Supplementary Materials

Supplemental Information included seven figures and supplemental material and methods and can be found with this article online.

Identification of miR-378 targets

Identification of miR-378 target genes was conducted as previously described with minor revision [5]. To identify genes with binding motifs for miR-378, we downloaded the target gene databases of miR-378 based on TargetScan [1], Pictar [3], and Starbase [7]. Only hits from TargetScan or PicTar algorithm that were confirmed by Ago HITS-CLIP (high-throughput sequencing of RNAs isolated by crosslinking immunoprecipitation (HITS-CLIP) from Argonaute protein complex) were selected. These three databases were compared using Microsoft Access 2000. We then carried out Gene Ontology analysis of potential targets using PathwayStudio software (Elsevier) and focused on obesity-related metabolic disorders [4], yielding 2 potential target genes whose 3'UTRs contain a miR-378 binding motif. Our prediction from *in silico* algorithms showed that 3' UTR of *Nrf1* mRNAs is 100% complementary to the miR-378 5' seed region exhibiting the highest prediction scores and binding energy. In addition, the seed regions are conserved between the species of mouse and human.

Expression vector construction

The Open Reading Frame (ORF) of murine *Nrf1* was amplified from mouse liver cDNA. We then inserted the ORF into the mini-circle parental plasmid. A transthyretin gene (*TTR*) promoter was used to ensure liver-specific expression of *Nrf1* [2]. This new construct was referred to as MC-*TTR*-Nrf1. To prepare the mini-circle, parental MC-*TTR*- Nrf1 vector was transformed into a

special host *E. coli* bacterial strain ZYCY10P3S2T (System Biosciences, Cat: MN900A-1). Mini-circles were made based on the manufacturer's instructions.

Reporter vector construction and luciferase assay

To generate the luciferase reporter vector of 3'UTR, 3' UTR of *Nrf1* was amplified from mouse cDNA using PCR, and inserted into the pMiR-Reporter vector (Ambion), referred as pMiR-*Nrf1*. Two bases of the binding sites for miR-378 within the 3'UTR of *Nrf1* were mutated using QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) per the manufacture's instruction, and referred to as pMiR-Mu-*Nrf1*. 24 hours before transfection, 5×10^4 Hepa1-6 cells were plated per well in a 24-well plate. Then, 200 ng of pMiR-*Nrf1*and miR-378 mimic (20 nM) as well as 30 ng of β-*gal* plasmid pSV-β-Galactosidase Vector (Promega) were transfected into Hepa1-6 cells using Lipofectamine 2000 (Invitrogen). Scrambled control (Dharmacon) was used as the control for miR-378 mimic. After 24 hours of transfection, luciferase and β-galactosidase assays were done using the Luciferase Assay System and Beta-Glo® Assay System (Promega). Luciferase activities were normalized to galactosidase activities; wells were transfected in triplicate; and each well was assayed in triplicate.

To generate the luciferase reporter vector of miR-378 promoter, the promoter of miR-378 (Supplementary Figure 7) was amplified from mouse genomic DNA using PCR, and inserted into the pGL3-basic (Promega), and referred to as pGL3-miR-378. Two bases of the binding site for Nrf1 within the promoter of miR-378 were mutated using QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) per the manufacture's instruction, and referred as pGL3-Mu-miR-378. 24 hours before transfection, 5×10^4 Hepa1-6 cells were plated per well in a 24-well plate. Then, pGL3-miR-378 (200 ng), MC-*TTR*-Nrf1 (200 ng) and 30 ng of β-*gal* plasmid pSV-β-Galactosidase were transfected into Hepa1-6 cells using Lipofectamine 2000 (Invitrogen).

Hepa1-6 cells treated with pGL3-miR-378 and empty vector was used as control. After 24 hours of transfection, luciferase and β-galactosidase assays were done using the Luciferase Assay System and Beta-Glo® Assay System (Promega). Luciferase activities were normalized to galactosidase activities; wells were transfected in triplicate; and each well was assayed in triplicate.

miRNA transfection and gene expression

5×10^4 of Hepa1-6 cells were seeded in a 24-well plate and allowed to adhere overnight. To determine the effects of miR-378 overexpression and knockdown on gene expression, Hepa1-6 cells cultured in the DMEM with 10% FBS were transfected with MC-*TTR*-miR-378 (500 ng/well) or MC-*TTR*-miR-378-MM (500 ng/well) using Lipofectamine 3000. 24 hours after transfection, cells were washed using cold PBS and the total RNA were isolated for gene expression analysis.

Histological analysis

Liver samples were embedded in Tissue-Tek OCT embedding compound, and frozen on dry ice. 8 μm-thick sections were cut with a Leica CM3050 S cryostat, air-dried, and fixed in 10% formalin. After washing, sections were stained with an Oil-Red-O (Sigma-Aldrich)/60% isopropanol solution (Fisher Scientific). Briefly, sections were rinsed with 60% isopropanol and stained for 20 min with prepared Oil Red O solution (0.5% in isopropanol followed by dilution to 60% with distilled water and filtered). After rinses in 60% isopropanol and distilled water, slides were counterstained with hematoxylin for 4 min, rinsed with water, and mounted.

RNA isolation and quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was isolated with miRNeasy Mini Kit (Qiagen). To assess gene expression, 1 μg RNA was used for cDNA synthesis with Superscript III reverse transcription reagent (Invitrogen). PCR amplification was performed at 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute in a 7900 real time-PCR system with SYBR green (Applied Biosystems). For each sample, we analyzed β-actin, GAPDH or 18S rRNA expression to normalize target gene expression. Primers for qRT-PCR were designed with Primer Express software (Applied Biosystems).

To determine levels of miRNA expression, 10 ng RNA were used for miRNA-specific cDNA synthesis with the TaqMan MicroRNA Reverse Transcription Kit and Taqman MicroRNA Assays (all Applied Biosystems). PCR amplification was performed at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute in a 7900 real time-PCR system (Applied Biosystems). The small RNA Sno202 and RNU6 were used to normalize target miRNA expression. Relative changes in gene and miRNA expression were determined using the $2^{-\Delta\Delta Ct}$ method [6].

Hepatic lipid analysis

Mouse liver (100 mg) was placed in 1 ml chloroform/methanol (2:1) mixture and incubated on mice for 10 minutes before homogenization. Lipids were extracted from liver homogenates through room temperature orbital shaking (2 hours) followed by centrifugation (5000 RPM for 5 minutes). Supernatants were collected and washed with 0.4 ml chloroform/methanol (2:1) mixture by centrifugation at 5000 RPM for 20 minutes (room temperature). New supernatants were washed with 0.2 volume of 0.9% NaCl. After centrifuging for 5 minutes at 5000 RPM, supernatants were removed and lower-phase was dried at 42°C. Dried lipids were re-suspended

in 2% Triton X-100. Liver triglycerides were quantified via a colorimetric assay using a triglyceride assay kit from Roche Diagnostics according to the manufacturer's protocols.

Western Blots and antibodies

Proteins were extracted from the cell and tissues using RIPA buffer (Cell Signaling Technology) with proteases inhibitors (Roche, Indianapolis, IN, USA). Protein concentration was measured using Pierce BCA Protein Assay Kit and 25~50 μg of total lysate was loaded and immunoblotted. Anti-NRF1 (Cat. NO.: ab34682) was purchased from Abcam. Anti-actin (NB600-501) was purchased from Novus Biologicals.

Figure legends

Supplementary Figure 1 HFD treatment induced hepatosteatosis. (A) Oil-Red staining of livers from mice treated with either standard diet (SD) (n=6) or high fat diet (HFD) (n=6) for 8 weeks. **(B**) Hepatic lipid content in mice after 8 weeks of SD or HFD treatment. (**C)** Levels of serum triglycerides in mice treated with SD or HFD. (**D**) Oil-Red Staining of Hepa1-6 cells treated with oleate (0.5 mM). Data represent mean ± SEM. Student *T* test was used to evaluate the statistical significance. *P* values are indicated.

Supplementary Figure 2 miR-378 impaired expression of genes controlling fatty acid oxidation but had no effects on mRNA levels of the lipogenic genes. (A) Expression levels of miR-378 and *Nrf1* in Hepa1-6 cells transfected with either scramble (control), miR-378-ASO, MC-*TTR*-Nrf1-shRNA, or a combination of miR-378-ASO and MC-*TTR*-Nrf1-shRNA. (**B**) Levels of miR-378 and *Nrf1* in Hepa1-6 cells transfected with MC-*TTR*-miR-378-MM (control), MC-*TTR*-miR-378, MC-*TTR*-Nrf1, or a combination of MC-*TTR*-miR-378 and MC-*TTR*-Nrf1. (C) mRNA levels of *Cpt1α*, *Pparα*, *Acox1*, *Acad1* and *Vlcad* in Hepa1-6 cells treated with MC-*TTR*-miR-378-MM (control), MC-*TTR*-miR-378, or a combination of MC-*TTR*-miR-378 and

MC-*TTR*-Nrf1. (**D**) mRNA levels of *Cpt1α*, *Pparα*, *Acox1*, *Acad1* and *Vlcad* in Hepa1-6 cells treated with either scramble (control), miR-378-ASO, or a combination of miR-378-ASO and MC-*TTR*-Nrf1 shRNA. (**E**) mRNA levels of the lipogenic genes including *Srebp1c, Fasn, Scd1 and Gpat* in Hepa1-6 cells treated with MC-*TTR*-miR-378-MM (control), MC-*TTR*-miR-378, or a combination of MC-*TTR*-miR-378 and MC-*TTR*-Nrf1. (**F**) mRNA levels of the lipogenic genes including *Srebp1c, Fasn, Scd1 and Gpat* in Hepa1-6 cells treated with either scramble (control), miR-378-ASO, or a combination of miR-378-ASO and MC-*TTR-Nrf1* shRNA. Hepa1-6 cells were maintained in the DMEM medium containing 0.5 mM oleate. Student *T* test was used to evaluate the statistical significance. Data represent mean ± SEM. NS: no significance; **p* < 0.05; ***p* < 0.01; and ****p* < 0.001.

Supplementary Figure 3 MC-*TTR*-miR-378 treatment had no effect on mRNA levels of the lipogenic genes. (A) Reduced mRNA levels of *Nrf1* in livers of mice treated with MC-*TTR*-miR-378 (*n*=10) compared to the mice treated with MC-*TTR*-miR-378-MM (*n*=10). (**B**) MC-*TTR*-miR-378 treatment had no effect on mRNA levels of lipogenic genes including *Srebp1c*, *Fasn*, *Scd1*, and *Gpat*. (**C-D**) Levels of miR-378 and *Nrf1* in livers of HFD-treated mice administered with scramble control (miR-378-ASO-MM), miR-378-ASO or a combination of miR-378-ASO and MC-*TTR*-Nrf1-shRNA. (**E**) Liver to body weight ratio of mice administered with either scramble control (miR-378-ASO-MM), miR-378-ASO or a combination of miR-378-ASO and MC-*TTR*-Nrf1-shRNA. (**F**) mRNA levels of the lipogenic genes including *Srebp1c*, *Fasn*, *Scd1*, and *Gpat* in livers of the above three groups of mice. qRT-PCR was used to determine mRNA levels of these genes. Data represent mean ± SEM. **p* < 0.05; ***p* < 0.01; and ****p* < 0.001.

Supplementary Figure 4 Hepatic lipid accumulation impaired expression of *Nrf1* in livers of dietary obese mice. mRNA levels of *Nrf1* in livers of mice treated with SD or HFD for 8

weeks. Data represent mean ± SEM. Mann-Whitney test was used to evaluate the statistical significance for mouse

Supplementary Figure 5 HFD treatment reduced Nrf1 occupancy over the promoter of miR-378. *In vivo* ChIP assays were performed using genomic DNA isolated from mouse livers treated with HFD (*n*=3) or SD (*n*=3); and the binding of Nrf1 to the endogenous promoter of miR-378 was detected using a specific Nrf1 antibody; N.S.: non-specific control, which is located 10 kb downstream of predicted Nrf1 binding site. qPCR was used to determine percentage of DNA precipitated by Nrf1 antibody relative to the amount of input DNA (% Input). Student's *t* test was used for statistical analysis. The data shown are representative of an experiment repeated three times and conducted in triplicate. Data represent mean ± SEM.

Supplementary Figure 6 *Nrf1* shRNA treatment reduced protein and mRNA levels of *Nrf1* in livers of mice. Eight-week-old wild-type male C57Bl/6 mice (*n*=6) were maintained on a high fat diet (Open Source D12492: 60% Kcal fat) for 8 weeks. After 8 weeks of HFD administration, mice were injected with either MC-*TTR* empty vector (Control, *n*=10) or MC-*TTR*-Nrf1 shRNA (*n*=10) weekly for another 8 weeks. Mini-circle vectors were injected by tail-vein in a volume of 100 μl saline (1.5 μg/g body weight). (**A**) mRNA levels of *Nrf1* in livers of two groups of mice and (**B**) protein levels of *Nrf1*. Student *T* test was used to evaluate the statistical significance. Data represent mean ± SEM. ***$p < 0.001$.

Supplementary Figure 7 Promoter sequence of miR-378 and the primers used for the promoter cloning

References

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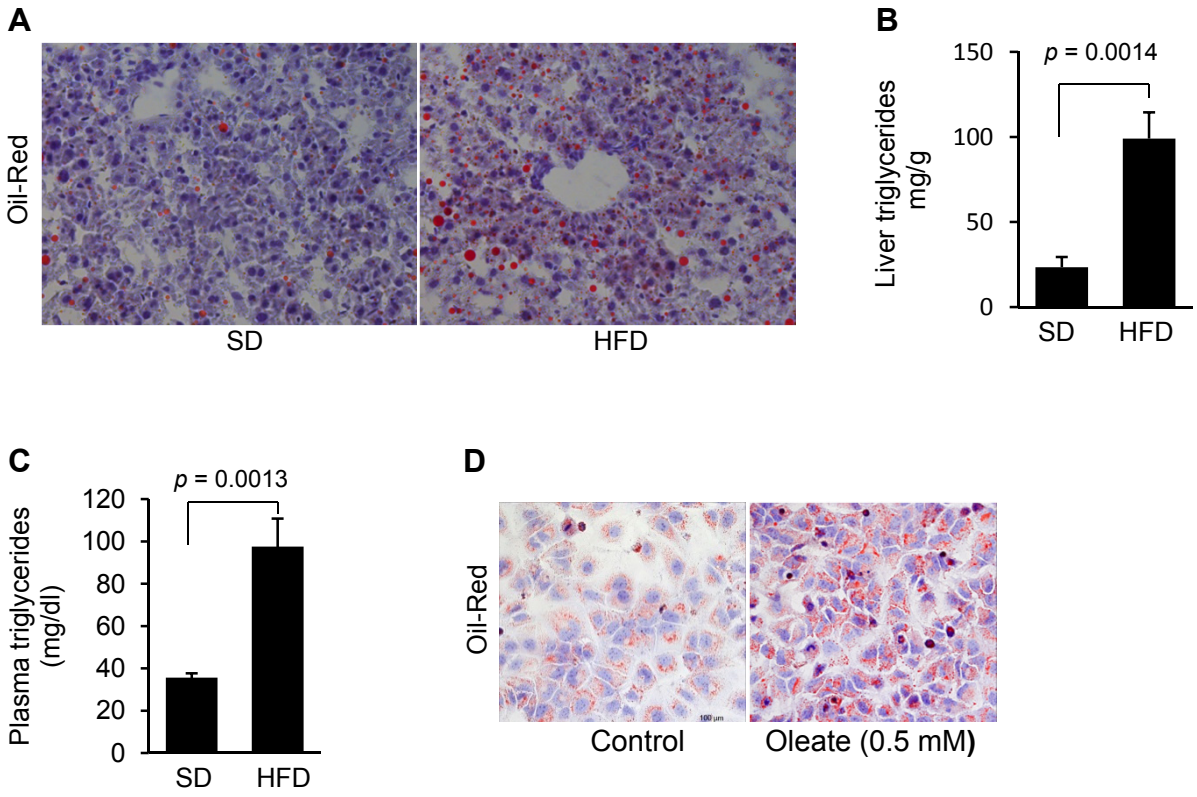
Supplementary Figure 1



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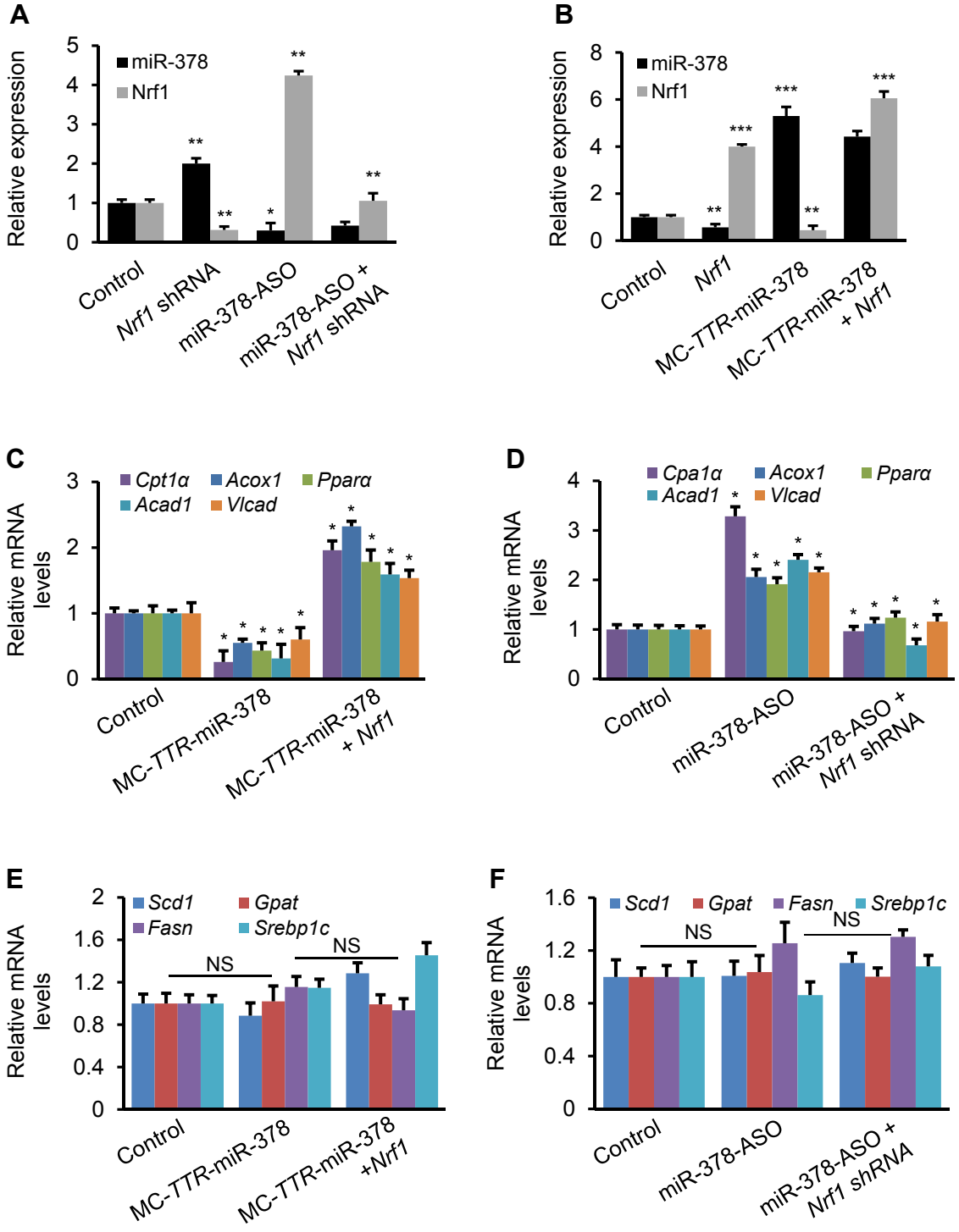

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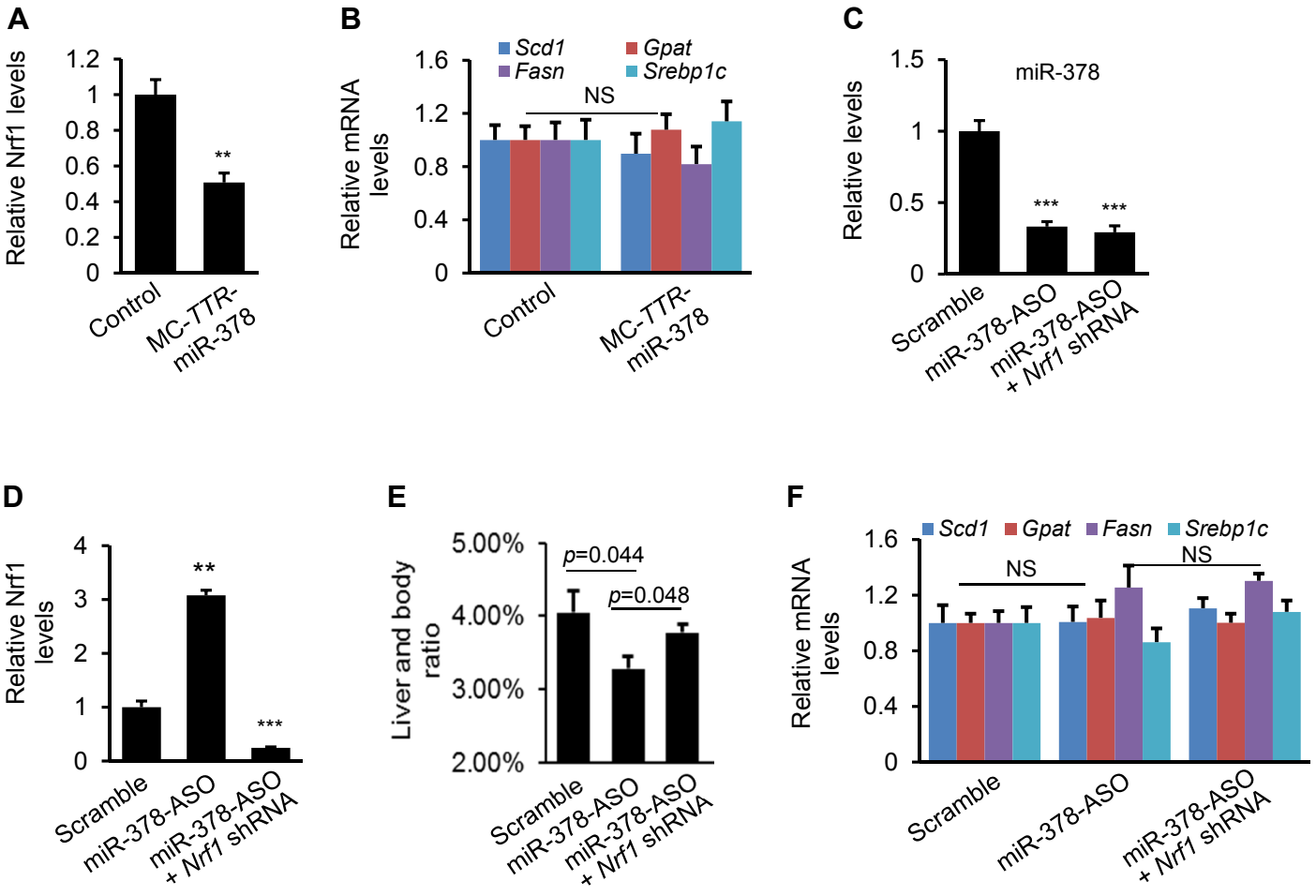

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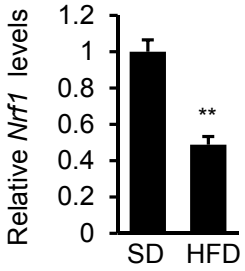

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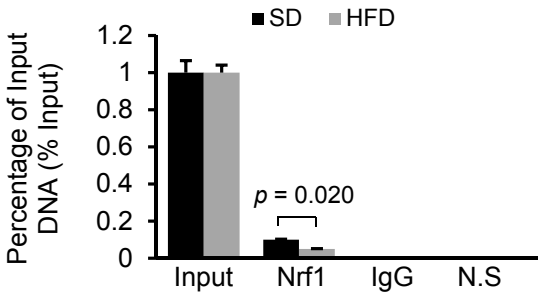
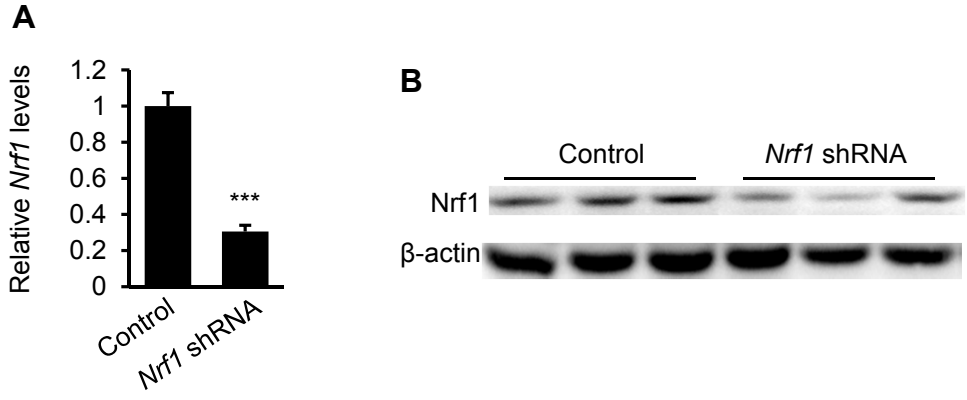


Supplementary Figure 6



Supplementary Figure 7

miR-378 promoter region

CTGCTGGCCCGGGCACCGCCACCTGGCTGCCGGAGCTTTTTTTTTTTCCCCTGCGGGGGGCTGCGAGACCTGGTCCCTGCGC
TAGACTAGGAGCAGGAAAAGCCGGGGGTGCGACCCCAGGCTCGCGCGGCCCCGCCCCCGCAGCCAGCCCGCCTGCCGCAGCC
GGGGGCCCGAGCTCCGCCCTCCACCCGCCCGCCGGGCAGCCTCCTCCCTCCTCCTCGCTCGCTTGCTCGCTCGCTCGCTCCC
TCCCCCGGGCGGGCTCGGCGCTGACTCCGCCGCACGCTGCAGCCGCGGCTGGAAGATGGCGGGGAACGACTGCGGCGCGCTG
CTGGATGAAGAGCTCTCGTCCTTCTTCCTCAACTATCTCTCTGACACGCAGGTACTGCCAGCAGGGAACGCAGGCCGGGGAC
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CCGGGCGGAAGGACTATCTAGGGGGTGCCTGGGTCCCCCTGCGGCGCGCGTTC

Transcription start site (TTS) was highlighted in pink and Nrf1 binding site was highlighted in yellow.

Cloning primers of miR-378 promoter:

Forward primer: CTGCTGGCCCGGGCACCGCCACCTG
Reverse primer: CCAGGCACCCCCTAGATAGTCCTT