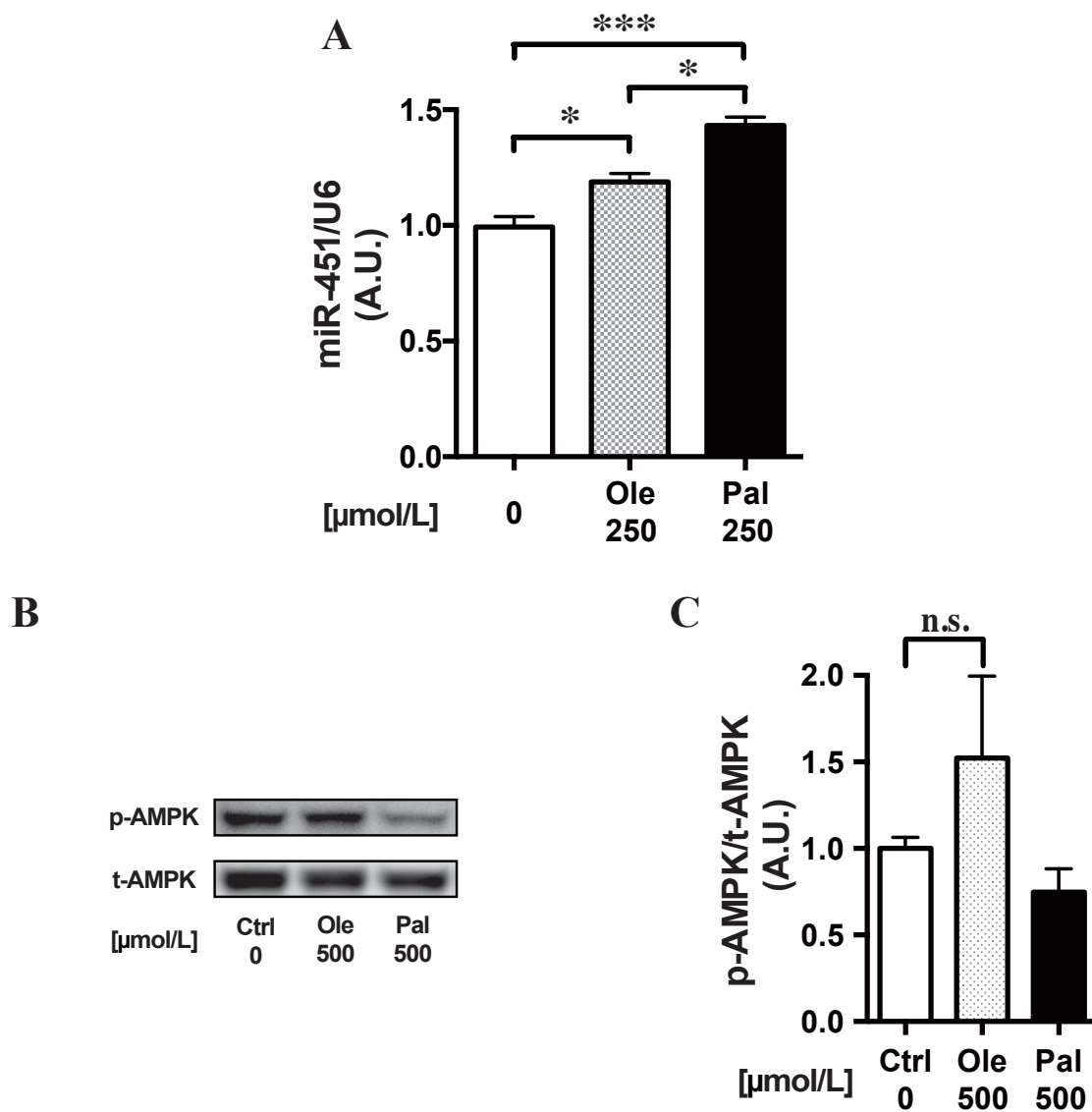
Data are presented as mean $\pm$ SEM. *, p < 0.05.

Online Figure III

A**B**

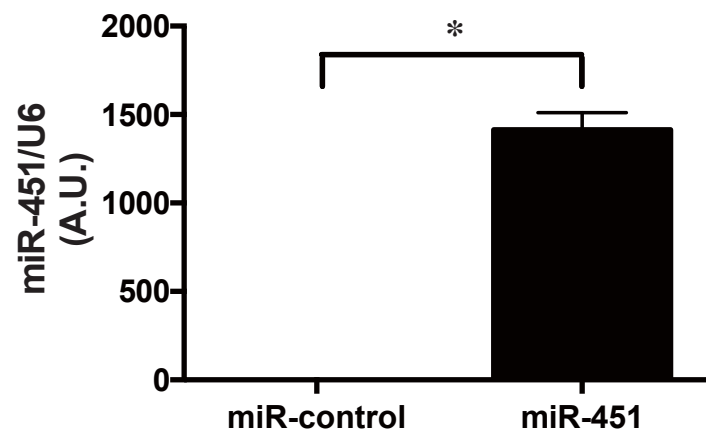
Online Figure IV. Isolating erythrocytes by FACS sorting, and miR-451 levels in cardiomyocytes and erythrocytes.

A, FACS analysis of neonatal mouse peripheral blood after staining with anti-mouse Ter-119 conjugated with FITC. B, miR-451 levels per cell for cardiomyocytes and erythrocytes. n=4. A.U.: arbitrary unit. Data are presented as mean $\pm$ SEM. *, $p < 0.05$.



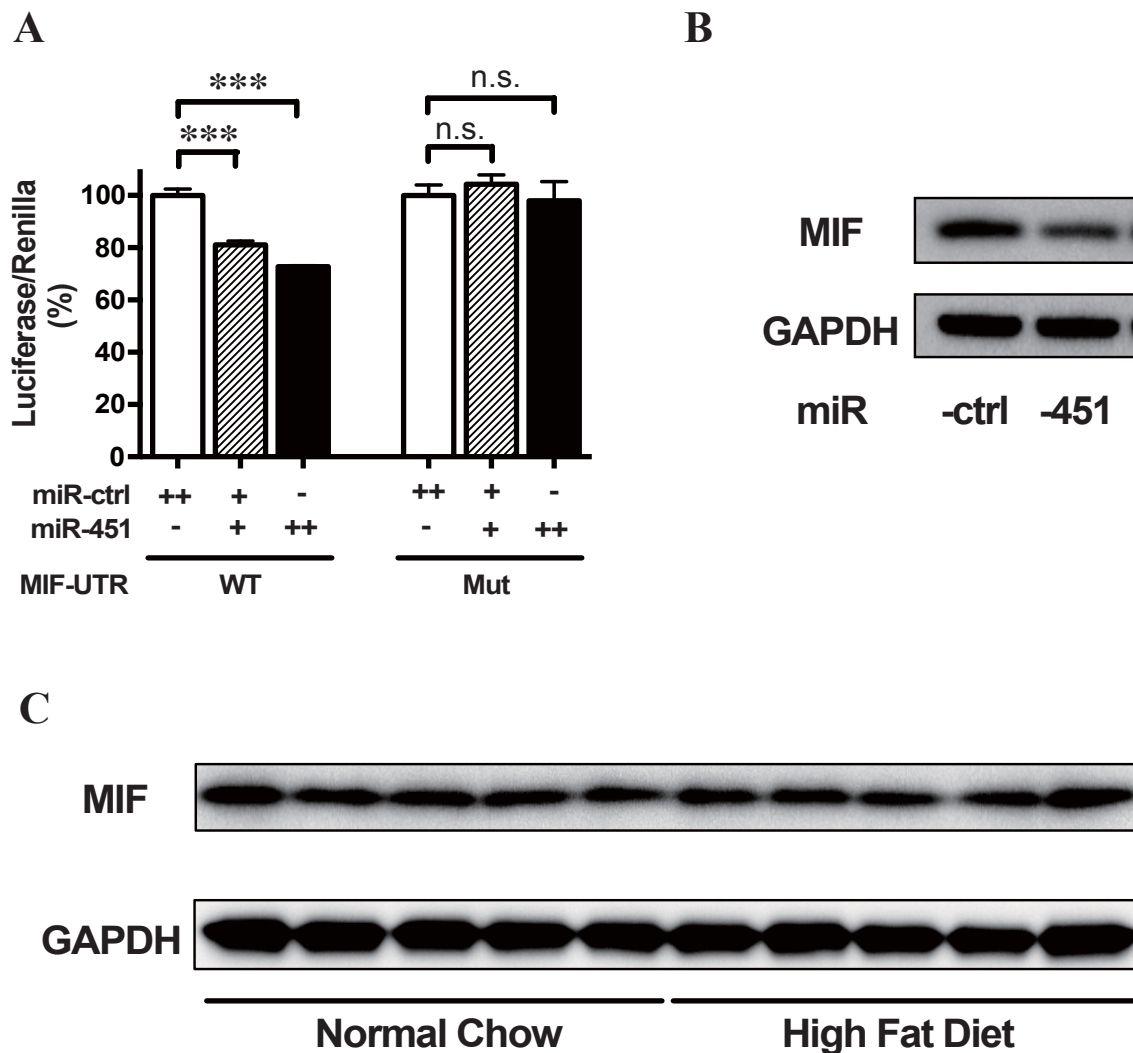
Online Figure V. miR-451 levels in NRCMs stimulated with oleic acid.

A, miR-451 levels in NRCMs after stimulation with 250 $\mu\text{mol/L}$ of the indicated acid for 24 hours. $n=4-5$. Ole: oleic acid; Pal: palmitic acid. B, Representative western blotting images for p-AMPK and t-AMPK after oleic acid or palmitic acid stimulation in NRCMs for 24 hours. C, Quantitative AMPK phosphorylation results by densitometry, $n=6$. A.U.: arbitrary units. Data are presented as mean $\pm$ SEM. *, $p < 0.05$; ***, $p < 0.001$.



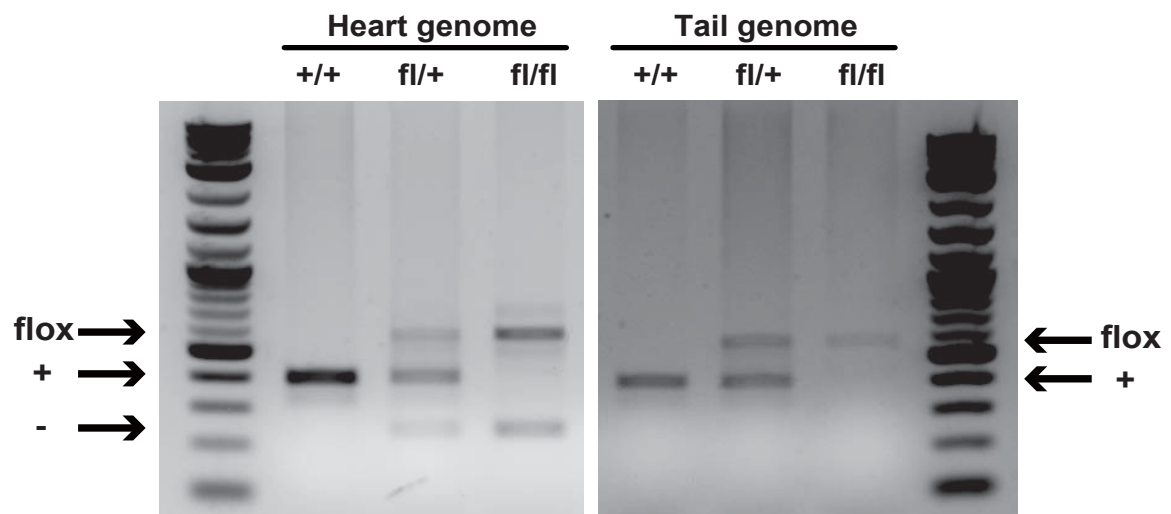
Online Figure VI. miR-451 level in NRCMs infected with miR-451 overexpression vector.

miR-451 levels were measured using Taqman microRNA real-time PCR. U6 indicates U6 small nuclear RNA used as an internal control, n=4. A.U.: arbitrary units. Data are presented as mean±SEM. *, $p < 0.05$.

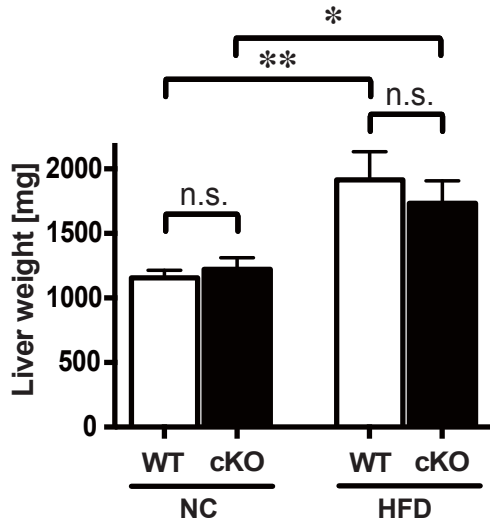
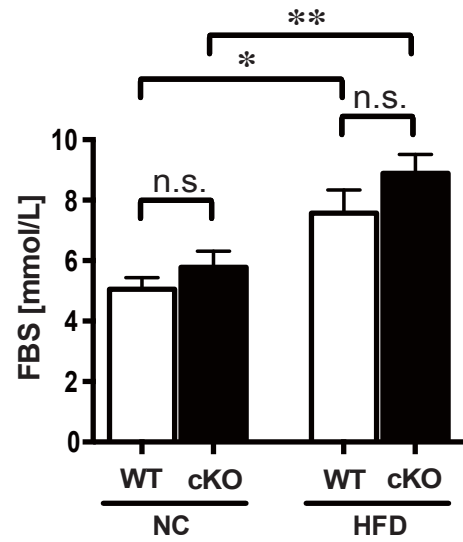
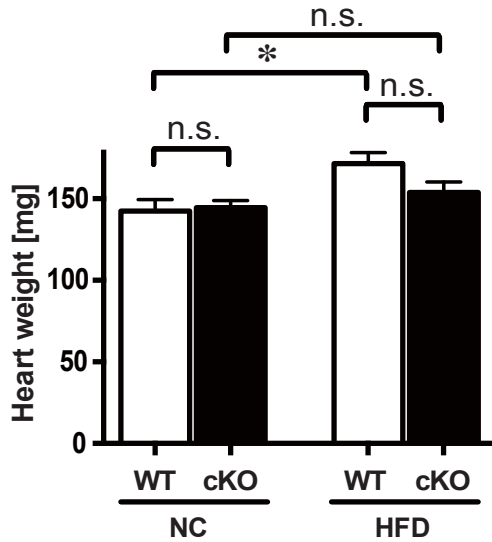
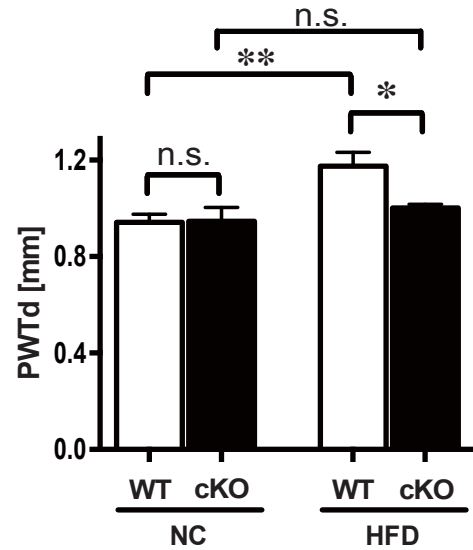


Online Figure VII. Macrophage inhibitory factor (MIF) is a direct target in neonatal rat cardiomyocytes (NRCMs).

A, Dual luciferase analysis to examine if MIF is a direct target of miR-451. 293T cells were transfected with the miR-control (miR-ctrl) or a miR-451 expression plasmid, and luciferase plasmid-harboring WT MIF 3'UTR or Mut MIF 3'UTR. n=4. B, Western blotting of MIF in NRCMs infected with the miR-ctrl or miR-451 expression vectors. C, Western blotting of MIF in the hearts of mice fed 10 kcal% fat-containing NC or 45 kcal% fat-containing HFD for 20 weeks. GAPDH: glyceraldehyde-3-phosphate dehydrogenase (as a loading control). Data are presented as mean±SEM. ***, $p < 0.001$.



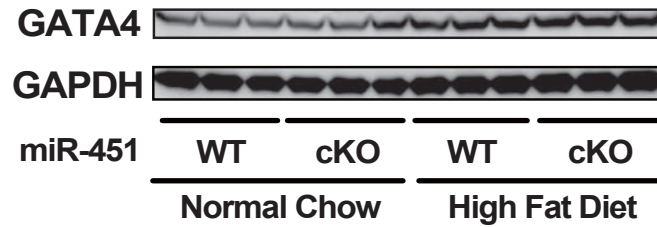
Online Figure VIII. Verification of miR-451 recombination in the heart genome of cardiomyocyte-specific miR-451 knockout (miR-451 cKO) mice.
 Validation of miR-451 recombination by electrophoresis of PCR products amplified from the heart and tail genomes.

A**B****C****D**

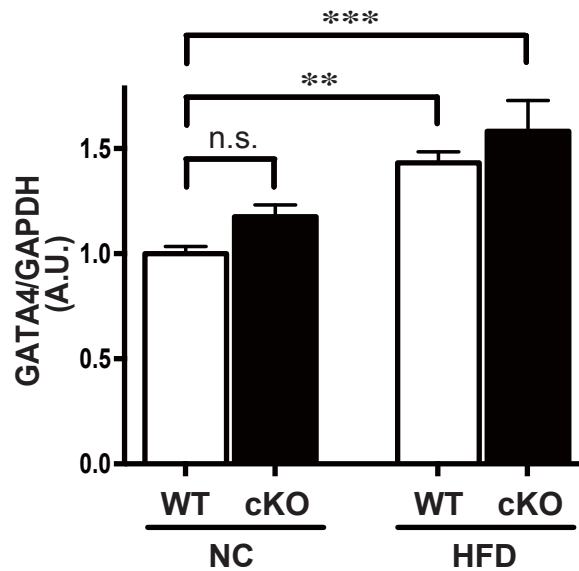
Online Figure IX. The phenotypes of cardiomyocyte-specific miR-451 knockout (miR-451 cKO) mice.

A–D, liver weights (A, n=9–10), fasting blood sugar (FBS) concentrations (B, n=10–14), heart weights (C, n=11–15), and posterior wall thickness at diastolic phase (PWTd) (D, n=6–9) of indicated mice fed NC or HFD for 20 weeks. WT and cKO indicate α MHC-Cre;miR-451^{+/+} and α MHC-Cre;miR-451^{fl/fl}, respectively. Data are presented as mean±SEM. *, p < 0.05; **, p < 0.01.

A



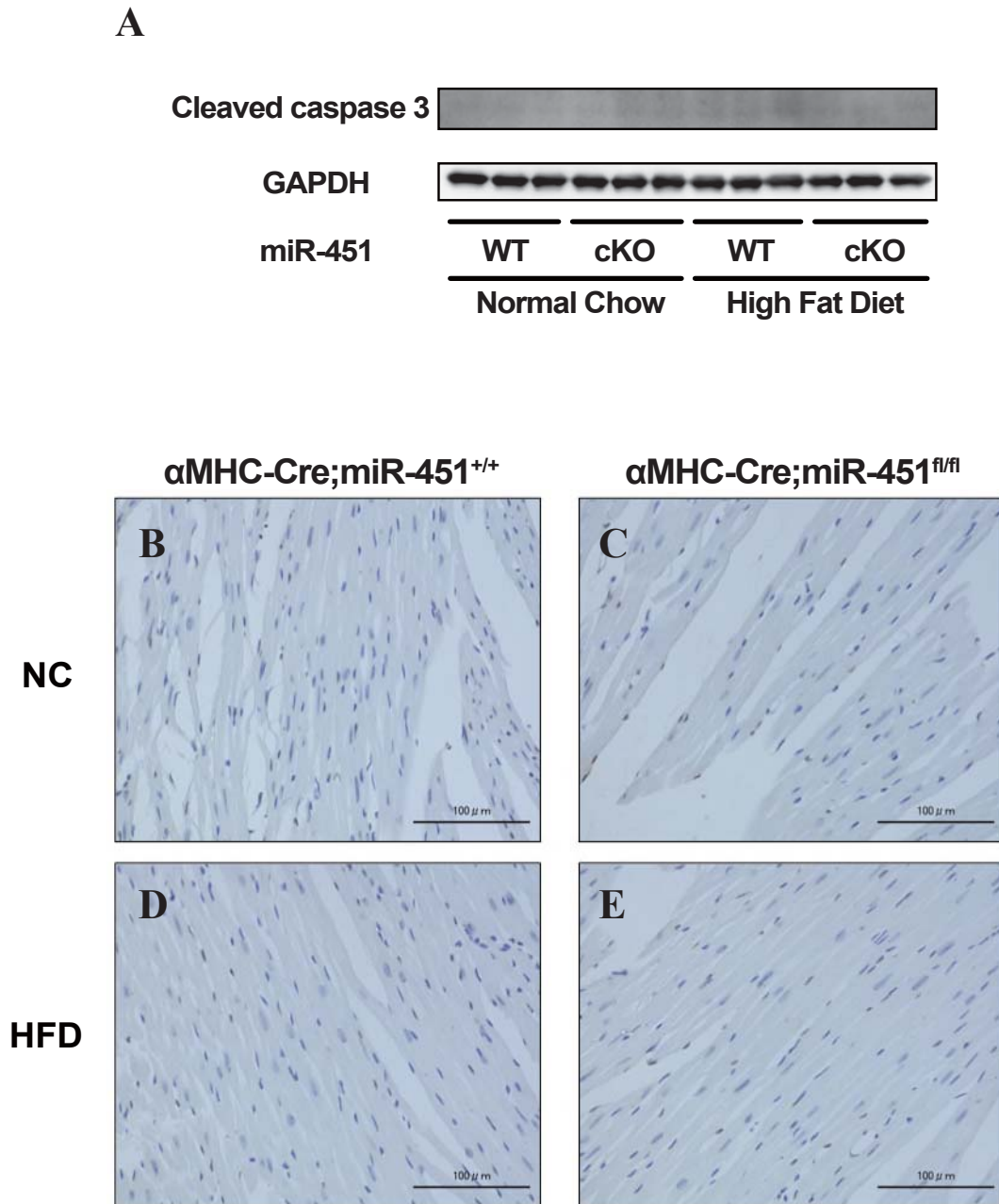
B



Online Figure X. GATA4 expression in control and cKO mouse hearts fed NC or a HFD for 20 weeks.

A, Representative western blotting images for GATA4. GAPDH: glyceraldehyde-3-phosphate dehydrogenase was used as a loading control. B, Quantitative results for GATA4 levels by densitometry, n=6. A.U.: arbitrary units. Data are presented as mean±SEM. **, p < 0.01; ***, p < 0.001.

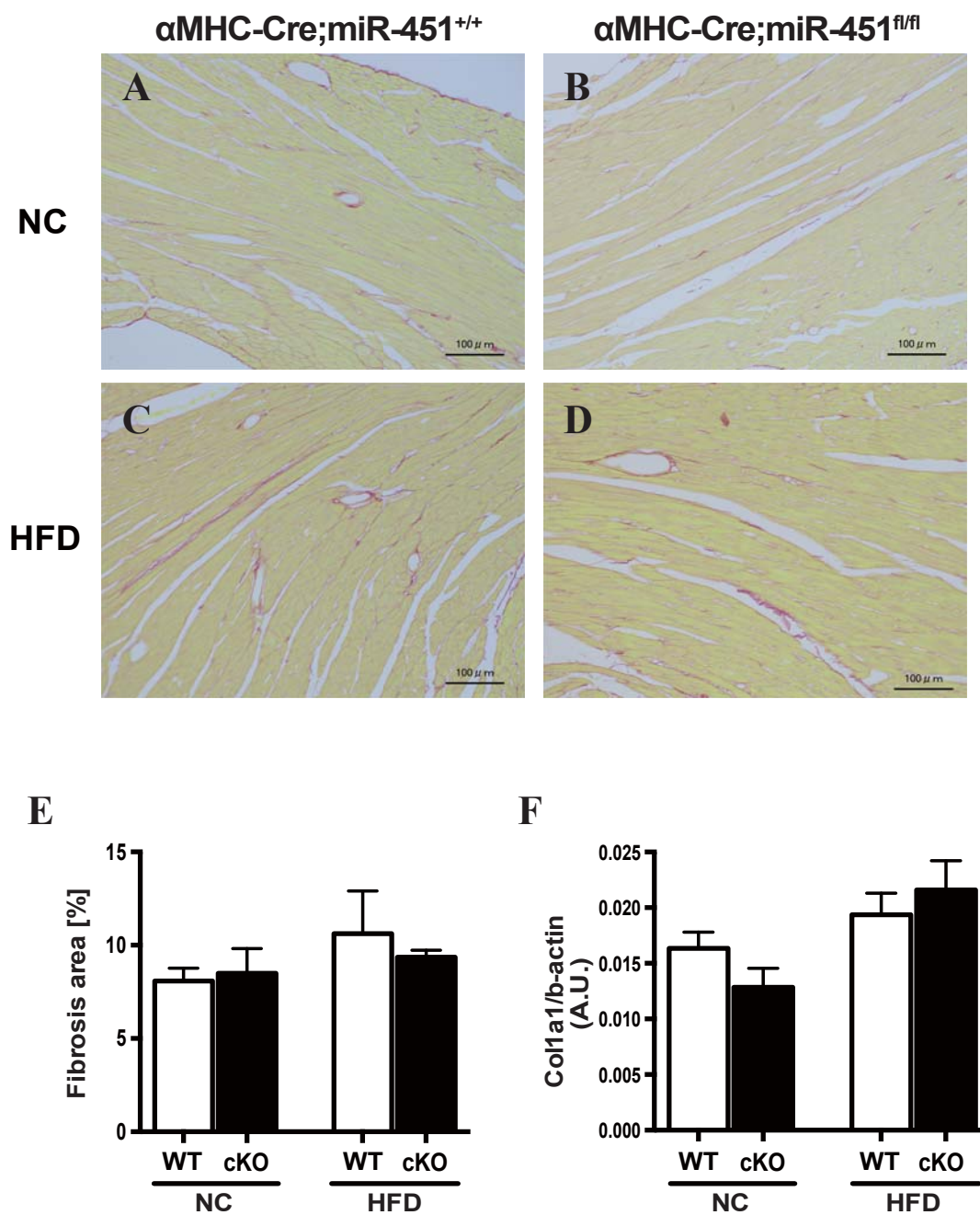
Online Figure X



Online Figure XI. Evaluation of cell toxicity for NC or HFD-fed mouse hearts.

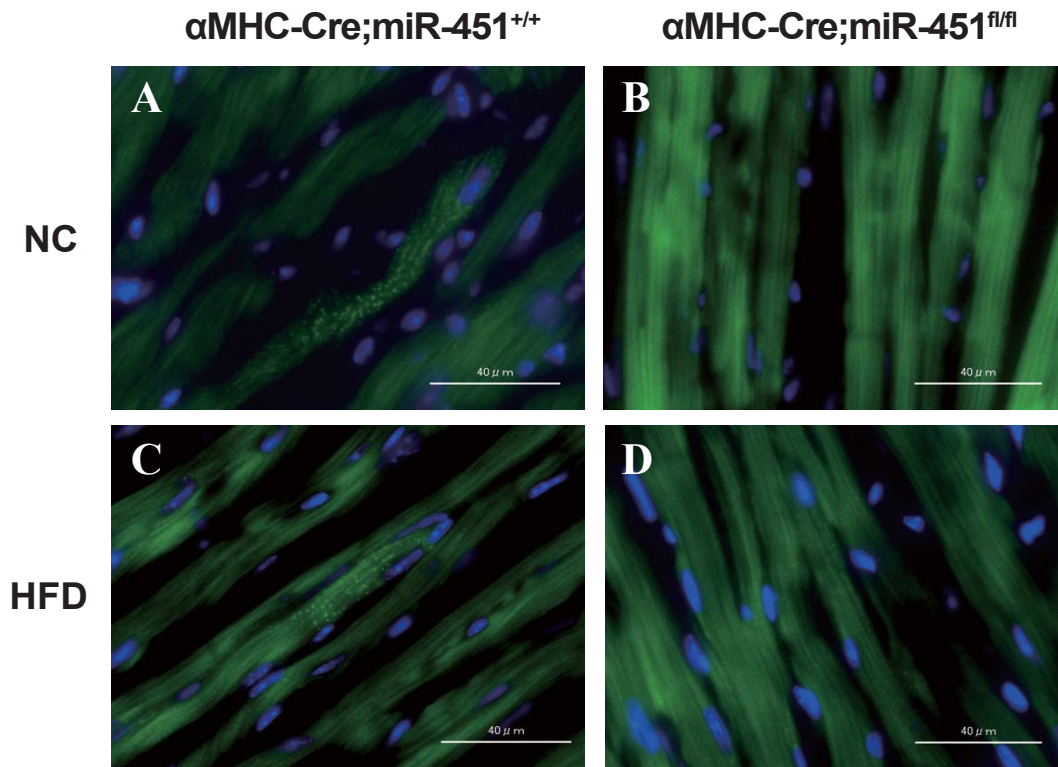
A, Western blotting for cleaved caspase-3. GAPDH: glyceraldehyde-3-phosphate dehydrogenase was used as a loading control. WT and cKO indicate α MHC-Cre;miR-451^{+/+} and α MHC-Cre;miR-451^{fl/fl}, respectively. B–E, Representative images of single strand DNA staining. Control and miR-451 cKO mice were fed NC or HFD for 20 weeks. Black bars represent 100 μ m.

Online Figure XI



Online Figure XII. Fibrosis in control and miR-451 cKO mice fed NC or HFD.

A–D, Representative images of control and miR-451 cKO mice that were fed NC or HFD for 20 weeks. Black bars represent 100 μ m. E, Quantitative results of fibrosis areas determined using picrosirius red staining, n=3–6. F, Col1a1 mRNA levels, n=5–7. WT and cKO indicate α MHC-Cre;miR-451^{+/+} and α MHC-Cre;miR-451^{fl/fl}, respectively. Data are presented as mean $\pm$ SEM.



Online Figure XIII. Neutral lipid staining of control and miR-451 cKO mice fed NC or HFD using BODIPY 505/515 neutral lipid probes.

A–D, Representative images of neutral lipid staining. Control and miR-451 cKO mice were fed NC or HFD for 20 weeks. White bars represent 40 μm .

ARTICLE

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MicroRNA-33 regulates sterol regulatory element-binding protein 1 expression in mice

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MicroRNAs (miRs) are small non-protein-coding RNAs that bind to specific mRNAs and inhibit translation or promote mRNA degradation. Recent reports have indicated that miR-33, which is located within the intron of sterol regulatory element-binding protein (SREBP) 2, controls cholesterol homeostasis and may be a potential therapeutic target for the treatment of atherosclerosis. Here we show that deletion of miR-33 results in marked worsening of high-fat diet-induced obesity and liver steatosis. Using miR-33^{-/-} *Srebp1*^{+/-} mice, we demonstrate that SREBP-1 is a target of miR-33 and that the mechanisms leading to obesity and liver steatosis in miR-33^{-/-} mice involve enhanced expression of SREBP-1. These results elucidate a novel interaction between SREBP-1 and SREBP-2 mediated by miR-33 *in vivo*.

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Sterol regulatory element-binding proteins (SREBPs) are the predominant transcription factors controlling the synthesis of cholesterol and fatty acids in the liver¹. The family of SREBPs essentially encompasses two isoforms, SREBP-1 and SREBP-2, encoded by the corresponding genes *SREBF1* and *SREBF2* (refs 2,3). In contrast to SREBP-2, SREBP-1 is transcribed into two major splicing variants, SREBP-1a and SREBP-1c, which differ only in their first exon through the use of alternative promoters^{2,3}. Although there is some functional overlap among the three SREBP isoforms⁴, these proteins regulate different metabolic pathways. SREBP-2 is the master regulator of cholesterol synthesis and metabolism, whereas SREBP-1c controls fatty acid synthesis in the liver and adipose tissue⁵. In replicating tumour cell lines, SREBP-1a mostly transactivates both lipogenic and cholesterogenic genes. Although SREBP-1a and SREBP-1c share the same bHLH and regulatory domains, SREBP-1a is a stronger activator than SREBP-1c owing to a longer amino-terminal transactivation domain⁶. Therefore, SREBP-1a, -1c and -2 have specific roles in the regulation of cholesterol and fatty acids. In order to fine-tune cellular metabolism efficiently, it may be important to regulate their functions in an interdependent manner. However, limited evidence has been obtained about the potential interactions between SREBP-1 and SREBP-2 until date.

MicroRNAs (miRs) are small non-protein-coding RNAs that bind to specific mRNAs and inhibit translation or promote mRNA degradation. miR-33 is encoded in an intron of *SREBF2*. The sequence of miR-33 is identical, and the stem-loop of the pre-miRNA is highly conserved in mammals^{7–10}. Recent reports, including ours, have indicated that miR-33 controls ABCA1 expression and reduces HDL-C levels, and that miR-33 is a potential target for the treatment of atherosclerosis^{11,12}. To determine the organ/cell type-specific function of miRs in the long term *in vivo*, studies on miRNA-deficient mice and analysis of specific organ/cell types from these mice are needed. Therefore, we generated miR-33-deficient mice and studied their phenotypes. We noted that miR-33-deficient mice gradually gained more weight than control mice, and the obese phenotype was evident after 26 weeks of age when receiving normal chow (NC). When we fed them a high-fat diet (HFD), miR-33-deficient mice became severely obese and suffered from liver steatosis. Microarray analysis showed that genes involved in fatty acid metabolism were upregulated in miR-33^{-/-} mice fed NC before becoming obese. We searched for potential target genes of miR-33 in a public database (TargetScan, <http://www.targetscan.org>),

and found that one of the targets of miR-33 is SREBP-1. *In vitro* experiments indicated that SREBP-1 is a likely target of miR-33. We further intercrossed miR-33^{-/-} mice with *Srebfl*^{+/-} mice and fed them HFD. The difference in body weight (BW) between miR-33^{-/-}*Srebfl*^{+/-} mice and miR-33^{-/-}*Srebfl*^{+/-} mice decreased and hepatic steatosis was reversed in miR-33^{-/-}*Srebfl*^{+/-} mice compared with miR-33^{-/-}*Srebfl*^{+/-} mice under pair-feeding conditions. These data demonstrate that miR-33 targets SREBP-1 *in vivo*.

In the present study, we demonstrated that miR-33 deficiency increases SREBP-1 levels, fatty acid synthesis, and fatty acid accumulation in the liver and adipose tissue. These results indicate a novel relationship between SREBP-1 and SREBP-2 through miR-33.

Results

miR-33-KO mice become obese and develop hepatic steatosis.

Twenty-six-week-old male miR-33-knockout mice weighed more than wild-type (WT) littermates after being fed NC (Fig. 1a). Up to 24 weeks of age, the BWs of the miR-33^{-/-} mice and those of age- and sex-matched miR-33^{+/+} control mice did not differ, but 26-week-old male miR-33^{-/-} mice were 20% heavier than controls. After feeding with HFD from 8 to 20 weeks of age, miR-33^{-/-} mice become markedly obese compared with controls of both genders (Fig. 1b,c). Computed tomography (CT) of 20-week-old miR-33^{+/+} and miR-33^{-/-} mice fed HFD showed a severe increase in body fat of miR-33^{-/-} mice compared with miR-33^{+/+} mice (Fig. 1d). We estimated fat weight from CT values because there is a good correlation of visceral fat weight and calculated weight from CT values (Supplementary Fig. S1a). Both visceral and subcutaneous fat weights were higher in miR-33^{-/-} mice fed HFD (Supplementary Fig. S1b). Figure 2a indicates that the increased BW was caused by an increase in liver and adipose tissue weight. The livers of miR-33^{-/-} mice fed HFD were severely enlarged and pale in colour (Fig. 1c). Histological examination revealed that miR-33^{-/-} mice fed HFD developed severe fatty liver with the accumulation of lipid droplets (Fig. 2b). We measured total cholesterol and triglyceride levels in the liver and found that triglyceride levels were significantly increased in the liver of miR-33^{-/-} mice fed HFD compared with miR-33^{+/+} mice fed HFD and mice fed NC (Fig. 2c right). On the other hand, cholesterol levels in the liver were increased in mice fed HFD compared with mice fed NC and there was no difference between miR-33^{+/+} and

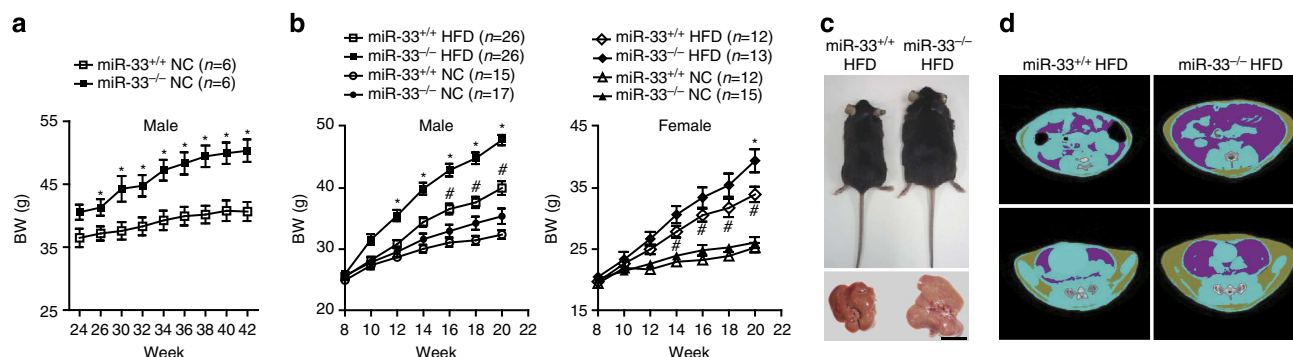


Figure 1 | miR-33^{-/-} mice become obese and develop hepatic steatosis. (a) Development of BW of miR-33^{+/+} and miR-33^{-/-} male mice fed NC. **P* < 0.05 versus NC-fed miR-33^{+/+} mice. Statistical comparisons were made by Student's *t*-test. (b) Development of BW of miR-33^{+/+} and miR-33^{-/-} mice fed or not fed HFD. **P* < 0.05 versus HFD-fed miR-33^{+/+} mice, #*P* < 0.05 versus NC-fed miR-33^{+/+} mice. Statistical comparisons were made by one-way analysis of variance test. (c) Representative image of miR-33^{+/+} and miR-33^{-/-} mice fed with a HFD. Lower images show the livers of these mice. Scale bars, 1.0 cm. (d) Representative CT images of miR-33^{+/+} and miR-33^{-/-} mice fed HFD. Values are the means ± s.e.m.

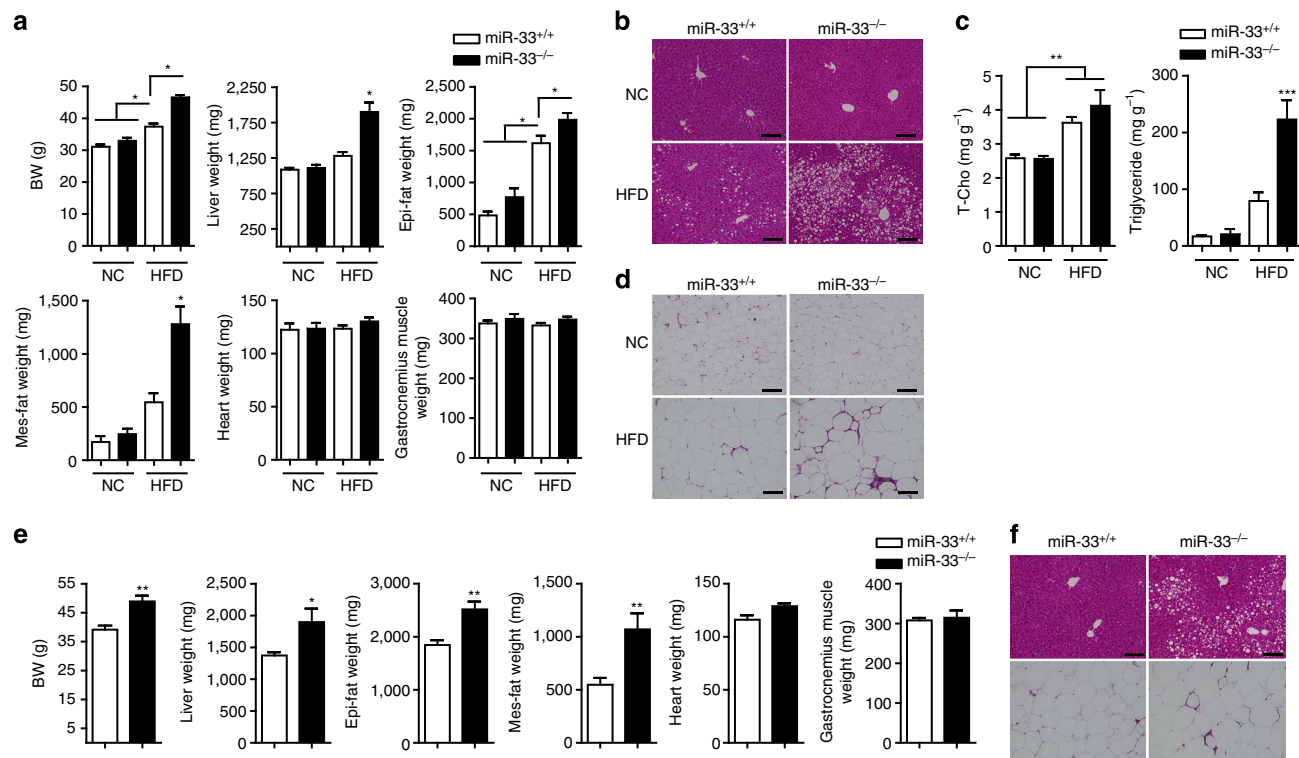


Figure 2 | Pathophysiological features of miR-33^{-/-} mice. (a) Body, liver, adipose tissue (epi-fat; epididymal fat, meso-fat; mesenteric fat), heart and muscle weights of miR-33^{+/+} and miR-33^{-/-} mice fed or not fed HFD ($n=12-13$ for NC, $n=21$ for HFD each, $*P<0.05$ in one-way analysis of variance test). (b) Representative microscopic images of the livers of miR-33^{+/+} and miR-33^{-/-} mice fed or not fed HFD. Scale bars, 200 μ m. (c) Cholesterol and triglyceride levels in the livers of miR-33^{+/+} and miR-33^{-/-} mice fed or not fed HFD ($n=5$ each, $**P<0.01$, $***P<0.001$ in one-way analysis of variance test). (d) Representative microscopic images of the adipose tissue (epididymal fat) of miR-33^{+/+} and miR-33^{-/-} mice fed or not fed HFD. Scale bars, 200 μ m. (e) Body, liver, adipose tissue, heart and muscle weights of miR-33^{+/+} and miR-33^{-/-} mice fed NC at the age of 50 weeks ($n=6$ each, $*P<0.05$, $**P<0.01$ in Student's t -test). (f) Representative microscopic images of the liver and the adipose tissue of miR-33^{+/+} and miR-33^{-/-} mice fed NC at the age of 50 weeks. Scale bars, 200 μ m. Values are the means $\pm$ s.e.m.

miR-33^{-/-} mice (Fig. 2c left). Figure 2d shows the increase in adipocyte size with the accumulation of infiltrated cells in white adipose tissue in miR-33^{-/-} mice fed HFD. It is of note that the same phenotypes as those of miR-33^{-/-} mice fed HFD were also observed in miR-33^{-/-} mice fed NC at 50 weeks of age (Fig. 2e,f). Thus, genetic ablation of miR-33 induces obesity and hepatic steatosis.

miR-33-KO mice have abnormal glucose and insulin tolerance. miR-33^{-/-} mice fed HFD from 8 weeks to 20 weeks of age showed higher fasting glucose levels and severely impaired glucose tolerance at 20 weeks (Fig. 3a,b).

However, miR-33^{-/-} mice fed NC showed the same glucose levels as miR-33^{+/+} mice at this age. Baseline glucose levels of NC-fed miR-33^{+/+} mice, NC-fed miR-33^{-/-} mice, HFD-fed miR-33^{+/+} mice and HFD-fed miR-33^{-/-} mice were 110.5 ± 8.3 , 122 ± 2.5 , 120.5 ± 5.6 and 155.6 ± 6.7 mg dl⁻¹, respectively (All values represent mean $\pm$ s.e.m.). Impaired insulin tolerance was observed only in miR-33^{-/-} mice fed HFD (Fig. 3c-f). Insulin levels in intraperitoneal glucose tolerance test (IPGTT) were significantly elevated in miR-33^{-/-} mice fed HFD (Fig. 3g,h). Plasma leptin levels were also elevated in miR-33^{-/-} mice fed HFD (Fig. 3i). Impaired glucose tolerance and insulin tolerance were also evident at the age of 50 weeks even in mice fed NC (Fig. 3j-m).

Serum levels of ALP, T-cho and HDL-C were elevated in miR-33^{-/-} mice compared with that in WT mice at the age of

20 weeks, as indicated in our previous report (Table 1)¹⁰. When these mice were fed HFD from 8 to 20 weeks of age, increases in serum levels of AST, ALT, NEFA and LDL-C became evident (Table 1). Similar elevation of T-cho was observed in miR-33^{-/-} mice fed NC at the age of 50 weeks compared with controls (Supplementary Table S2).

miR-33-KO mice find HFD more palatable. Food intake, as analysed by housing in metabolic cages, was higher in miR-33^{-/-} mice fed HFD than that in their control counterparts (Fig. 4a). The difference in food intake was only observed when they were fed with HFD (Supplementary Fig. S2a, b), which suggests that miR-33^{-/-} mice find HFD more palatable. These mice showed similar body temperatures (37.38°C versus 37.27°C) and O₂ consumption rate or activity did not differ between these strains during the day or night at the age of 16 weeks when fed NC (Fig. 4c-f). Moreover, urinary excretion of adrenaline, noradrenaline and dopamine were also the same between these strains at the same age (Fig. 4g).

miR-33 regulates SREBP-1 expression *in vivo*. In order to determine the cause of the phenotypic changes observed in miR-33^{-/-} mice fed HFD or in older miR-33^{-/-} mice, we analysed the gene expression profiles by microarray analysis using the livers of miR-33^{+/+} and miR-33^{-/-} mice fed NC at the age of 16 weeks when their weights were the same. The pathways altered in the livers of miR-33^{-/-} mice were determined by

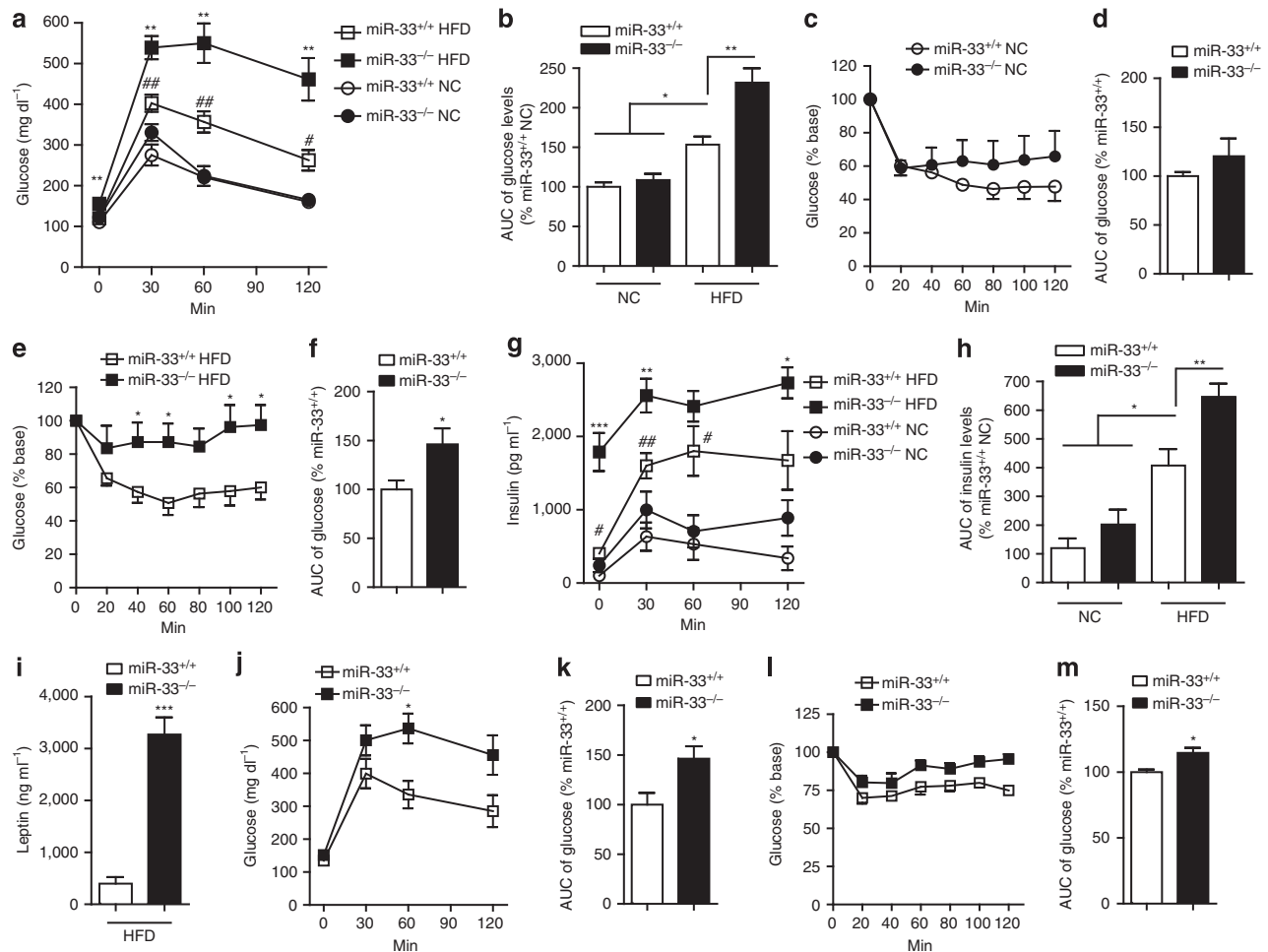


Figure 3 | Analysis of glucose and insulin tolerance. (a,b) Serial changes in glucose levels (a) and area under curve (AUC) of glucose levels (b) after intraperitoneal injection of glucose in miR-33^{+/+} and miR-33^{-/-} mice fed or not fed HFD ($n=6$ for NC, $n=11-12$ for HFD each. $*P<0.05$ versus mice fed NC. $**P<0.01$ versus miR-33^{+/+} mice fed HFD. $\#P<0.05$ versus miR-33^{+/+} mice fed NC, $\#\#P<0.01$ versus miR-33^{+/+} mice fed NC in one-way analysis of variance test). (c,d) Serial changes in glucose levels (c) and AUC of glucose levels (d) after intraperitoneal injection of insulin in miR-33^{+/+} and miR-33^{-/-} mice fed NC ($n=5$ each). (e,f) Serial changes in glucose levels (e) and AUC of glucose levels (f) after intraperitoneal injection of insulin in miR-33^{+/+} and miR-33^{-/-} mice fed HFD ($n=9$ each, $*P<0.05$ in Student's *t*-test). (g) Serial changes in insulin levels after intraperitoneal injection of glucose in miR-33^{+/+} and miR-33^{-/-} mice fed or not fed HFD ($n=6$ for NC, $n=11-12$ for HFD each. $*P<0.05$ versus miR-33^{+/+} mice fed HFD, $**P<0.01$ versus miR-33^{+/+} mice fed HFD, $***P<0.001$ versus miR-33^{+/+} mice fed HFD, $\#P<0.05$ versus miR-33^{+/+} mice fed NC, $\#\#P<0.01$ versus miR-33^{+/+} mice fed NC in one-way analysis of variance test). (h) AUC of insulin levels after intraperitoneal injection of glucose in miR-33^{+/+} and miR-33^{-/-} mice fed or not fed HFD ($n=6$ for NC, $n=11-12$ for HFD each, $*P<0.05$, $**P<0.01$ in one-way analysis of variance test). (i) Serum leptin levels in miR-33^{+/+} and miR-33^{-/-} mice fed HFD ($n=10$ for each, $***P<0.001$ in Student's *t*-test). (j,k) Serial changes in glucose levels (j) and AUC of glucose levels (k) after intraperitoneal injection of glucose in miR-33^{+/+} and miR-33^{-/-} mice fed NC at the age of 50 weeks ($n=6$ each, $*P<0.05$ in Student's *t*-test). (l,m) Serial changes in glucose levels (l) and AUC of glucose levels (m) after intraperitoneal injection of insulin in miR-33^{+/+} and miR-33^{-/-} mice fed NC at the age of 50 weeks ($n=6$ each, $*P<0.05$ in Student's *t*-test). Values are the means $\pm$ s.e.m.

Table 1 | Serum profile of miR-33^{+/+} and miR-33^{-/-} on NC or HFD.

	miR-33 ^{+/+} NC ($n=8$)	miR-33 ^{-/-} NC ($n=8$)	miR-33 ^{+/+} HFD ($n=5$)	miR-33 ^{-/-} HFD ($n=5$)
AST (IU l ⁻¹)	67.63 $\pm$ 5.43	56.50 $\pm$ 11.89	53.80 $\pm$ 2.89	134.40 $\pm$ 18.48**
ALT (IU l ⁻¹)	39.38 $\pm$ 6.02	41.00 $\pm$ 11.95	31.40 $\pm$ 3.92	167.40 $\pm$ 29.15**
ALP (IU l ⁻¹)	186.88 $\pm$ 15.29	238.13 $\pm$ 14.57*	138.80 $\pm$ 9.83	210.60 $\pm$ 20.79*
T-CHO (mg dl ⁻¹)	85.75 $\pm$ 3.83	110.25 $\pm$ 6.16**	157.40 $\pm$ 13.25	244.8 $\pm$ 21.42**
TG (mg dl ⁻¹)	35.63 $\pm$ 4.39	34.50 $\pm$ 4.82	21.80 $\pm$ 2.33	17.60 $\pm$ 3.72
NEFA (μ Eq l ⁻¹)	766.8 $\pm$ 30.89	867.1 $\pm$ 53.68	778 $\pm$ 61.21	979 $\pm$ 55.40*
LDL-C (mg dl ⁻¹)	5.50 $\pm$ 0.63	6.88 $\pm$ 0.72	11.40 $\pm$ 0.51	26.00 $\pm$ 5.30*
HDL-C (mg dl ⁻¹)	52.38 $\pm$ 2.08	66.13 $\pm$ 2.72**	79.00 $\pm$ 4.71	83.60 $\pm$ 1.25

Values are the means $\pm$ s.e.m. Statistical comparisons were made by Student's *t*-test ($*P<0.05$, $**P<0.01$).

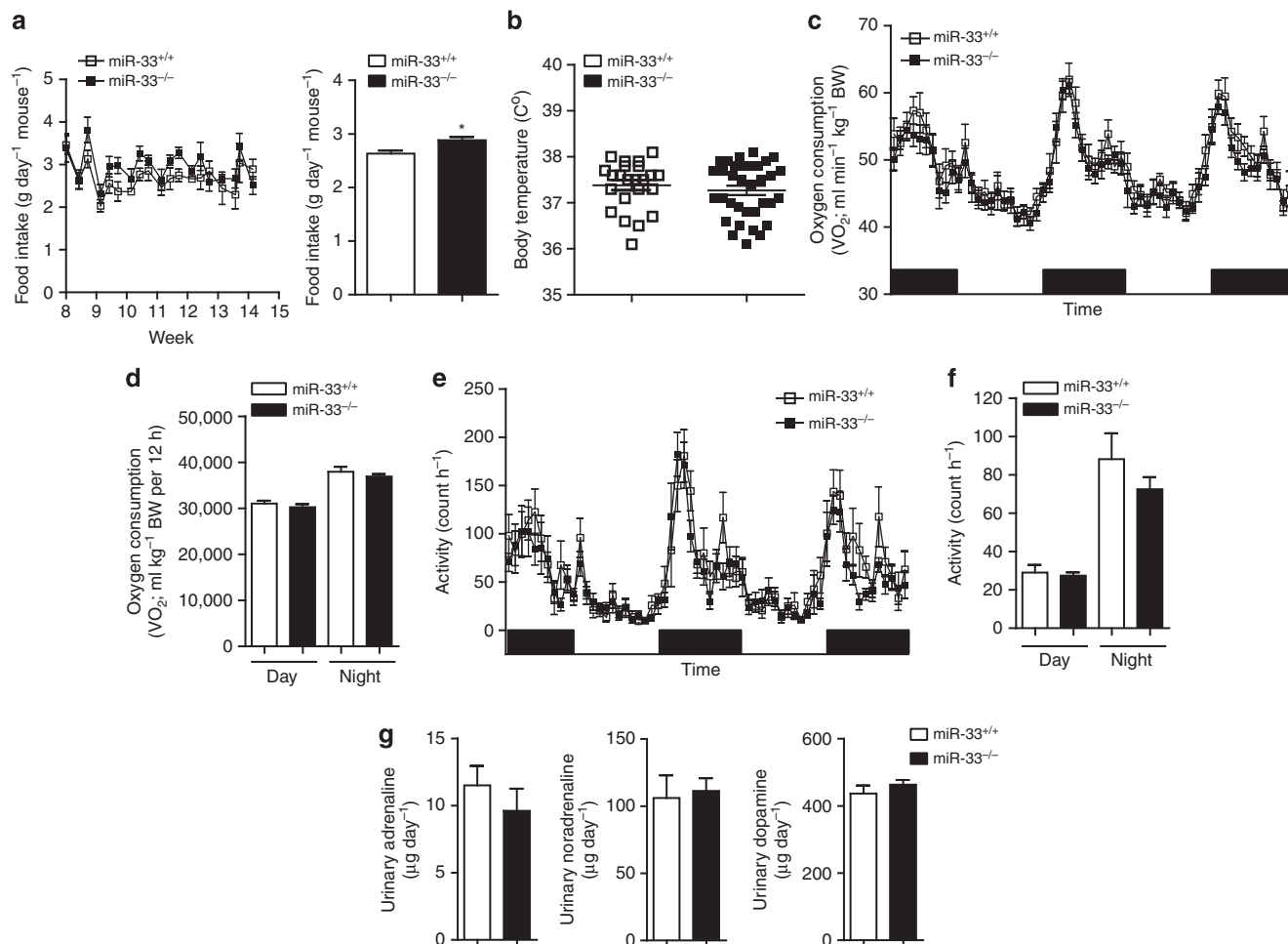


Figure 4 | Analysis of energy balance. (a) Serial changes in food intake of *miR-33*^{+/+} and *miR-33*^{-/-} mice fed HFD in metabolic cages ($n=5-6$ each, $*P<0.05$ in Student's t -test). (b) Body temperature of *miR-33*^{+/+} and *miR-33*^{-/-} mice at 16 weeks of age ($n=23, 34$ each). (c) Oxygen consumption rate of *miR-33*^{+/+} and *miR-33*^{-/-} mice fed NC at 16 weeks of age ($n=8$ each). (d) Oxygen consumption during 12 h by *miR-33*^{+/+} and *miR-33*^{-/-} mice fed NC at 16 weeks of age ($n=8$ each). (e) Serial changes in activity of *miR-33*^{+/+} and *miR-33*^{-/-} mice fed NC at 16 weeks of age ($n=8$ each). (f) Day and night activity of *miR-33*^{+/+} and *miR-33*^{-/-} mice fed NC at 16 weeks of age ($n=8$ each). (g) Urinary secretion of adrenaline, noradrenaline and dopamine ($n=3-4$ each). Values are the means $\pm$ s.e.m.

GenMAPP analysis (<http://www.genmapp.org/about.html>). Most strikingly, the fatty acid metabolism pathway showed the highest Z score (Supplementary Table S3). We picked up genes related to fatty acid metabolism and validated their expression levels in the liver by quantitative RT-PCR (PCR with reverse transcription). Interestingly, significant differences were observed in the expression levels of several lipogenic genes including *Srebf1*, *Pparg* and its downstream genes (Fig. 5a). We also measured *de novo* hepatic fatty acid synthesis rate, as previously described^{13,14}. It was increased significantly in the *miR-33*^{-/-} mice compared with that of the *miR-33*^{+/+} mice (Supplementary Fig. S3a). The *Srebf1* 3'UTR has a potential binding site for miR-33 in many species (TargetScan; <http://www.targetscan.org>; Fig. 5b). Overexpression of miR-33 reduced the luciferase activity of a reporter gene fused with *Srebf1* 3'UTR sequences from humans and mice (Fig. 5c). Moreover, miR-33 decreased luciferase activity dose-dependently, whereas miR-146a, which has no binding site in the *Srebf1* 3'UTR, could not (Fig. 5d). Mutation in this binding site abolished the reduction of luciferase activity in 293T cells (Fig. 5e). The same results were also obtained in COS-7 cells (Supplementary Fig. S3b, c). We also measured the activity of SREBP-1 by sterol regulatory element (SRE) and fatty acid synthase (FAS) promoter reporter analysis by the use of *Srebf1*

with or without the 3'UTR. Luciferase activity of the SRE and FAS reporter genes was significantly reduced by miR-33 expression when *Srebf1* with the 3'UTR was present. This reduction was not observed in the experiments conducted with *Srebf1* without the 3'UTR (Fig. 5f,g). Overexpression of miR-33 reduced protein levels of SREBP-1 and ABCA1 but not of IRS-2 in HepG2 cells (Fig. 5h and Supplementary Fig. S4a). The decrease in *SREBF1* expression was mainly caused by reduction in *SREBF1c* (Supplementary Fig. S4b). Overexpression of miR-33 also reduced the protein levels of SREBP-1 and ABCA1 but not of IRS-2 in *miR-33*^{+/+} primary hepatocytes (Fig. 5h and Supplementary Fig. S4c). It was confirmed that *miR-33*^{-/-} mice had higher protein expression levels of SREBP-1 and ABCA1 but not of IRS-2 (Fig. 5h and Supplementary Fig. S4d). We measured the expression levels of lipogenic genes in the primary hepatocyte transduced with miR-33 or the control. As shown in Supplementary Fig. S4e, expression levels of *Srebf1*, *Abca1* and several lipogenic genes were downregulated. Moreover, *Srebf1*, *Abca1* and several lipogenic genes were upregulated in *miR-33*^{-/-} primary hepatocytes compared with *miR-33*^{+/+} primary hepatocytes (Supplementary Fig. S4f). SREBP-1 levels were further enhanced in *miR-33*^{-/-} mice fed HFD (Supplementary Fig. S5a). We also measured the levels of

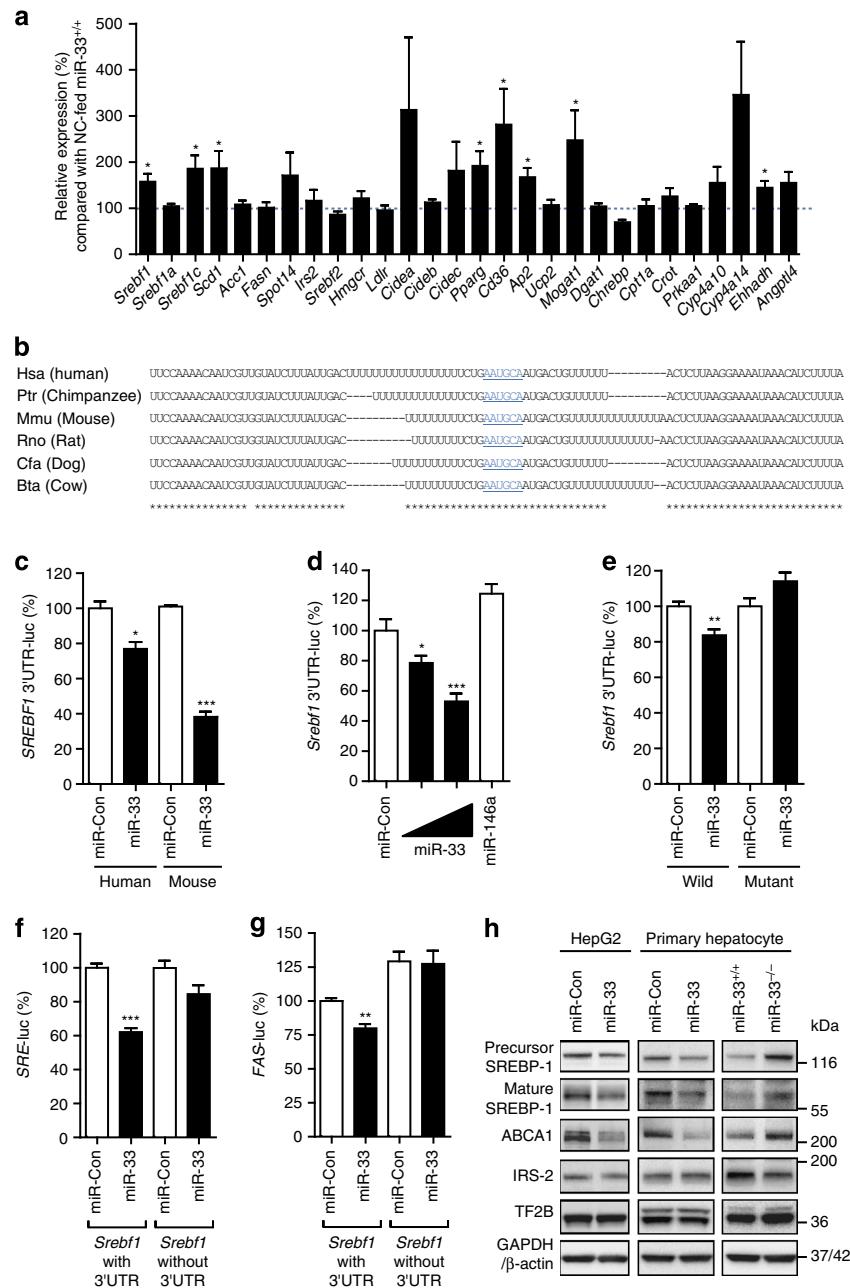


Figure 5 | *Srebfl* is a miR-33 target gene. (a) Relative changes in lipid metabolism-related genes in the livers of miR-33^{-/-} mice compared with miR-33^{+/+} mice fed NC at 16 weeks of age. (*n* = 5–8 each, **P* < 0.05 in Student's *t*-test). (b) Conservation of miR-33 target regions in the 3'UTR of *Srebfl*. Underlined sequences are the potential binding site of miR-33 seed sequences. * indicates the conservation among species. (c) 3'UTR reporter assay used to verify the target. Luciferase reporter activity of human and mouse SREBP-1 gene 3'UTR constructs in 293T cells overexpressing miR-control (miR-Con) and miR-33 (*n* = 4 each, **P* < 0.05 and ****P* < 0.001 in Student's *t*-test). (d) miR-33 dose-dependent changes in luciferase reporter activity of mouse *Srebfl* 3'UTR construct in 293T cells. miR-Con and miR-146a is used as a negative control (*n* = 4 each, **P* < 0.05 and ****P* < 0.001 in one-way analysis of variance test). (e) Luciferase reporter activity of the WT or mutant *Srebfl* 3'UTR at the potential miR-33 binding site in 293T cells (*n* = 4 each, ***P* < 0.01 in Student's *t*-test). (f,g) Luciferase reporter activity of *SRE*-promoter (f) or *FAS*-promoter (g) in 293T cells. 293T cells were co-transfected with mouse *Srebfl* with the full-length 3'UTR or without the 3'UTR, along with expression plasmids for miR-negative control, or miR-33. Values are the mean ± s.e. (*n* = 4 each, ***P* < 0.01 versus miR-Con. ****P* < 0.001 versus miR-Con in one-way analysis of variance test). (h) Western blotting analysis of SREBP-1, ABCA1, and IRS-2 in miR-33 transduced HepG2 cells and primary hepatocytes and hepatocytes prepared from miR-33^{+/+} and miR-33^{-/-} mice. Representative western blot images are shown (*n* = 4). Values are the means ± s.e.m.

AMPK α , previously described as a potential miR-33 target, but we could not detect any difference between miR-33^{+/+} and miR-33^{-/-} mice (Supplementary Fig. S5b). We further checked whether PPAR- γ is regulated by miR-33 in primary hepatocytes. Overexpression of miR-33 did not change the protein

expression level of PPAR- γ after transduction in HepG2 cells (Supplementary Fig. S5c) and primary hepatocytes (Supplementary Fig. S5d), and found non-enhancement in miR-33^{-/-} mice (Supplementary Fig. S5d). Moreover, we conducted peroxisome proliferator-activated receptor response

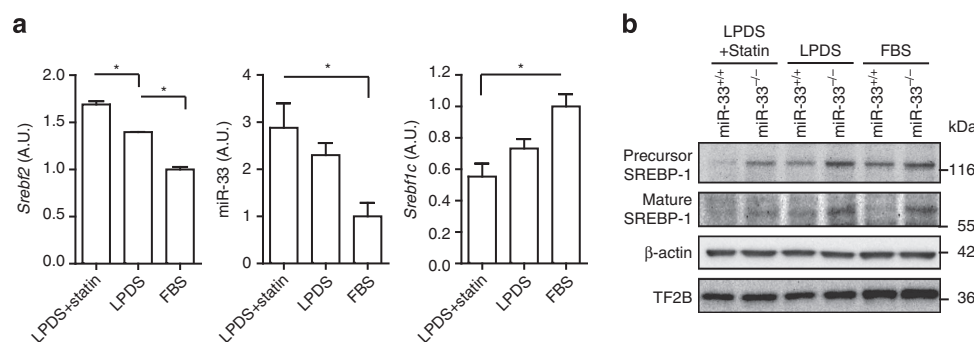


Figure 6 | SREBP-1 is regulated by endogenous changes in miR-33 in vitro. (a) RNA expression levels in *Srebf2*, *miR-33* and *Srebf1c* in primary hepatocytes cultured in DMEM supplemented with 5% FBS or 5% LPDS with or without statin treatment. Values are the mean $\pm$ s.e.m. ($n=3$ each for *Srebf2* and *Srebf1c*, $n=4-6$ each for *miR-33*, $*P<0.05$ in one-way analysis of variance test). (b) Protein levels of SREBP-1 in primary hepatocytes cultured in DMEM supplemented with 5% FBS or 5% LPDS with or without statin treatment. Representative western blot images are shown ($n=4$).

elements (PPRE)-driven luciferase assay in HepG2 cells. No difference was observed in PPRE activity in control and miR-33 transduced cells with or without PPAR ligand pioglitazone (Supplementary Fig. S5e). Therefore, these results indicate that *Pparg* is not a direct target of miR-33.

SREBP-1 is regulated by endogenous changes in miR-33 in vitro. We further attempted to confirm whether the expression of SREBP-1 was affected by endogenous changes in miR-33 expression via modulating the cellular cholesterol level in primary hepatocytes. When the cells were depleted of sterols by prior incubation in medium containing lipoprotein-deficient serum (LPDS) with or without pitavastatin, mRNA levels of *Srebf2* and *miR-33* were significantly increased in parallel (Fig. 6a). In this situation, *Srebf1c* and protein levels of SREBP-1 were decreased in miR-33^{+/+} primary hepatocytes, whereas they were still elevated in miR-33^{-/-} hepatocytes (Fig. 6b). There is a potential binding site of miR-33 in the 3'UTR of human SCAP. However, it is not conserved in mice and mRNA and protein levels of SCAP are the same in miR-33^{+/+} and miR-33^{-/-} mice (Supplementary Fig. S6a-c). Thus, the levels of precursor and mature forms of SREBP-1 are regulated in parallel.

Reduction of SREBP-1 reverses fatty liver in miR-33-KO mice.

To elucidate the role of SREBP-1 in the phenotypic changes in miR-33^{-/-} mice fed HFD, we generated miR-33^{-/-} mice that have SREBP-1 expression levels similar to WT mice. As shown in Fig. 7a, protein levels of SREBP-1 are the same in miR-33^{-/-} *Srebf1*^{+/+} and miR-33^{+/+} *Srebf1*^{+/+} mice. Aberrant bands of SREBP-1 were observed in *Srebf1*-deficient mice (Fig. 7a and Supplementary Fig. S7a)¹⁵. mRNA levels of *Srebf1* in these mice are shown in Fig. 8a. Because miR-33^{-/-} mice showed positive palatability for HFD compared with miR-33^{+/+} mice (Fig. 4a), we analysed these four groups of mice under pair-feeding conditions. miR-33^{+/+} *Srebf1*^{+/+}, miR-33^{-/-} *Srebf1*^{+/+} and miR-33^{-/-} *Srebf1*^{+/+} mice received HFD in amounts that matched the rate of food intake of miR-33^{+/+} *Srebf1*^{+/+} mice. As shown in Fig. 7b's left panel, miR-33^{-/-} *Srebf1*^{+/+} mice gained significantly more weight than miR-33^{+/+} *Srebf1*^{+/+} mice under these conditions. Therefore, the BW gain in miR-33^{-/-} mice compared with miR-33^{+/+} mice is not caused by a change in food intake. The BW increase caused by miR-33 deficiency was abolished in a *Srebf1*^{+/+} background (Fig. 7b right and Supplementary Fig. S7b). On the other hand, glucose tolerance was ameliorated in miR-33^{-/-} *Srebf1*^{+/+} compared with miR-33^{-/-} *Srebf1*^{+/+} mice (Fig. 7c,d). There was no

difference in BW or glucose tolerance among miR-33^{+/+} *Srebf1*^{+/+}, miR-33^{+/+} *Srebf1*^{+/+} and miR-33^{-/-} *Srebf1*^{+/+} mice (Fig. 7b-d). Serum insulin levels were reduced in miR-33^{-/-} *Srebf1*^{+/+} mice compared with miR-33^{-/-} *Srebf1*^{+/+} mice (Supplementary Fig. S7c, d). A striking difference was observed in the liver. Hepatic steatosis was reversed in miR-33^{-/-} *Srebf1*^{+/+} mice compared with that in miR-33^{-/-} *Srebf1*^{+/+} mice in both macro- and microscopic images and the liver triglyceride content of these mice was almost the same as that of miR-33^{+/+} *Srebf1*^{+/+} and miR-33^{+/+} *Srebf1*^{+/+} mice (Supplementary Fig. S7b and Fig. 7e,f). As shown in Fig. 7g, adipocyte size was partially reduced in miR-33^{-/-} *Srebf1*^{+/+} mice compared with miR-33^{-/-} *Srebf1*^{+/+} mice and there were still many infiltrating cells in miR-33^{-/-} *Srebf1*^{+/+} mice. Serum leptin levels also reduced to baseline levels in miR-33^{-/-} *Srebf1*^{+/+} mice (Fig. 7h). These results indicate that obesity and hepatic steatosis were ameliorated in miR-33^{-/-} *Srebf1*^{+/+} mice compared with miR-33^{-/-} *Srebf1*^{+/+} mice. There was no difference in the mRNA and protein levels of AMPK α and SIRT6, which are negative regulators of SREBP-1 and potential targets of miR-33, as shown in previous reports (Fig. 8c and Supplementary Fig. S7e). Finally, we examined the lipogenic gene profiles in the liver of these mice. As shown in Fig. 8a, the expression levels of *Srebf1* were significantly increased in miR-33^{-/-} *Srebf1*^{+/+} mice compared with miR-33^{+/+} *Srebf1*^{+/+} mice, and this was reversed in miR-33^{-/-} *Srebf1*^{+/+} mice. The same pattern was observed in *Scd1*. Although they were not statistically significant, the expression levels of *Fasn*, *Acc1* and *Pparg* were reduced in miR-33^{-/-} *Srebf1*^{+/+} mice compared with miR-33^{-/-} *Srebf1*^{+/+} mice (Fig. 8a,b). Serum data for these mice are summarized in Supplementary Table S4. Serum ALP levels were significantly elevated in miR-33^{-/-} *Srebf1*^{+/+} mice, which was reversed in miR-33^{-/-} *Srebf1*^{+/+} mice. *Srebf1* and other lipogenic genes were also increased in adipose tissue of miR-33^{-/-} mice compared with that of miR-33^{+/+} mice (Supplementary Fig. S8).

Discussion

In the current study, we showed that obesity and hepatic steatosis are observed in miR-33-deficient mice at the age of 50 weeks or when fed HFD for 12 weeks. We demonstrated that miR-33 targets SREBP-1, and miR-33^{-/-} mice had an enhanced expression of SREBP-1 in the liver. Study of miR-33^{-/-} *Srebf1*^{+/+} mice clearly showed that enhanced expression of SREBP-1 caused obesity and fatty liver in miR-33^{-/-} mice

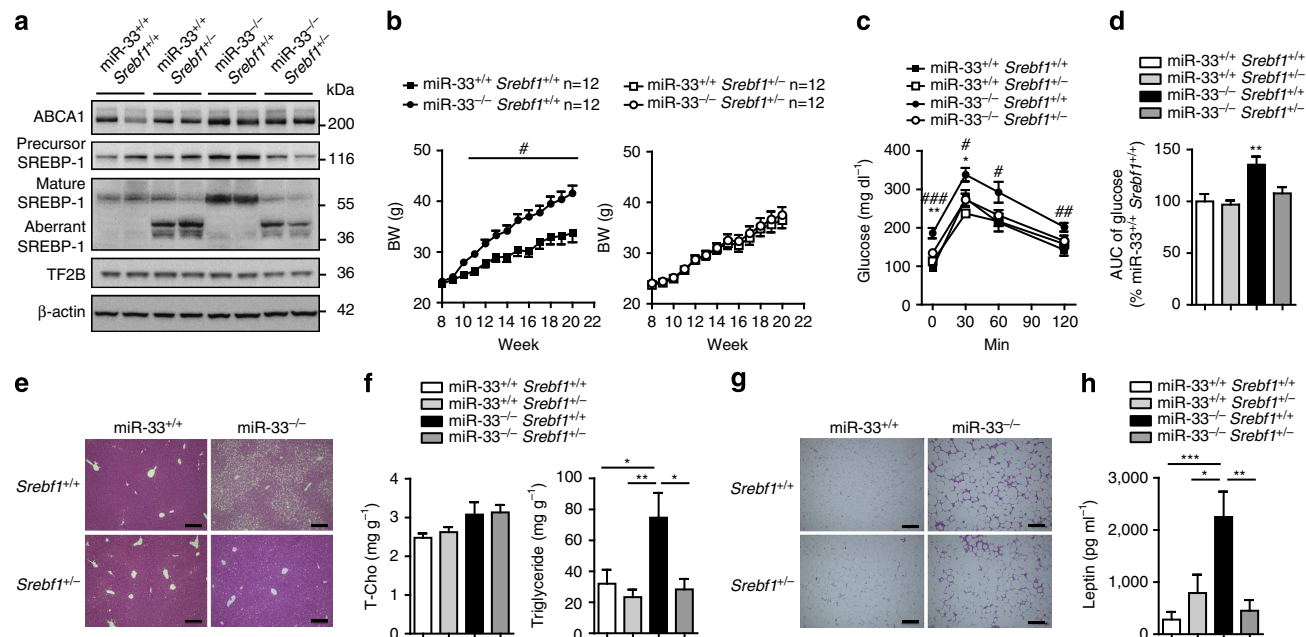


Figure 7 | Reversal of hepatic steatosis by the reduction of SREBP-1 levels. (a) Western blotting analysis of SREBP-1 and ABCA1 levels in the livers of *miR-33*^{+/+}*Srebf1*^{+/+}, *miR-33*^{+/+}*Srebf1*^{+/-}, *miR-33*^{-/-}*Srebf1*^{+/+} and *miR-33*^{-/-}*Srebf1*^{+/-} mice. Representative western blot images are shown ($n=4$). (b) Serial changes in BW levels of *miR-33*^{+/+}*Srebf1*^{+/+}, *miR-33*^{+/+}*Srebf1*^{+/-}, *miR-33*^{-/-}*Srebf1*^{+/+} and *miR-33*^{-/-}*Srebf1*^{+/-} mice fed HFD under pair-feeding condition ($n=12$ each). (c) Serial changes in glucose levels after intraperitoneal injection of glucose into *miR-33*^{+/+}*Srebf1*^{+/+}, *miR-33*^{+/+}*Srebf1*^{+/-}, *miR-33*^{-/-}*Srebf1*^{+/+} and *miR-33*^{-/-}*Srebf1*^{+/-} mice ($n=11-12$ each). * $P<0.05$ and ** $P<0.01$ versus *miR-33*^{-/-}*Srebf1*^{+/-} mice. # $P<0.05$, ## $P<0.01$ and ### $P<0.001$ versus *miR-33*^{+/+}*Srebf1*^{+/+} mice in one-way analysis of variance test. (d) AUC of glucose levels in glucose tolerance tests in *miR-33*^{+/+}*Srebf1*^{+/+}, *miR-33*^{+/+}*Srebf1*^{+/-}, *miR-33*^{-/-}*Srebf1*^{+/+} and *miR-33*^{-/-}*Srebf1*^{+/-} mice ($n=11-12$ each). ** $P<0.01$ versus *miR-33*^{-/-}*Srebf1*^{+/-} mice in one-way analysis of variance test. (e) Representative microscopic images of the liver of *miR-33*^{+/+}*Srebf1*^{+/+}, *miR-33*^{+/+}*Srebf1*^{+/-}, *miR-33*^{-/-}*Srebf1*^{+/+} and *miR-33*^{-/-}*Srebf1*^{+/-} mice fed HFD. Scale bars, 200 μ m. (f) Cholesterol and triglyceride levels in the liver of *miR-33*^{+/+}*Srebf1*^{+/+}, *miR-33*^{+/+}*Srebf1*^{+/-}, *miR-33*^{-/-}*Srebf1*^{+/+} and *miR-33*^{-/-}*Srebf1*^{+/-} mice fed HFD. * $P<0.05$ and ** $P<0.01$ in one-way analysis of variance test. (g) Representative microscopic images of the adipose tissue of *miR-33*^{+/+}*Srebf1*^{+/+}, *miR-33*^{+/+}*Srebf1*^{+/-}, *miR-33*^{-/-}*Srebf1*^{+/+} and *miR-33*^{-/-}*Srebf1*^{+/-} mice fed HFD. Scale bars, 200 μ m. (h) Serum leptin levels of *miR-33*^{+/+}*Srebf1*^{+/+}, *miR-33*^{+/+}*Srebf1*^{+/-}, *miR-33*^{-/-}*Srebf1*^{+/+} and *miR-33*^{-/-}*Srebf1*^{+/-} mice fed HFD. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ in one-way analysis of variance test. Values are the means $\pm$ s.e.m.

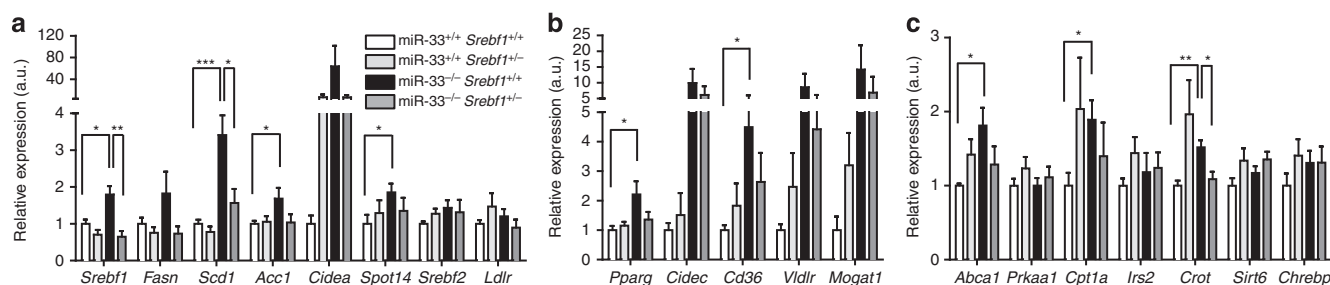


Figure 8 | Relative mRNA expression levels of lipid metabolism-related genes. (a) *Srebf1* and lipogenic genes. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ in one-way analysis of variance test ($n=6-8$ each). (b) *Pparg* and its downstream genes. * $P<0.05$ in one-way analysis of variance test ($n=6-8$ each). (c) Other lipid metabolism-related genes. * $P<0.05$ and ** $P<0.01$ in one-way analysis of variance test ($n=6-8$ each). Relative values of lipid metabolism-related genes in the livers of *miR-33*^{+/+}*Srebf1*^{+/+}, *miR-33*^{+/+}*Srebf1*^{+/-}, *miR-33*^{-/-}*Srebf1*^{+/+} and *miR-33*^{-/-}*Srebf1*^{+/-} mice fed with HFD. Values are the means $\pm$ s.e.m.

(Supplementary Fig. S9a). These results indicate a previously unrecognized relationship between SREBP-1 and SREBP-2 through miR-33 (Supplementary Fig. S9b left).

Until now, there has been little evidence for an interaction between SREBP-1 and SREBP-2. It is known that in cholesterol-rich dietary conditions, SREBP-2 is downregulated at the cleavage level and SREBP-1c is transcriptionally activated through the activation of liver X receptors (LXRs) by the binding of

oxysterols¹⁶⁻¹⁹. However, in sterol-depleted conditions, SREBP-2 is cleaved in the Golgi and the active N-terminal region translocates to the nucleus. Reduction in oxysterol levels leads to the inactivation of liver X receptors, which results in a decrease in SREBP-1c mRNA levels. Recently, it was shown that statin treatment induced hepatic miR-33 expression and at the same time decreased mRNA levels of miR-33 targets, including *Abca1* (ref. 8), *Abcb11* and *Atp8b1* (ref. 20) in mice. Thus, not

only the activation by proteolytic cleavage but also its transcriptional regulation of SREBP-2/miR-33 is important *in vivo*. Our data showed that miR-33 targeted the 3'UTR of *Srebf1* and the upregulation of miR-33 by cholesterol depletion, considerably affecting the reduction of SREBP-1 expression. Therefore, based upon our findings that miR-33 regulates SREBP-1, miR-33 in the intron of *SREBP-2* may amplify reduction in SREBP-1 levels in sterol-depleted conditions (Supplementary Fig. S9b centre). SREBP-1c activates transcription of genes involved in fatty acid and triglyceride synthesis, such as genes encoding acetyl-CoA carboxylase, fatty acid synthase and *elovl-6* and stearoyl-CoA desaturase. Therefore, it is possible that in sterol-depleted conditions, acetyl-CoA is preferred as a substrate for cholesterol production and not for fatty acid production, through the upregulation of miR-33. On the other hand, in cholesterol-rich conditions, miR-33 levels decrease⁷ and its negative regulation of SREBP-1 may be reduced. Thus, in this situation, acetyl-CoA is preferred as a substrate for fatty acid production (Supplementary Fig. S9b right).

Previously, several different SREBP-1 transgenic (TG) mice have been produced. Liver-specific transgenic mice using a phosphoenolpyruvate carboxykinase (PEPCK) promoter indicated that the livers of the SREBP-1a TG mice (PEPCK-SREBP-1a) were massively enlarged, owing to the accumulation of triglycerides and cholesterol⁶. PEPCK-SREBP-1c TG livers were only slightly enlarged with a moderate increase in triglycerides but not cholesterol. It is interesting that epididymal fat weight was not increased in these mice⁶. However, other liver-specific TG mice lines overexpressing human SREBP-1a and SREBP-1c under the control of the albumin promoter showed a vast accumulation of lipids in the liver and obesity as observed in miR-33^{-/-} mice^{21,22}. Therefore, overexpression of SREBP-1 does not show consistent phenotypes and these changes may depend on the different promoters and expression patterns in organs of each transgenic line. Because SREBP-2 and miR-33 are expressed ubiquitously, miR-33^{-/-} mice may have mildly enhanced expression of SREBP-1 throughout the body. Previously developed SREBP-1 TG mice do not resemble miR-33-deficient mice in this context. Thus, obesity and hepatic steatosis developed slowly when fed NC and became prominent when fed HFD. Although SREBP-1c-deficient mice do not show any change in BW, it is possible that some compensatory mechanisms may have occurred in these mice²³. Because miR-33^{-/-} mice showed a slight but significant increase in food intake when fed HFD, we conducted our crossbreeding experiments in pair-feeding conditions, which may have enabled us to clearly observe the changes. It is interesting that *Ldlr*^{-/-} mice that received anti-miR-33 oligonucleotides once a week for 14 weeks gained weight although the change was not statistically significant^{24,25}. Therefore, reduction of miR-33 levels is related to unwanted consequences such as elevated BW. It should also be noted that the expression of *Pparg* and its downstream signalling molecules were enhanced in miR-33^{-/-} *Srebf1*^{+/+} mice compared with WT control mice^{26–29}. Because these molecules are attenuated in miR-33^{-/-} *Srebf1*^{+/+} mice, enhanced expression of *Pparg* may be related to the increase in SREBP-1 levels associated with miR-33 deficiency^{30,31}.

The phenotype of miR-33^{-/-} *Srebf1*^{+/+} mice is not completely the same as that of WT mice, and this may be because of other miR-33 target genes. *Abca1* mRNA expression and serum HDL-C levels still tend to be higher in miR-33^{-/-} *Srebf1*^{+/+} mice than those in miR-33^{+/+} *Srebf1*^{+/+} mice. Moreover, RIP140, another miR-33 target^{12,32}, promotes the activity of NF-κB and upregulates the expression of genes implicated in inflammation such as TNFα and IL-6 in macrophages. Enhanced expression of RIP140 by miR-33

deficiency may affect the inflammatory conditions in adipose tissue.

It is important to explain why did not compare *Srebf1*^{+/+} with *Srebf1*^{+/+} mice, but only compared *miR-33*^{-/-} *Srebf1*^{+/+} and *miR-33*^{-/-} *Srebf1*^{+/+} in our experiments. The feedback system of SREBP-2 guarantees appropriate levels of cellular cholesterol. Meanwhile, excess glucose cumulatively activates SREBP-1 and increases triglyceride storage. The latter can be achieved by the fact that *Srebf1* expression is enhanced by SREBP-1 itself and that cleavage of SREBP-1 is less sensitive to sterol-suppression than SREBP-2 (refs 33–36). Therefore, we hypothesize that the differences in the expression of SREBP-1 and its downstream molecules between *Srebf1*^{+/+} and *Srebf1*^{+/+} would be enhanced when SREBP-1 levels are increased by intercrossing with miR-33^{-/-} mice. The *Srebf1* levels are reduced in *Lep*^{ob/ob} × *Srebf1*^{+/+} mice compared with *Lep*^{ob/ob} mice³⁷. However, in *Lep*^{ob/ob} mice, the *Srebf1* levels are not considerably enhanced in the liver, but are rather decreased in adipose tissue and the phenotype is different between *Lep*^{ob/ob} and miR-33^{-/-} mice. Because *Srebf1* may be more enhanced in miR-33^{-/-} mice than *Lep*^{ob/ob} mice, intercrossing with *Srebf1*^{+/+} had more effect in miR-33^{-/-} mice than *Lep*^{ob/ob} mice. Similarly, phenotypic difference between *Srebf1*^{+/+} and *Srebf1*^{+/+} mice with WT background may become small because *Srebf1* is not enhanced. Although a threshold may exist for the SREBP-1 levels that distinguishes between the normal condition and lipotoxicity caused by positive energy imbalance, the threshold level of SREBP-1 can only be achieved by feeding the *miR-33*^{-/-} *Srebf1*^{+/+} mice HFD or at an older age. This can also explain the fact that not much difference is observed between the *Srebf1*^{+/+} and *Srebf1*^{+/+} mice at the basal condition. For this reason, one copy of *Srebf1* can rescue the phenotype when miR-33 is absent. Recently, miRNAs have been recognized as therapeutic targets. It seems that it would be efficient to inhibit the function of a small number of miRNAs that have many different targets with similar functions at the same time. However, it is estimated that one miRNA may have hundreds of different target genes, and unpredicted results may be obtained by complete and long-term inhibition of an miRNA. It is also known that the results obtained by antisense oligonucleotide-based medicine are sometimes different from those obtained in miR-deficient mice. For example, the administration of an miR-21 antagonist prevented pressure-overload-induced cardiac hypertrophy and fibrosis in mice³⁸; however, miR-21-deficient mice did not show any cardiac differences compared with WT mice under pressure overload³⁹. As for miR-33, many target genes have been reported by computer algorithm and *in vitro* experiments such as luciferase-based 3'UTR analysis, only some of which show enhanced expression in miR-33-deficient mice.

A recent report indicated that the inhibition of miR-33a/b in non-human primates increases plasma HDL-C and lowers very-low-density lipoprotein triglycerides⁴⁰. The authors showed a 50% decrease in *SREBF1* mRNA and protein in anti-miR-33-treated monkeys at 12 weeks. They speculated that the decrease in SREBP-1 may result from the derepression of negative regulators of this pathway such as AMPK, which is targeted by miR-33. They actually observed an increase in AMPK (*PRKAA1*) mRNA levels. However, there was no change in AMPKα levels in our experiment, and this point should be clarified in future experiments. It is true that in humans, and not in rodents, there is miR-33b in *SREBF1*. Because miR-33a and miR-33b have the same seed sequence, their targets would be the same. This may explain the differences in AMPKα levels. Further studies may be required to clarify whether there is autoregulation of SREBP-1 by miR-33b. In contrast, the experiment on monkeys

was designed to administer antisense miR-33 for a limited time period. In the present study, we demonstrated that miR-33 deficiency serves to raise SREBP-1, increase fatty acid synthesis and promote fatty acid accumulation in the body. Therefore, long-term therapeutic modulation of miR-33 to cure metabolic diseases requires caution for obesity and related diseases, as miRNAs have potentially many target genes and we cannot detect all of them by computer analysis. Moreover, many genes are affected by these secondary or tertiary target genes. Careful observation of miR-deficient mice enables us to detect overall functions of miRNAs *in vivo*.

In conclusion, these results unravel a previously unrecognized interaction between SREBP-1 and SREBP-2 by the way of miR-33. It will be important to establish the tissue- and time-specific regulation of miR-33 to avoid unexpected side effects.

Methods

Materials. The antibodies used were anti-ABCA1 (NB400-105) (Novus Biologicals, Littleton, CO, USA), anti-GAPDH (14C10; no. 2118S), anti-IRS-2 (no. 4502S), anti-AMPK α (no. 2532), anti-SIRT6 (D8D12; no. 12486) (Cell Signaling Technology, Beverly, MA, USA), anti- β actin (AC-15; A5441, Sigma-Aldrich, St Louis, MO, USA), anti-SREBP-1 (sc-13551, sc-8984), anti-TF2B (sc-225), anti-PPAR γ (sc-7273), anti-SCAP (sc-48671) (Santa Cruz Biotechnology, California, USA), antibodies. Anti-rabbit, anti-mouse and anti-goat IgG HRP-linked antibodies were purchased from GE Healthcare (Amersham, UK). *N*-acetyl-leu-leu-norleucinal (Calpain inhibitor; ALLN) and Complete Mini (Protease inhibitor cocktail) were from Roche. Pitavastatin (NK-104) was kindly provided by Kowa (Japan). *PPRE*-luciferase promoter plasmid (*PPRE*-luc) was kindly gifted by Dr Kelly-DP. *FAS*-luciferase promoter plasmid (*FAS*-luc) was obtained from Addgene. *SRE*-luciferase promoter plasmid (*SRE*-luc) was from Dr Yahagi-N. Mouse *Srebf1* was obtained from the FANTOM (functional annotation of the mouse) full-length mouse cDNA clone set, and Mouse *Srebf1* with or without the 3'UTR was cloned into pcDNA3.1.

Cell culture. HepG2 cells were cultured Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Japan) supplemented with 10% fetal bovine serum (FBS). Mouse primary hepatocytes were obtained from male miR-33^{+/+} or miR-33^{-/-} mice at 8–10 weeks of age by the two-step collagenase perfusion method⁴¹. In brief, hepatocyte suspensions were obtained by passing collagenase type II (Gibco BRL, Life Technologies Inc., Rockville, MD, USA) digested liver through a 70 μ m cell strainer, followed by centrifugation to collect the mature hepatocytes. After isolation, hepatocytes were resuspended in DMEM supplemented with 5% FBS, and seeded on collagen type I-coated dishes (Iwaki Asahi Glass Co. Ltd., Japan) at a density of 7×10^4 cells ml⁻¹. After incubation for 24 h, cells were used for experiments. For sterol-depleted experiments, cells were washed twice with DMEM without FBS two times and switched to DMEM containing 5% LPDS (Sigma-Aldrich) with or without pitavastatin (1 μ M).

Generation of miR-33^{-/-} *Srebf1*^{+/+} mice. To obtain reduced levels of SREBP-1 in miR-33-deficient mice (miR-33^{-/-} *Srebf1*^{+/+}), miR-33^{-/-} mice were mated with *Srebf1*^{+/+} mice, which were a kind gift from Dr Shimano¹⁵. After being weaned at 4 weeks of age, mice were fed NC containing 4.5% fat until 8 weeks of age, and then switched to HFD (D12451; 45% fat by kcal; Research Diet Inc. New Brunswick, NJ, USA) or kept on NC for the next 12 weeks. All of the experimental protocols were approved by the Ethics Committee for Animal Experiments of Kyoto University. Primers for genotyping were as follows.

WT/KO (miR-33) sense; GGCCTACTTCTGATCCTTC
WT (miR-33) antisense; CAACTACAATGCACCACAGCTG
KO (miR-33) antisense; TTGGGATCCAGAATTCGTGATTAA
WT (*Srebf1*) sense; TGTGTCTGACCTGCAATCCT
WT (*Srebf1*) antisense; AGGCCAACACTAGTAGTCCATTG
KO (*Srebf1*) sense; AGGATCTCTGTCATCTCACC
KO (*Srebf1*) antisense; GCCAACGCTATGTCTCTGATA

Western blotting. Western blotting was performed using standard procedures as described previously⁴². For cell experiments, ALLN (12.5 μ g ml⁻¹) was added to the cells 2 h before collection. Samples were lysed in lysis buffer consisting of 100 mM Tris-HCl, pH 7.4, 75 mM NaCl and 1% Triton X-100 (Nacalai Tesque). The lysis buffer was supplemented with complete mini protease inhibitor (Roche), ALLN (25 μ g ml⁻¹), 0.5 mM NaF and 10 μ M Na₃VO₄ just before use. Nuclear protein was extracted using the CelLytic NuCLEAR Extraction Kit (Sigma-Aldrich) in accordance with the manufacturer's instructions. The protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Bio-Rad). All samples (20 μ g of protein) were suspended in lysis buffer, fractionated using NuPAGE 4–12% Bis-Tris (Invitrogen) gels and transferred to a Protran nitrocellulose transfer membrane (Whatman). The membrane was blocked using

1 $\times$ phosphate-buffered saline (PBS) containing 5% non-fat milk for 1 h and incubated with the primary antibody (anti-ABCA1; 1:1,000, anti-ABCG1; 1:1,000, anti-IRS-2; 1:500, anti-AMPK α ; 1:1,000, anti-SREBP-1; 1:250 (Supplementary Fig. S10), anti-TF2B; 1:1,000, anti-PPAR γ ; 1:250, anti- β actin; 1:3,000, anti-GAPDH; 1:3,000, anti-SIRT6; 1:1,000 and anti-SCAP; 1:200) overnight at 4 °C. Following a washing step in PBS-0.05% Tween 20 (0.05% T-PBS), the membrane was incubated with the secondary antibody (anti-rabbit, anti-mouse or anti-goat IgG HRP-linked; 1:2,000) for 1 h at 4 °C. The membrane was then washed in 0.05% T-PBS and detected by ECL Western Blotting Detection Reagent (GE Healthcare), using an LAS-1000 system (Fuji Film). Full-length images on immunoblots are shown in Supplementary Figs S11–S13.

RNA extraction and qRT-PCR. Total RNA was isolated and purified using TriPure Isolation Reagent (Roche), and cDNA was synthesized from 1 μ g of total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche) in accordance with the manufacturer's instructions. For quantitative RT-PCR, specific genes were amplified by 40 cycles using SYBR[®] Green PCR Master Mix (Applied Biosystems). Expression was normalized to the housekeeping gene β -actin. Gene-specific primers are listed in Supplementary Table S1.

Quantitative PCR for miRNAs. Total RNA was isolated using TriPure Isolation Reagent (Roche). miR-33 was measured in accordance with the TaqMan MicroRNA assays (Applied Biosystems) protocol, and the products were analysed using a thermal cycler (ABI Prism7900HT sequence detection system). Samples were normalized by U6 snRNA expression.

Measurement of fatty acid synthesis. Following 4 h of fasting, mice were intraperitoneally injected with 10 μ Ci [1-¹⁴C]-sodium acetate (PerkinElmer Co., Ltd.). Male mice at 10 weeks of age were sacrificed 30 min after injection and livers were rinsed in ice-cold 1 $\times$ PBS. Liver samples were saponified by heating in 3 ml of 30% KOH (w/v) at 70 °C for 15 min, followed by the addition of 3 ml of 95% ethanol (v/v) and continued heating at 70 °C for a further 2 h. Saponified fatty acids were acidified with 3 ml of 9 M H₂SO₄ and extracted with petroleum ether^{13,14}.

Dual luciferase assays. Full-length PCR fragments of the 3'UTR of *SREBF1* were amplified from human or mouse cDNAs and subcloned downstream of a CMV-driven Firefly luciferase cassette in a pMIR-REPORT vector (Ambion). To create WT or mutant 3'UTR luciferase reporter genes, a fragment of the *Srebf1* 3'UTR as follows was inserted into a pMIR-REPORT vector:

Wild type;

CTTCCAAACAATCGTGGTATCTTTATTGACTTTTTTTTTTCTGAATG
CAATGACTGTTTTTTTTTTTTTTAAAC

Mutant;

CTTCCAAACAATCGTGGTATCTTTATTGACTTTTTTTTTTCTGA
TACGTATGACTGTTTTTTTTTTTTTTAAAC

For *SRE* and *FAS* promoter assay, 293T cells were co-transfected with mouse *Srebf1* with full-length 3'UTR or without 3'UTR, along with expression plasmids for miR-control (negative control), or miR-33. An internal control reporter, *Renilla reniformis* luciferase, driven by the thymidine kinase (TK) promoter (pRL-TK; Promega) was also co-transfected to normalize the transfection efficiency. Luciferase activities were measured using a dual luciferase kit (PicaGene dual kit, Toyo Ink Co.). The relative luciferase activity of each construct (arbitrary unit) was reported as the fold induction.⁴²

Lentivirus production and DNA transduction. We produced lentiviral stocks in 293FT cells in accordance with the manufacturer's protocol (Invitrogen). In brief, virus-containing medium was collected 48 h post transfection and filtered through a 0.45- μ m filter. One round of lentiviral infection was performed by replacing the medium with virus-containing medium (containing 8 μ g of Polybrene per ml), followed by centrifugation at 2,500 rpm for 30 min at 32 °C. Cells were used for analysis 3 days after DNA transduction.

IPGTT and insulin tolerance test. For IPGTT, after overnight fasting, male mice at 20 or 50 weeks of age were injected with 1.5 g kg⁻¹ glucose intraperitoneally. For insulin tolerance test, after a 4-h fast, mice were injected intraperitoneally with insulin (0.75 u kg⁻¹ and 1.0 u kg⁻¹ for NC and HFD, respectively, Humulin R; Eli Lilly Japan KK). Blood was obtained from the orbital vein and glucose levels were measured using a glucose sensor.

Measurement of serum insulin and leptin levels. We quantified serum levels of insulin and leptin in male mice at 20 weeks of age using an ELISA assay kit for mouse insulin and leptin in accordance with the manufacturer's instructions (Shigayagi Co. Ltd, Shibukawa, Japan).

Biochemical analysis of serum. After mice (male at 20 or 50 weeks of age) were fasted for 4–6 h, blood was obtained from the inferior vena cava of anaesthetised mice, and serum was separated by centrifugation at 4 °C and stored at –80 °C. Biochemical data were measured by standard methods using a Hitachi 7180 Auto Analyzer (Nagahama Life Science Laboratory, Nagahama, Japan).

Measurement of cholesterol and triglyceride in the liver. Lipids in the liver were extracted by the Folch procedure⁴³. In brief, lipids were extracted by addition of ice-cold MeOH followed by the addition of ice-cold CHCl₃. High purity water was added and the sample kept on ice for an additional 10 min with occasional mixing. The sample was centrifuged for 5 min at 2,000 g and the upper (aqueous) phase was removed and reextracted by addition of ice-cold CHCl₃:MeOH (2:1, v/v) as above. The upper phase was discarded and both organic phases were combined, dried under nitrogen stream. Lipids were quantified using standard enzymatic colorimetric methods (Sky Light Biotech, Akita, Japan).

Measurement of fat body mass by CT. CT scans were obtained and fat body mass and lean body mass were analysed using a Lathia LTC-100 (Aloka, Tokyo, Japan) under pentobarbital anaesthesia.

DNA microarray analysis. Five liver RNA samples from miR-33^{+/+} or miR-33^{-/-} male mice at 16 weeks of age receiving NC were pooled and analysed using a DNA microarray (3D-Gene Mouse Oligo chip 24k, Toray, Tokyo, Japan).

Pair feeding. Every other day, male mice received the same amount of food consumed by miR-33^{+/+} *Srebp1*^{+/+} mice (WT) on the previous 2 days, from 8 to 20 weeks of age.

Assessment of metabolic rate and activity. Oxygen consumption and activity of male mice at 16 weeks of age were measured with an indirect calorimetric system. In brief, room air was pumped through an acrylic metabolic chamber, and the expired gas was filtered through thin cotton, dried and subjected to gas analysis (model RL-600; Alco System, Tokyo, Japan)⁴⁴.

Statistics. Data are presented as means ± s.e.m. Statistical comparisons were performed using unpaired two-tailed Student's *t*-tests or a one-way analysis of variance with the Bonferroni *post hoc* test where appropriate, with a probability value of <0.05 taken to indicate significance.

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Author contributions

T.H., T.N. and K.O. designed the project; T.H., T.N., O.B., Y.K., T.N., M.N., S.U., M.I., M.S. and S.M. performed experiments; T.H., T.N., N.Y., H.S., K.I., H.M., T.M., K.H., N.K., M.Y., T.K., T.K. and K.O. analysed and interpreted data; N.Y. and H.S. contributed materials; and T.H., T.N. and K.O. prepared the manuscript.

Additional information

Supplementary Information accompanies this paper at <http://www.nature.com/naturecommunications>

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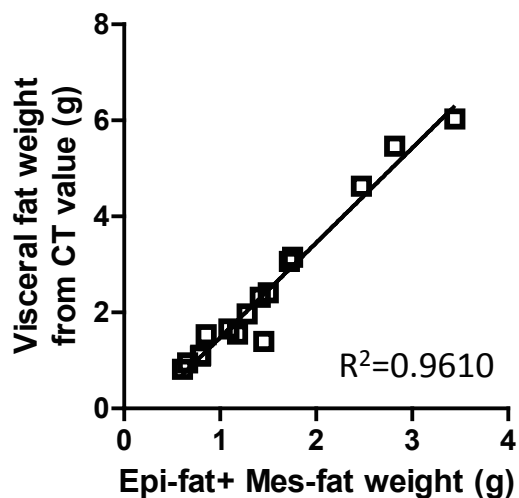
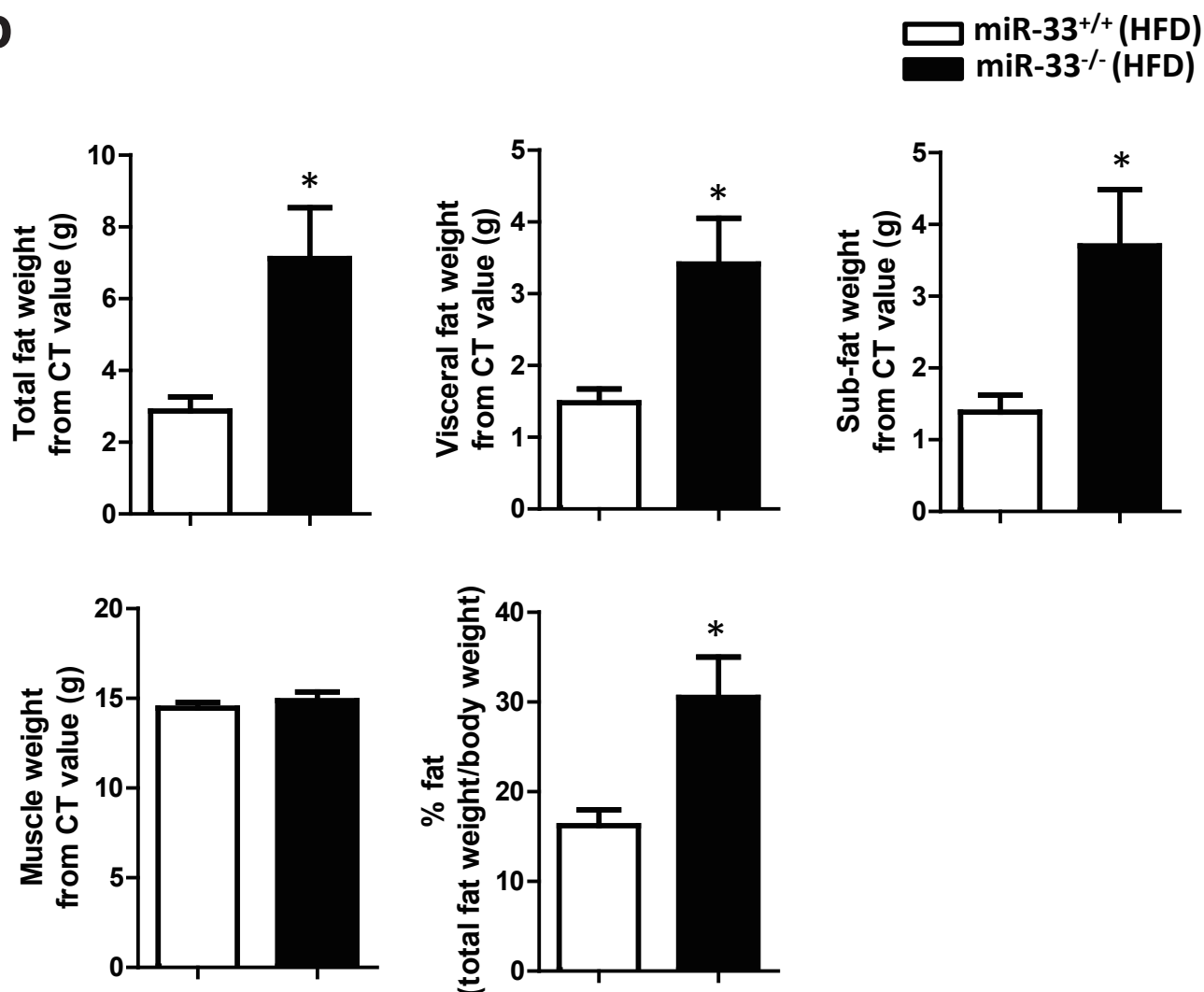


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Supplementary Information

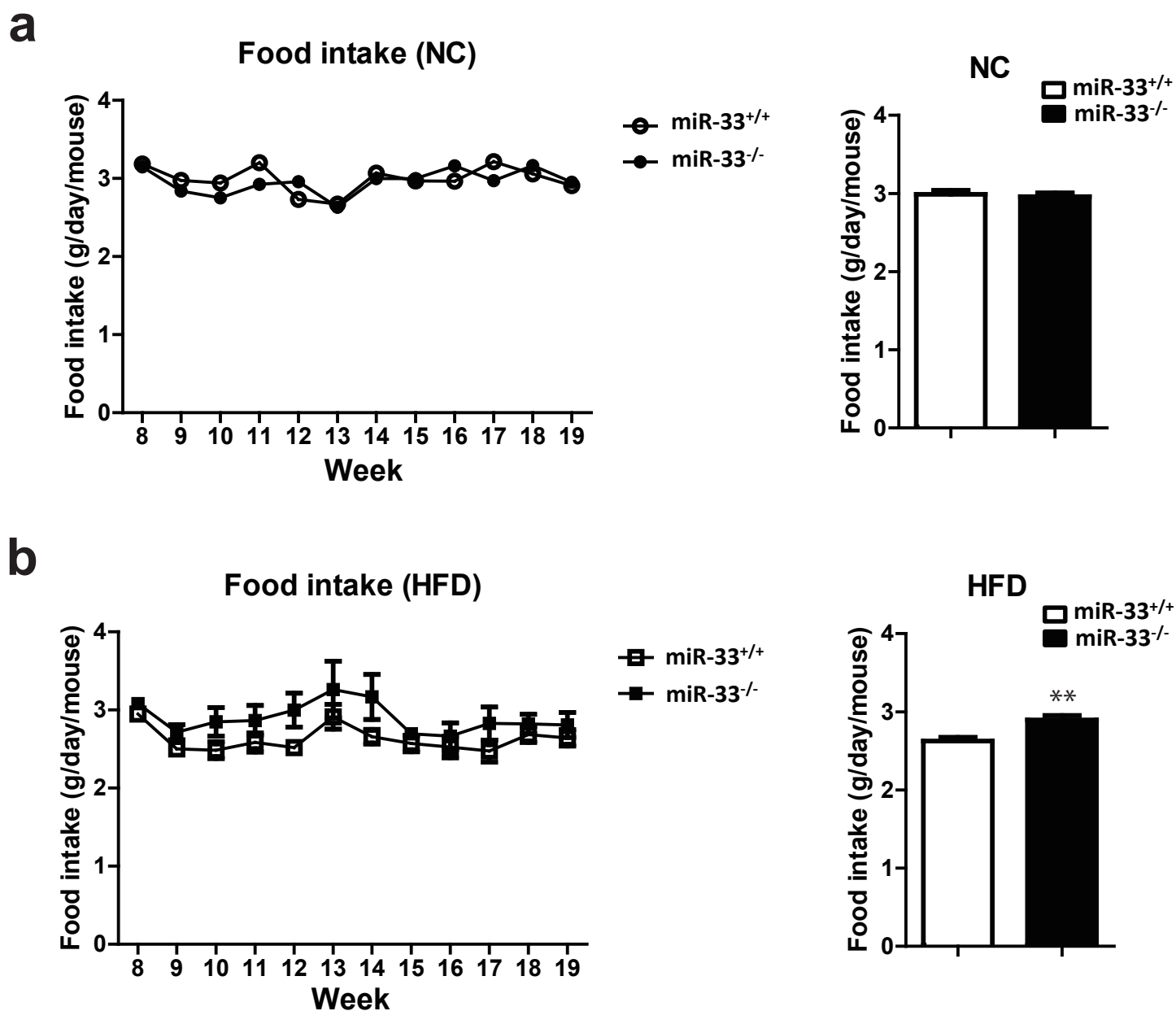
MicroRNA-33 regulates sterol regulatory element-binding protein 1 expression in mice

Takahiro Horie, Tomohiro Nishino, Osamu Baba, Yasuhide Kuwabara, Tetsushi Nakao, Masataka Nishiga, Shunsuke Usami, Masayasu Izuhara, Naoya Sowa, 1Naoya Yahagi, Hitoshi Shimano, Shigenobu Matsumura, Kazuo Inoue, Hiroyuki Marusawa, Tomoyuki Nakamura, Koji Hasegawa, Noriaki Kume, Masayuki Yokode, Toru Kita, Takeshi Kimura, and Koh Ono

a**b**

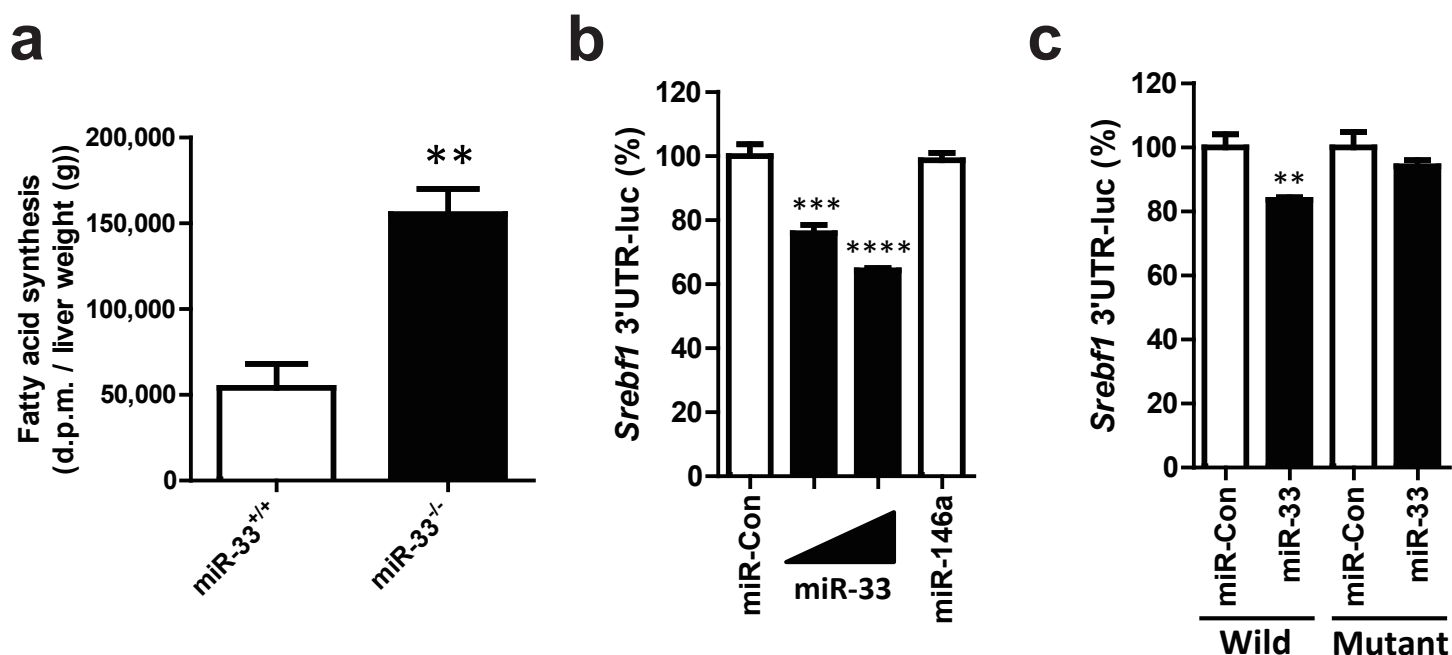
Supplementary Figure S1. Estimation of fat weight from CT images.

(a) Relationship between estimated fat weight and actual weight. **(b)** Estimated fat weight of miR-33^{+/+} and miR-33^{-/-} mice fed HFD. Values are the means $\pm$ s.e.m. ($n = 8$ each, $*p < 0.05$ in Student's t-test).



Supplementary Figure S2. Food intake of miR-33^{+/+} and miR-33^{-/-} mice.

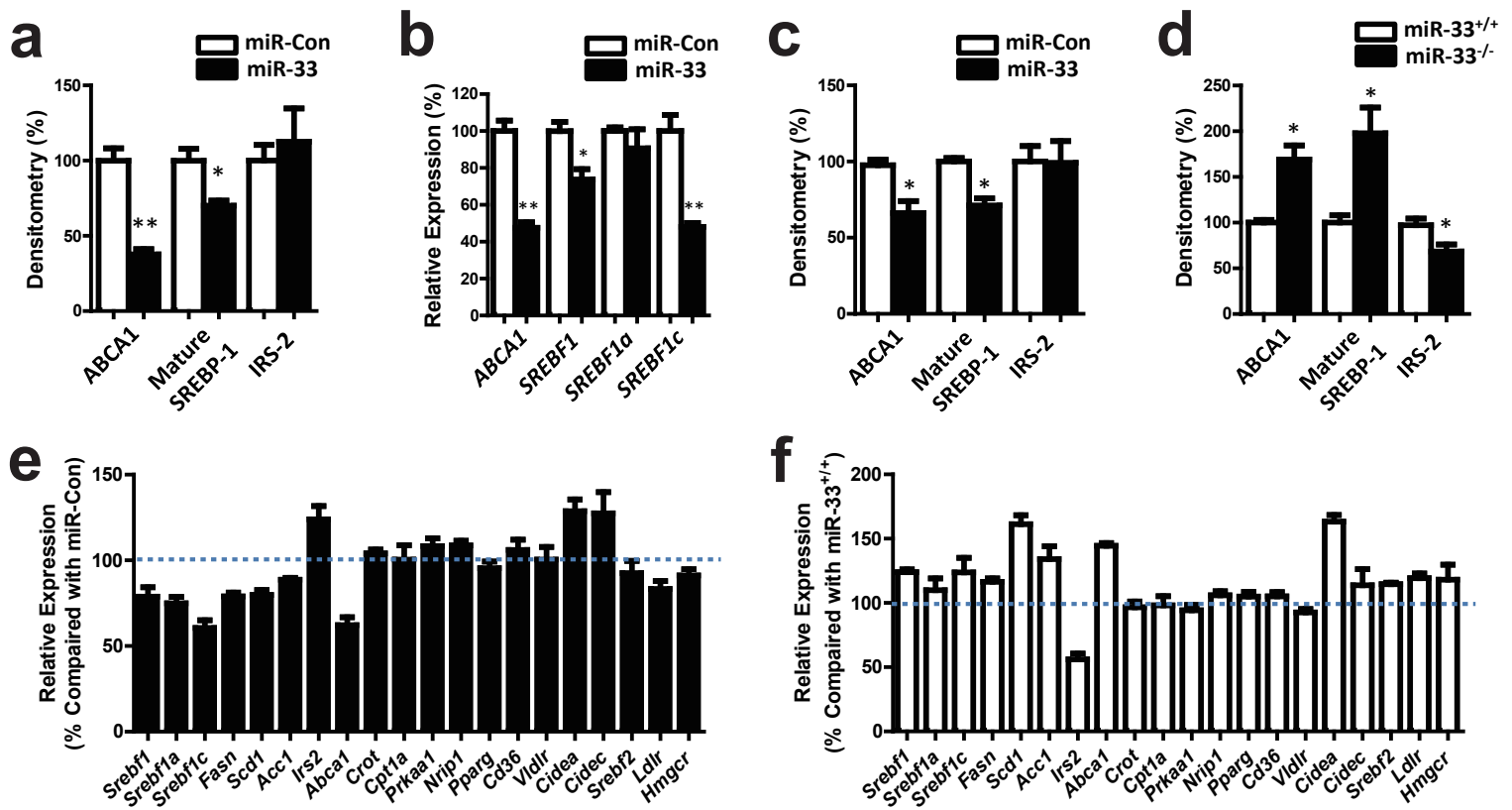
(a) Serial changes in food intake associated with NC feeding. **(b)** Serial changes of food intake associated with HFD feeding. Values are the means $\pm$ s.e.m. Statistical comparisons were made by Student's t-test ($n=5$, $**p < 0.01$).



Supplementary Figure S3. miR-33 deficiency increased fatty acid synthesis *in vivo*

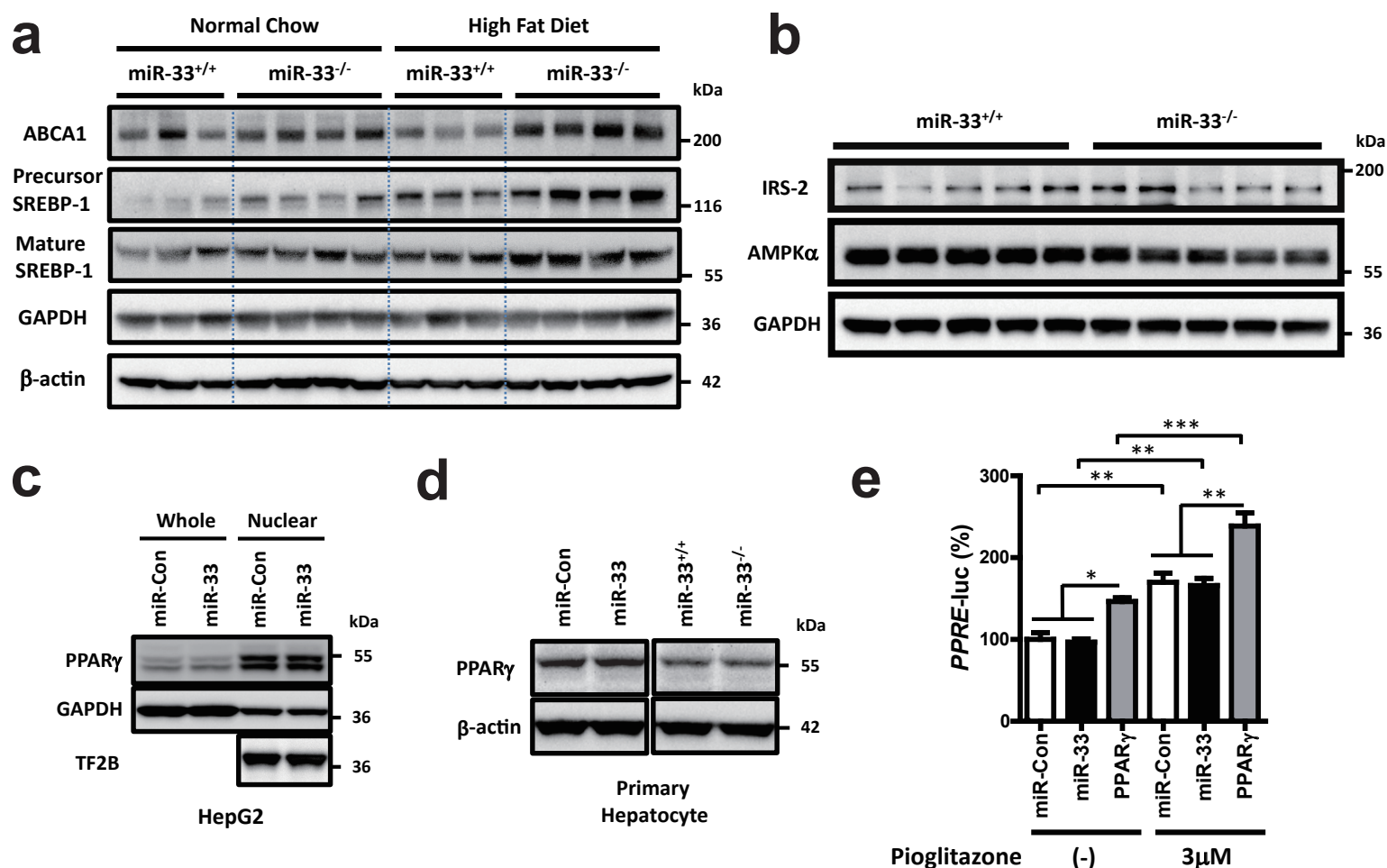
and miR-33 targets the 3'UTR of *Srebf1* in COS-7 cells.

(a) *De novo* fatty acid synthesis in the livers of miR-33^{+/+} and miR-33^{-/-} mice (n = 4 each, **p < 0.01 in Student's t-test). **(b)** Luciferase reporter activity of mouse *Srebf1* 3'UTR in COS-7 cells. miR-control (miR-Con) and miR-146a were used as negative controls (n = 4 each, ***p < 0.001, ****p < 0.0001 in one-way analysis of variance test) **(c)** Luciferase reporter activity of wild-type or mutant *Srebf1* 3'UTR at the potential miR-33 binding site in COS-7 cells (n = 4 each, **p < 0.01 in Student's t-test). Values are the mean ± s.e.m.



Supplementary Figure S4. The expressions of lipid metabolism-associated genes in HepG2 cells and primary hepatocytes transduced with miR-Con or miR-33 expression vector and in miR-33^{+/+} and miR-33^{-/-} mice.

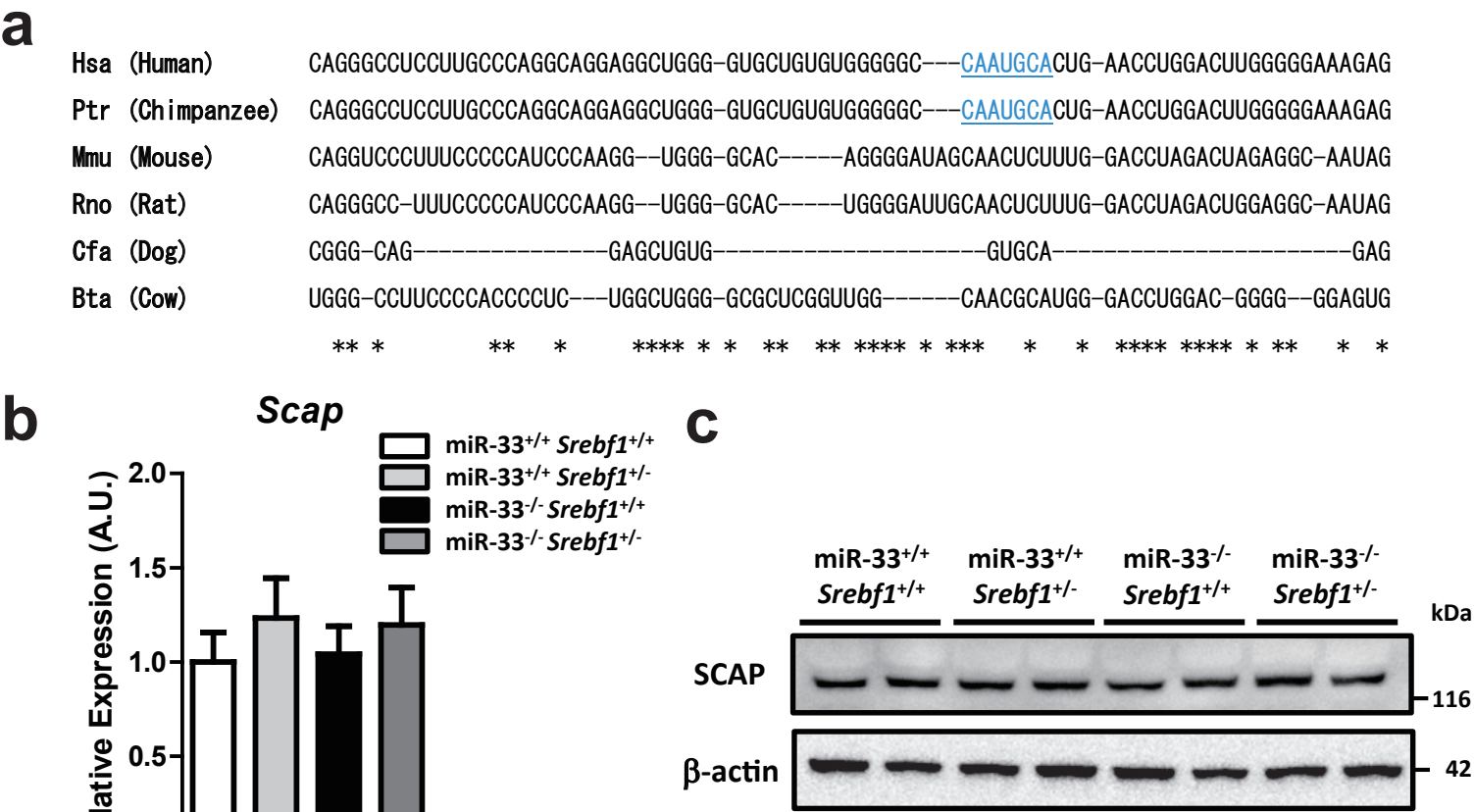
(a) ABCA1, SREBP-1, and IRS-2 expression levels in HepG2 cells transduced with miR-Con or miR-33 expression vector. **(b)** ABCA1, SREBF1, SREBF1a, and SREBF1c expression levels in HepG2 cells transduced with miR-Con or miR-33 expression vector *p<0.05, **p<0.01. **(c)** ABCA1, SREBP-1, and IRS-2 expression levels in primary hepatocytes transduced with miR-Con or miR-33 expression vector. *p<0.05. **(d)** ABCA1, SREBP-1, and IRS-2 expression levels in primary hepatocytes prepared from miR-33^{+/+} and miR-33^{-/-} mice. *p<0.05. **(e)** Relative expression levels of lipid metabolism-related genes in primary hepatocytes transduced with miR-Con or miR-33 expression vector. **(f)** Relative expression levels of lipid metabolism-related genes in primary hepatocytes prepared from miR-33^{+/+} and miR-33^{-/-} mice. Values are the means $\pm$ s.e.m. Statistical comparisons were made by Student's t-test.



Supplementary Figure S5. Western blotting analysis of ABCA1, SREBP-1, IRS-2,

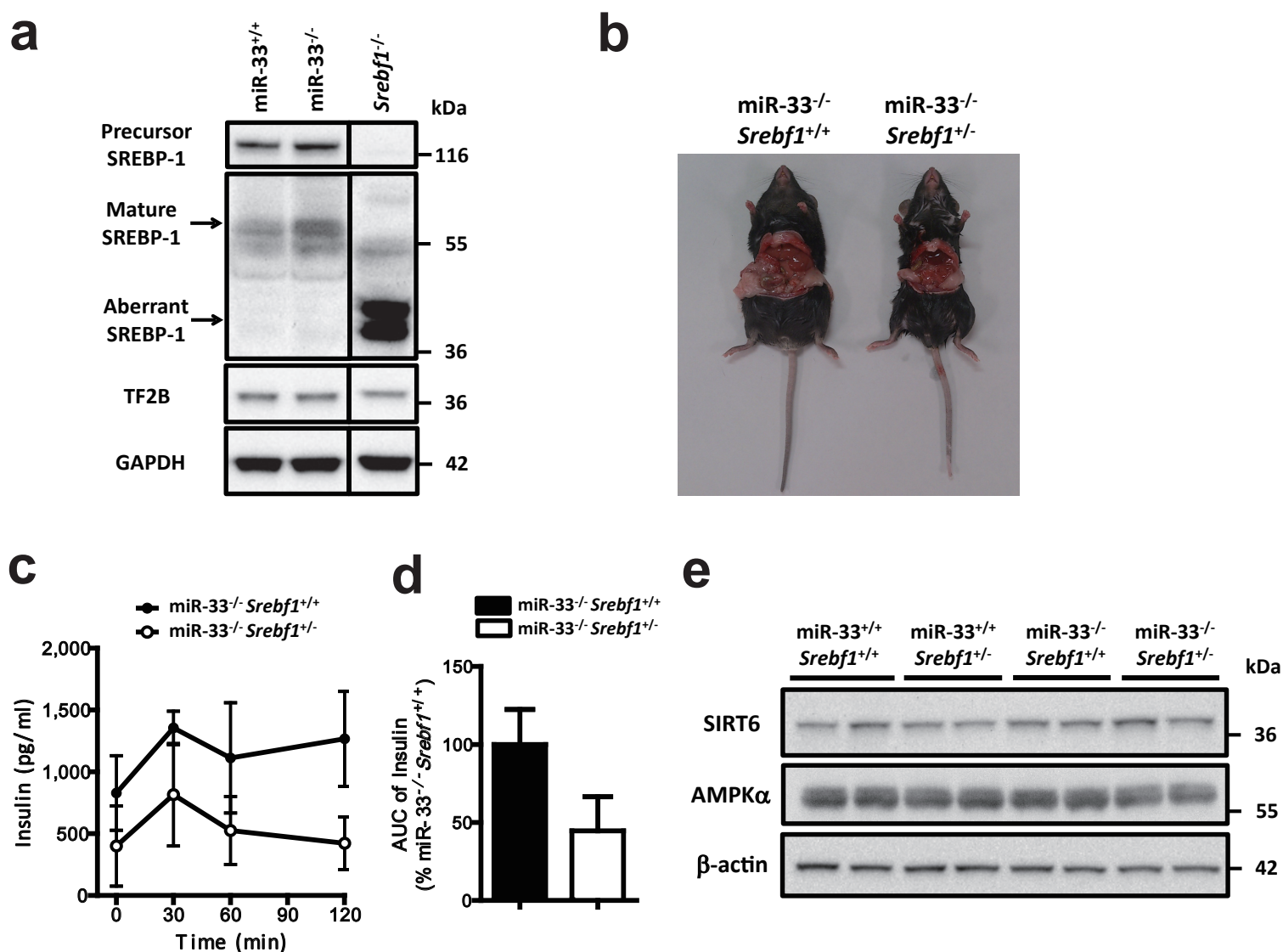
AMPKα, and PPARγ.

(a) ABCA1 and SREBP-1 protein levels in the livers of miR-33^{+/+} and miR-33^{-/-} mice fed NC or HFD. **(b)** AMPKα and IRS-2 protein levels in the livers of miR-33^{+/+} and miR-33^{-/-} mice. **(c)** PPARγ protein levels in HepG2 cells transduced with miR-Con or miR-33 expression vector. **(d)** PPARγ protein levels in primary hepatocytes transduced with miR-Con or miR-33 expression vector and primary hepatocytes prepared from miR-33^{+/+} and miR-33^{-/-} mice. **(e)** Luciferase reporter activity of *PPRE* in HepG2 cells. HepG2 cells with or without 3 μM pioglitazone treatment were transfected with the *PPRE* luciferase construct, along with expression plasmids for miR-Con (negative control), miR-33, or PPARγ (positive control). Values are the mean ± s.e.m. Statistical comparisons were made by one-way analysis of variance test (n = 4 each, *p < 0.05, **p < 0.01, ***p < 0.001).



Supplementary Figure S6. The expression of SCAP in miR-33^{+/+} *Srebf1*^{+/+}, miR-33^{+/+} *Srebf1*^{+/-}, miR-33^{-/-} *Srebf1*^{+/+}, and miR-33^{-/-} *Srebf1*^{+/-} mice fed HFD.

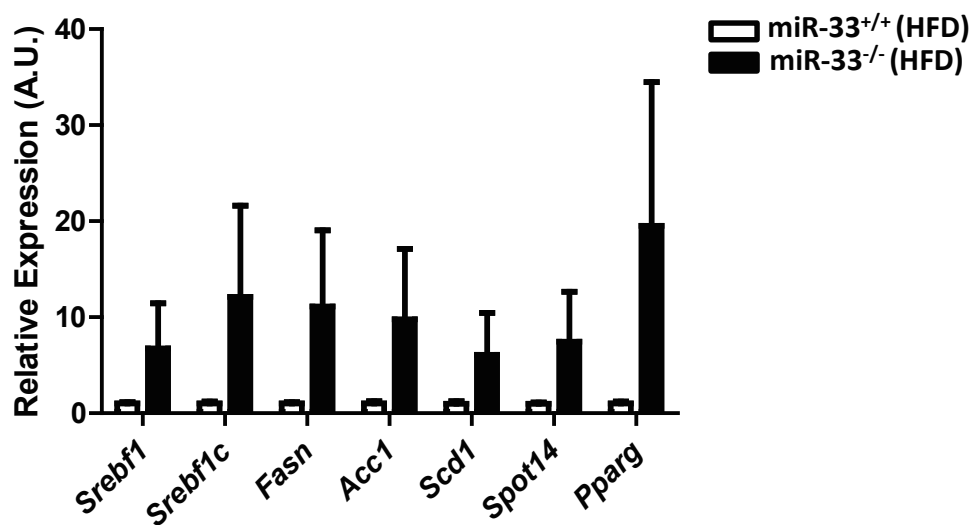
(a) Sequence alignment of *Scap* 3'UTR at the potential miR-33 binding site. * indicates the conservation among species. **(b)** Quantitative real-time PCR analysis of *Scap*. Values are the mean ± s.e.m. (n = 6–8 each). **(c)** Western blotting analysis of SCAP levels. β-actin was used as a loading control.



Supplementary Figure S7. Phenotypic changes by the reduction of SREBP-1 levels

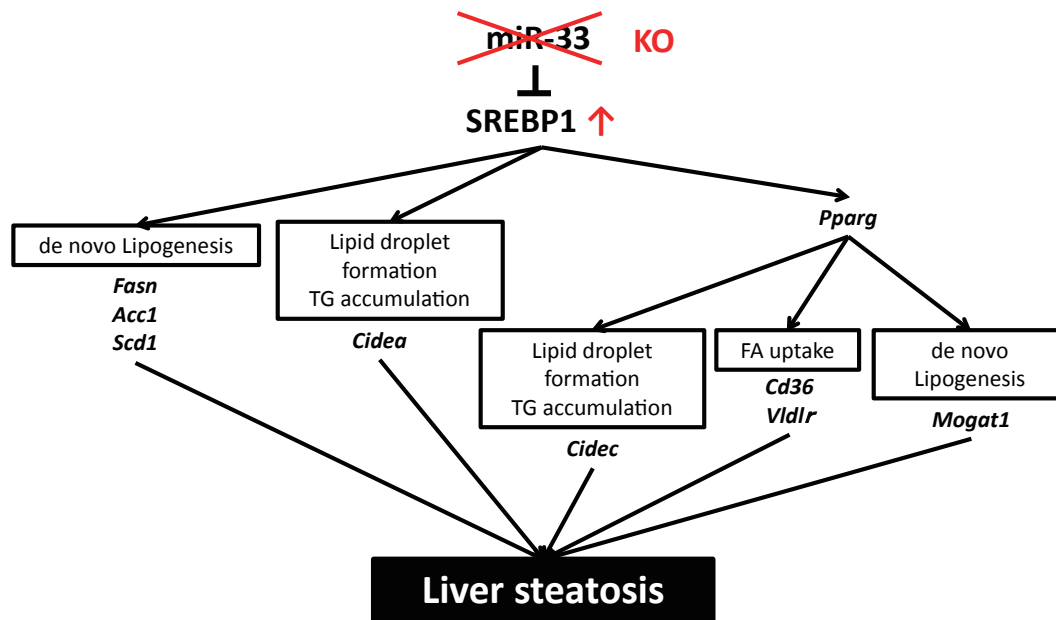
in miR-33^{-/-} mice.

(a) Western blotting analysis of SREBP-1 in the liver of miR-33^{+/+}, miR-33^{-/-} and *Srebf1*^{-/-} mice fed NC. **(b)** Representative picture of miR-33^{-/-} *Srebf1*^{+/+} and miR-33^{-/-} *Srebf1*^{+/-} mice fed HFD. **(c)** Serial changes of insulin levels after intraperitoneal injection of glucose in miR-33^{-/-} *Srebf1*^{+/+} and miR-33^{-/-} *Srebf1*^{+/-} mice fed HFD (n = 5 each). **(d)** AUC of insulin levels after intraperitoneal injection of glucose in miR-33^{-/-} *Srebf1*^{+/+} and miR-33^{-/-} *Srebf1*^{+/-} mice fed HFD (n = 5 each). **(e)** Western blotting analysis of SIRT6 and AMPKα in miR-33^{+/+} *Srebf1*^{+/+}, miR-33^{+/+} *Srebf1*^{+/-}, miR-33^{-/-} *Srebf1*^{+/+}, and miR-33^{-/-} *Srebf1*^{+/-} mice fed HFD. β-actin was used as a loading control. Values are the means ± s.e.m.



Supplementary Figure S8. Relative mRNA expression levels of lipid metabolism -related genes in epididymal fat pads from miR-33^{+/+} and miR-33^{-/-} mice fed HFD.

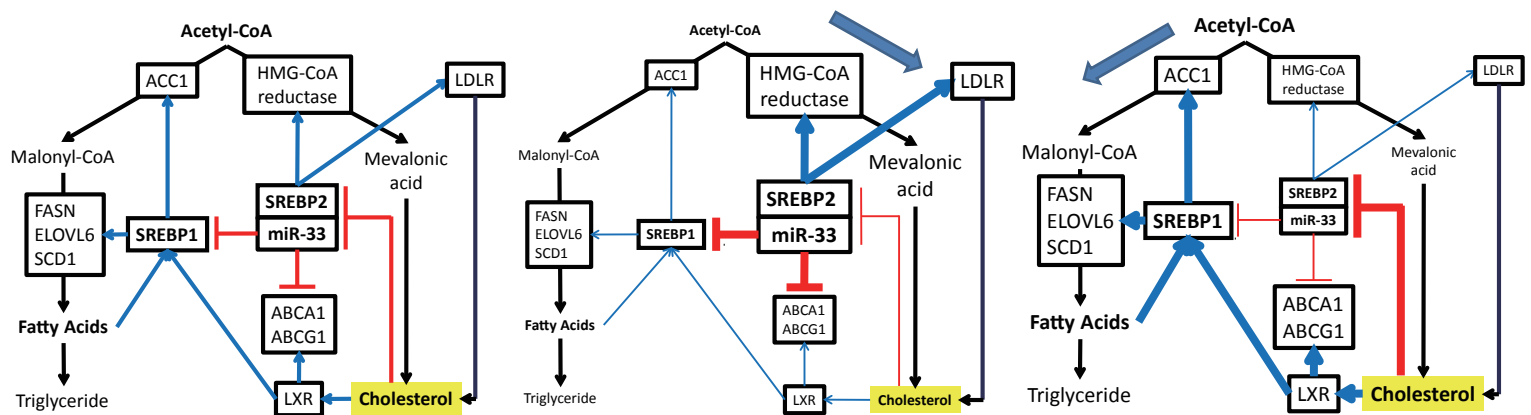
Values are the means $\pm$ s.e.m. (n=5).

a**b**

Regulation of cholesterol and fatty acid synthesis by miR-33

Cholesterol-depleted or fasting states

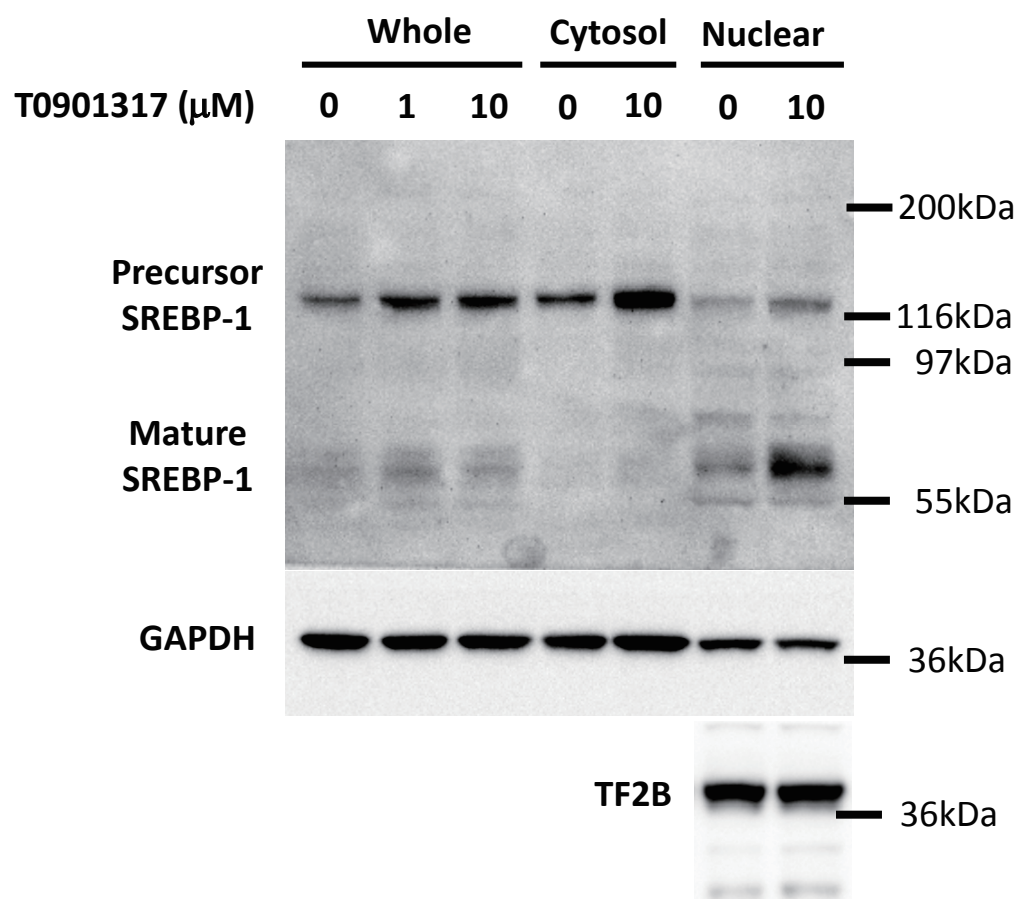
Cholesterol-loaded or overeating states



Supplementary Figure S9. Schematic overview of the function of miR-33 in the regulation of SREBP-1 expression levels.

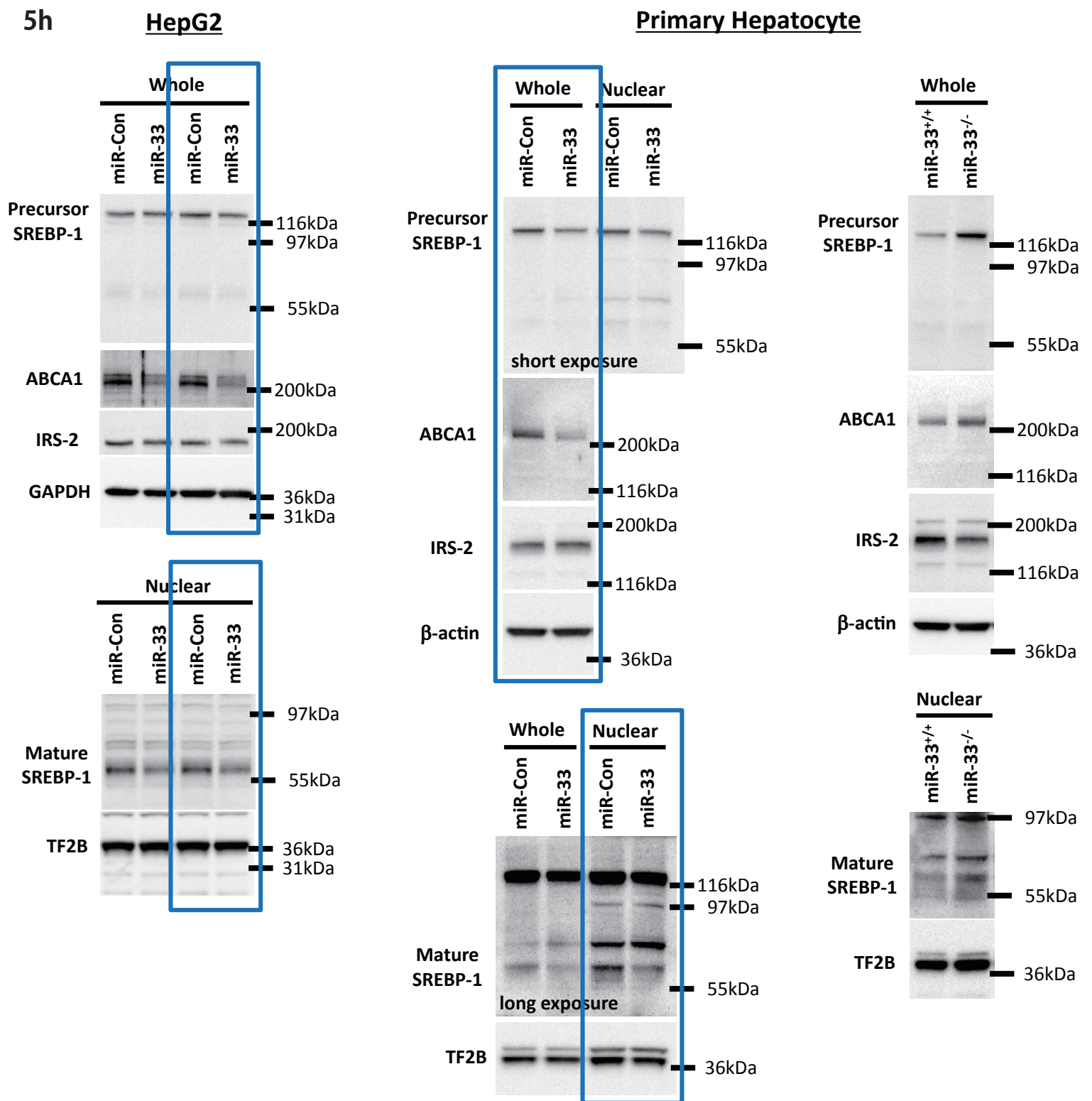
(a) Overview of the function of miR-33 deficiency on liver steatosis.

(b) Context-dependent change of the role of miR-33 on lipid metabolism.

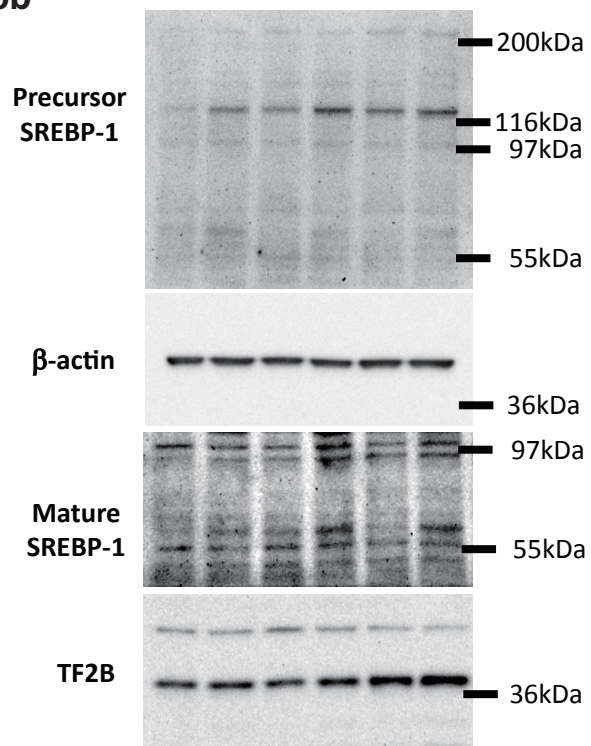
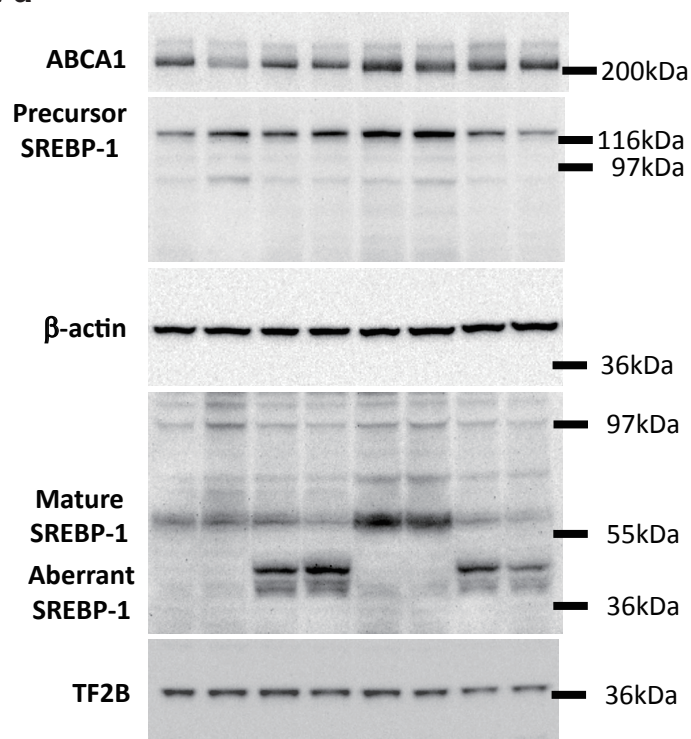


Supplementary Figure S10. Western blotting for SREBP-1, GAPDH, TF2B in T0901317-stimulated HepG2 cells.

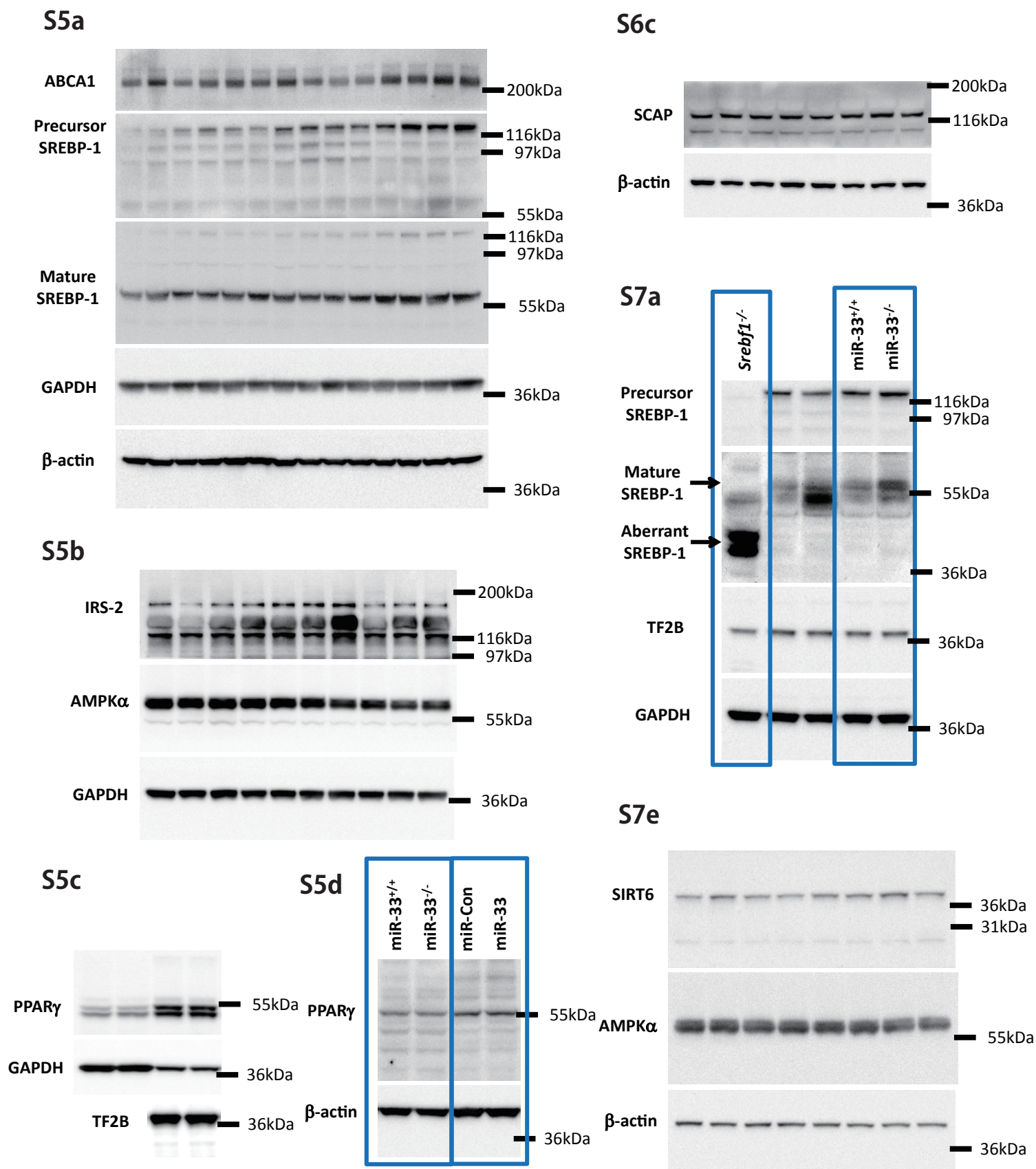
HepG2 cells were stimulated with the indicated concentrations of T0901317 for 24 h.



Supplementary Figure S11. Scans of the original blots in Figure 5h

6b**7a**

Supplementary Figure S12. Scans of the original blots in Figure 6b and 7a



Supplementary Figure S13. Scans of the original blots in Supplementary Figure

S5a-d, S6c, S7a and S7e

Supplementary Table S1

Used gene-specific oligonucleotide primer sequences

Gene	Species	Forward / Reverse
<i>SREBF1</i>	Human	5'-AACAGTCCCACTGGTCGTAGAT-3' / 5'-TGTTGCAGAAAGCGAATGTAGT-3'
<i>SREBF1a</i>	Human	5'-ATGGACGAGCCACCCTTC-3' / 5'-GCCAGGGAAGTCACTGTCTTG-3'
<i>SREBF1c</i>	Human	5'-GCGCCTTGACAGGTGAAGTC-3' / 5'-GCCAGGGAAGTCACTGTCTTG-3'
<i>ABCA1</i>	Human	5'-GTCCTCTTTCCCGCATTATCTGG-3' / 5'-AGTTCCTGGAAGGTCTTGTTAC-3'
<i>IRS2</i>	Human	5'-AGAGGACCTACTCCCTGACCAC-3' / 5'-GATCTCGATGTCTCCGTAGTCC-3'
<i>ACTB</i>	Human	5'-AGGCACTCTTCCAGCCTTCC-3' / 5'-GCACTGTGTTGGCGTACAGG-3'
<i>Srebf1</i>	Mouse	5'-TAGAGCATATCCCCAGGTG-3' / 5'-GGTACGGGCCACAAGAAGTA-3'
<i>Srebf1a</i>	Mouse	5'-GGCCGAGATGTGCGAACT-3' / 5'-TTGTTGATGAGCTGGAGCATGT-3'
<i>Srebf1c</i>	Mouse	5'-GGAGCCATGGATTGCACATT-3' / 5'-GAAGTCACTGTCTTGTTGTTG-3'
<i>Srebf2</i>	Mouse	5'-GTGGAGCAGTCTCAACGTCA-3' / 5'-TGGTAGGTCTACCCAGGAG-3'
<i>Scd1</i>	Mouse	5'-GCGATACACTCTGGTGCTCA-3' / 5'-CCCAGGGAAACCAGGATATT-3'
<i>Acaca (Acc1)</i>	Mouse	5'-AGAAACCCGAACAGTGGAAC-3' / 5'-AGGTAGCCCTTACGGTTAAA-3'
<i>Fasn</i>	Mouse	5'-GGGTTCTAGCCAGCAGAGTCTA-3' / 5'-TGAGATGTGGATACCACCAGAG-3'
<i>Thrsp (Spot14)</i>	Mouse	5'-TGACAGTCATGGATCGGTACTC-3' / 5'-TGGTCCACTTCTACACAGATGC-3'
<i>Irs2</i>	Mouse	5'-ACAACCTATCGTGGCACCTC-3' / 5'-CCATGAGACTTAGCCGCTTC-3'
<i>Hmgcr</i>	Mouse	5'-CGTAACCCAAAGGTCAAGA-3' / 5'-GACCCAAGGAAACCTTAGCC-3'
<i>Ldlr</i>	Mouse	5'-ATTGGGTTGATTCCAAACTCC-3' / 5'-ATTCACATCTGAACCCGTGAG-3'
<i>Cidea</i>	Mouse	5'-ATGATCTTGAAAAAGGGACAGA-3' / 5'-GCCTGTATAGGTGGAAGGTGAC-3'
<i>Cideb</i>	Mouse	5'-GCCTGCTAAGGTCAGTATCCAC-3' / 5'-CAGTGTAGCACTCCACGTAGC-3'
<i>Cidec</i>	Mouse	5'-GACCCAACAGCTGGTGTCTAA-3' / 5'-TTCAAGATGTCCTGGACCTTG-3'
<i>Pparg</i>	Mouse	5'-CCCACCAACTTCGGAATCAG-3' / 5'-TGCTGGAGAAATCAACTGTGGTA-3'
<i>Cd36</i>	Mouse	5'-GGCCAAGCTATTGCGACAT-3' / 5'-CAGATCCGAACACAGCGTAGA-3'
<i>Ap2</i>	Mouse	5'-GTGATGCCTTTGTGGGAACCTGGAAG-3' / 5'-TCATAAACTCTTGTGGAAGTCACGCC-3'
<i>Ucp2</i>	Mouse	5'-CAGGTCACTGTGCCCTTACCAT-3' / 5'-CACTACGTTCCAGGATCCCCAAG-3'
<i>Vldlr</i>	Mouse	5'-TGTAGTCGTGGCTATCAAATGG-3' / 5'-TCTCTCTAGGCCAATCTTCCTG-3'
<i>Mogat1</i>	Mouse	5'-CCTGGCTTTACATCGTATCTCC-3' / 5'-GCTCAGCACATGAGACAAACTC-3'
<i>Dgat1</i>	Mouse	5'-CCAACCATCTGATCTGGCTTA-3' / 5'-CAGCATTCCACCAATCTCTGT-3'
<i>Mlxipl (Chrebp)</i>	Mouse	5'-CCTCACTTCACTGTGCCTCA-3' / 5'-ACAGGGGTTGTTGTCTCTGG-3'
<i>Cpt1a</i>	Mouse	5'-GATCTACAATTCCCCCTCTGCTCT-3' / 5'-TAGAGCCAGACCTTGAAGTAACG-3'
<i>Crot</i>	Mouse	5'-TACTTTTACCACGGCCGAAC-3' / 5'-GACGGTCAAATCCTTTTCCA-3'
<i>Prkaa1</i>	Mouse	5'-AGAGGGCCGCAATAAAAGAT-3' / 5'-TGTTGTACAGGCAGCTGAGG-3'
<i>Cyp4a10</i>	Mouse	5'-ATTTATGGTCTCCACCACAACC-3' / 5'-CACAATCACCTTCAGCTCACTC-3'
<i>Cyp4a14</i>	Mouse	5'-CTCAGTCACTGTACCTTC-3' / 5'-ATCCTCCTGAGAATGGCAGAT-3'
<i>Ehhadh</i>	Mouse	5'-GCCTTACTGGACCGTCTTAC-3' / 5'-ACCCAGTGGCTTGTCACTACTG-3'
<i>Angptl4</i>	Mouse	5'-CTTCTCTACTTGGGACCAAGACC-3' / 5'-CGTGGGATAGAGTGGAAGTATTG-3'
<i>Abca1</i>	Mouse	5'-AACAGTTTGTGGCCCTTTTG-3' / 5'-AGTTCAGGCTGGGGTACTT-3'
<i>Actb</i>	Mouse	5'-GATCTGGCACCACACCTTCT-3' / 5'-GGGGTGTGAAGGTCTCAA-3'
<i>Scap</i>	Mouse	5'-GATGTGTTCCGGTCACCTCT-3' / 5'-TTGGTCCCTGAGCTGTCTCT-3'
<i>Sirt6</i>	Mouse	5'-GGCTACGTGGATGAGGTGAT-3' / 5'-GGCTCAGCCTTGAGTGCTAC-3'

Supplementary Table S2

Serum profile of NC-fed aged mice

	miR-33^{+/+} NC 50W (n=5)	miR-33^{-/-} NC 50W (n=5)	
AST (IU/L)	53.40±4.11	62.00±8.07	
ALT (IU/L)	36.20±3.65	63.20±16.83	
ALP (IU/L)	201.80±12.28	237.00±19.77	
T-CHO (mg/dL)	89.60±4.70	137.60±8.42	**
TG (mg/dL)	25.00±1.52	18.80±1.66	*
NEFA (μEq/L)	807.8±72.94	976.2±53.30	
LDL-C (mg/dL)	5.40±0.40	11.2±1.28	**
HDL-C (mg/dL)	55.80±3.01	66.20±2.35	*

Values are the means ± s.e.m. Statistical comparisons were made by Student's t-test (*p < 0.05, **p < 0.01).

Supplementary Table S3

Pathway analysis using GenMAPP software. Up-regulated pathways in miR-33^{-/-} liver

Pathway	miR-33 ^{-/-} /miR-33 ^{+/+}	
	> 2	
	gene	Z Score
Mm_Fatty_acid_metabolism	5	7.449
Mm_gamma_Hexachlorocyclohexane_degradation	4	6.88
Mm_Tryptophan_metabolism	5	6.748
Mm_Complement_and_Coagulation_Cascades_KEGG_WP449_33690	3	3.73
Mm_Striated_Muscle_Contraction_WP216_33380	2	3.166
Mm_Ascorbate_and_aldarate_metabolism	1	2.799
Mm_beta_Alanine_metabolism	1	2.799
Mm_Propanoate_metabolism	1	2.674
Mm_Complement_Activation,_Classical_Pathway_WP200_32694	1	2.457
Mm_Endochondral_Ossification_WP1270_32869	2	2.35
Mm_Glutathione_metabolism	1	2.274
Mm_Eicosanoid_Synthesis_WP318_35469	1	2.274
Mm_Statin_Pathway(PharmGKB)_WP1_35542	1	2.193
Mm_selenium_WP1272_34380	1	2.193
Mm_Folic_Acid_Network_WP1273_34393	1	2.193

Supplementary Table S4

Serum profile of mice with indicated genotype

	miR-33^{+/+} <i>Srebf1</i> ^{+/+}	miR-33^{+/+} <i>Srebf1</i> ^{+/-}	miR-33^{-/-} <i>Srebf1</i> ^{+/+}	miR-33^{-/-} <i>Srebf1</i> ^{+/-}
AST (IU/L)	60.2±3.94	66.17±4.09	101.67±33.60	53.50±3.10
ALT (IU/L)	22.67±2.53	37.67±4.76	73.33±45.79	25.50±5.38
ALP (IU/L)	144.83±8.77	144.33±5.06	188.33±10.93*	144.83±12.93
T-CHO (mg/dL)	140.33±15.88	141.83±16.32	164.00±20.30	167.83±8.74
TG (mg/dL)	18.67±4.89	14.00±1.84	17.83±1.74	15.00±2.18
NEFA (μEq/L)	505.33±60.80	473.83±45.38	480.33±65.29	421.67±36.62
LDL-C (mg/dL)	12.83±2.80	10.00±1.37	13.83±2.83	13.67±1.48
HDL-C (mg/dL)	68.17±5.36	70.00±5.54	76.50±7.12	73.00±2.07

Values are the means ± s.e.m. Statistical comparisons were made by one-way analysis of valiance test (n= 6 each, *p < 0.05).