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Supplementary Materials for

CRISPR-mediated BMP9 ablation promotes liver steatosis via the down-regulation of PPARα expression

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Supplementary Materials

Materials and Methods

Animal experiments

Male C57BL/6 and db/db mice aged 8-12 weeks were purchased from the Shanghai Laboratory Animal Company (SLAC, Shanghai). BMP9 KO mice were generated by CRISPR/Cas9 on a C57BL/6 background, and KO mice were compared to WT littermates. We commissioned CYAGEN Bioscience Co., Ltd. to create the BMP9-KO mice by CRISPR/Cas-mediated genome engineering. The mouse BMP9 gene (GenBank accession number: NM_019506.4; Ensemble: ENSMUSG00000072625) is located on mouse chromosome 14. Mouse BMP9 contains 2 exons. Paired CRISPR gRNA1 (TGTACAAGTCGATCATGTACTGG) and gRNA2 (GCTGAAGCTCCGCACGATGTTGG) were designed and used to target sites in BMP9 exon 1. Cas9 mRNA and guide RNA (gRNA) generated by *in vitro* transcription were coinjected into fertilized eggs for KO mouse production. The positive pups were bred to the next generation, which was genotyped by PCR and DNA sequencing analysis.

All mice were housed in a temperature-controlled room (at 22°C) with a 12-h light and dark cycle with free access to food and water. HFD-fed mice were maintained with free access to high-fat chow (SLACOM) and drinking water. The HFD contained 60% kcal from fat, 20% kcal from carbohydrate, and 20% kcal from protein. All animal procedures were approved by the Shanghai Jiao Tong University Animal Care and Use Committee.

Hepatic and cellular TG measurements

Liver tissues were homogenized in chloroform/methanol (2:1 v:v) using a tissue grinder (PRO Scientific, PRO200). Lipid extracts were prepared by the classical Folch method. The extracts were dissolved in isopropanol. For the *in vitro* model of cellular steatosis, Hepa 1-6 cells were exposed to 10 ng/ml recombinant BMP9 protein (Bio-Techne, SUS0215121) or PBS vehicle control. The protein concentration was measured by a BCA Protein Quantitation kit (Beyotime, P0010). The TG content was measured using a commercial Triglyceride Assay kit (Dongou, V1103) according to the manufacturer's instructions.

Insulin tolerance test (ITT) and glucose tolerance test (GTT)

For the ITT, awake mice were fasted for 5 h. Fed mice were intraperitoneally injected with insulin (Novo Nordisk, Novolin R) at a dose of 0.75 IU/kg body weight. Glycemia was measured in tail vein blood samples at the indicated time points by Glucometer (ONETOUCH UltraEasy, SN-CFD8413CR) combined with Glucose Test Strips (ONETOUCH Ultra, 4251884). For the GTT, mice were fasted overnight (16 h) and administered an intraperitoneal injection of glucose (Sigma-Aldrich, G8270) at 1 g/kg body weight. Glycemia was measured in tail vein blood samples. Insulin and glucose were diluted in 0.9% sodium chloride injection buffer (Baxter, A6E1323).

Western blot analysis

The cells were washed with phosphate-buffered saline (Invitrogen, AM9624) and harvested, and cell extracts were prepared with lysis buffer and centrifuged at 12,000 rpm for 20 min at 4°C. The protein concentration of each sample was determined, and the protein samples were separated by SDS-PAGE on 15% polyacrylamide gels (GENSHARE BIOLOGICAL, JC-PE022) and transferred to a polyvinylidene fluoride membrane (Millipore, IEVH08100). The membrane was blocked with 5% BSA for 1 h at room temperature and incubated with primary antibodies (dilution ratio 1:1000) of BMP9 (Bio-Techne, MAB3209-100), PPARα (Abcam, ab24509), PPARβ/δ (Abcam, ab23673), PPARγ (Cell Signaling Technology, C26H12) , FGF21(Abcam, ab171941), Akt (Cell Signaling Technology, 2920S) , and Insulin Signaling Pathway Antibody Sampler Kit

（Cell Signaling Technology, 42022）, overnight at 4°C, and followed by a secondary antibody conjugated to a fluorescent tag (Cell Signaling Technology, 5151P; dilution ratio 1:5000). β-actin (Proteintech, 66009-1-lg; dilution ratio 1:2000) was used as the control protein. Antibodies were diluted in Universal Antibody Diluent (NCM Biotech, WB100D). The band signals were visualized and quantified using the Odyssey Infrared Imagining System (LI-COR, ODYSSEY CLx).

RNA extraction and real-time PCR

Cellular and total tissue RNA were extracted using TRIzol reagent (Invitrogen, 15596026) according to the manufacturer's instructions. cDNA was synthesized using a PrimeScript^{TM} RT Reagent kit (Takara, RR037A) and PowerUP^{TM} SYBR Green Master Mix (Thermo Fisher Scientific, 1708040) with the following cycling conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Real-time PCR was performed using the QuantStudio^{TM} 6 Flex Real-Time PCR System (Applied Biosystems, 4484642). 18s mRNA was used as the control. Relative changes in mRNA expression were calculated using the comparative cycle method ($2^{-\Delta\Delta Ct}$). Real-time PCR Primers (Table S2) were obtained from the online primer database PrimerBank (https://pga.mgh.harvard.edu/primerbank/).

BMP9 shRNA cell line construction

For BMP9 knockdown, shRNA sequences (BMP9-sh1: GGAGAACATGAAGGTGGATTT and BMP9-sh2: GGAAGGAAGCATGGTCGTTTA) targeting BMP9 were obtained by PCR using Bam HI-EcoR I sites and then cloned into Bam HI-EcoR I sites in the pLenR-GPH vector (Biolink, Shanghai). For lentiviral packaging, 1×10^7 293T cells (Cobioer, CBP60439) were cultured in DMEM supplemented with 10% FBS, maintained at 37°C, and transfected with 6 μg of pLenR-GPH (Biolink, Shanghai) empty vector, pLenR-GPH-BMP9-sh1, pLenR-GPH-BMP9-sh2, 6 μg of pRsv-REV (Biolink, Shanghai), 3 μg of pMDlg-pRRE (Biolink, Shanghai) and 3 μg of pMD2G (Biolink, Shanghai) using Lipofectamine 2000 reagent (Invitrogen, 11668019). Twenty-four hours after transfection, the medium was replaced with 10 ml of fresh medium. After 48 h and 72 h, the viral supernatants were collected and filtered through a 0.45 μm cellulose acetate filter (Sartorius, Germany). Viral supernatants were concentrated with Centrifugal Filter Units (Millipore, USA) and spun at 3040 × g for 20 min. Hepa 1-6 cells were transfected with lentivirus for 48 h and then screened with medium supplemented with 4 μg/ml puromycin (Sigma-Aldrich, P8833) for 2 weeks.

Gene microarray analysis

Three independent 16-week-old littermate WT and BMP9-KO mouse livers were collected and frozen in liquid nitrogen for microarray analysis. The Agilent SurePrint G3 Mouse GE V2.0 Microarray (8*60K, Design ID: 074809) was used in this experiment. Total RNA was quantified by a NanoDrop ND-2000 (Thermo Fisher Scientific), and the RNA integrity was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies). Sample labeling, microarray hybridization and washing were performed based on the manufacturer's standard protocols. Briefly, total RNA was transcribed to double-stranded cDNA, then synthesized into cRNA and labeled with Cyanine-3-CTP. The labeled cRNAs were hybridized onto the microarray. After being washed, the arrays were scanned by an Agilent Scanner G2505C (Agilent Technologies). Feature Extraction Software (version 10.7.1.1, Agilent Technologies) was used to analyze array images to obtain raw data. Genespring (version 13.1, Agilent Technologies) was used to finish the basic analysis of the raw data. First, the raw data were normalized with the quantile algorithm. The probes with 100% of the values in any 1 out of all conditions flagged as "Detected" were chosen for further data analysis. Differentially expressed genes were then identified through the fold change (FC) and the P value calculated by t-test. The threshold for up- and

downregulated genes was an FC ≥ 2.0 and a P value ≤ 0.05. Subsequently, GO and KEGG analyses were used to determine the roles of the differentially expressed mRNAs. Finally, hierarchical clustering was performed to display the distinguishable gene expression patterns among the samples.

Promoter dual-luciferase reporter assay

First, the PPARα promoter sequence (1800 bp) was inserted into the pGL3 basic vector (Addgene, 64784), which contains firefly luciferase. The pRL-TK vector (Addgene, 11313) containing renilla luciferase was used as a control. Hepa 1-6 cells were seeded at a density of 10,000 cells per well in flat-bottomed 24-well plates. The cells were cultured in DMEM supplemented with 10% FBS, maintained at 37°C and transfected with 2 μg pGL3 basic vector and 0.1 μg pRL-TK vector using Lipofectamine 2000 reagent (Invitrogen, 11668019). 24 h after transfection, the medium was replaced with 200 μl of fresh DMEM. Then, the Hepa 1-6 cells were treated with (0, 2, 10, or 40 ng/ml) recombinant BMP9 protein for 24 h. The relative luciferase activity was determined using a Dual-Luciferase® Reporter Assay System (Promega, E1910) according to the manufacturer's instructions. Signals were measured using a Microplate System (Promega, GloMax E5311). For further PPARα antagonist treatment, GW6471 (MCE, HY-15372) was dissolved in DMSO and kept at 10 mM. Where necessary, Hepa 1-6 cells were treated with 50 μM GW6471.

ChIP analysis

PPARα promoter was divided into six 300 bp regions. Furthermore, we investigated the precise TF binding sites of the PPARα promoter. We utilized an online promoter prediction tool (http://www.fruitfly.org/cgi-bin/seq_tools/promoter.pl) to generate 3 potential PPARα promoter TF binding sites per 50 bp. Promoter predictions for 1 eukaryotic sequence with score cutoff 0.80. ChIP assays were performed using a Chromatin Immunoprecipitation kit (Merck Millipore, 17-371) according to the manufacturer's instructions. Hepa 1-6 cells were treated with 0 or 10 ng/ml recombinant BMP9 protein for 24 h. After treatment, the cells were lysed by ultrasonic processing using an Ultrasonic Cell Crusher (SONICS Uibra Cell, VCX130). Thereafter, a smad 1/5 Antibody Sampler kit (Cell Signaling Technology, 12656) antibody was added to the cell lysate for immunoprecipitation. The immunoprecipitation products were collected for further semi-quantitative PCR followed by 2% agarose gel electrophoresis analysis and qRT-PCR. Primers for the ChIP assay were designed by the online primer tool Primer3Plus (http://www.primer3plus.com/cgi-bin/dev/primer3plus.cgi) (Table S3).

Histology staining

Mouse liver tissue samples were fixed in 5% formaldehyde solution overnight and then embedded in paraffin. The tissue sections were stained with hematoxylin and eosin (H&E), Oil Red O, Masson, Sirius Red for the evaluation of the tissue morphology, according to standardized protocols. Cellular lipid droplets were visualized by an Oil Red O Staining kit (Servicebio, G1015L).

Metabolic cage studies to calculate energy expenditure in mice

Based on their home cages, mice were housed in a monitoring system that measures physical activity, food intake, oxygen and carbon dioxide concentrations. Sample data were collected at 14 min intervals for a 4-day period with light (7 a.m.) and dark (7 p.m.) cycle every 24 hours. Oxygen consumption and carbon dioxide production are exported as ml per kg lean mass per h by using an adjusted mean lean mass.

Nuclear magnetic resonance (NMR) analysis

Mouse fat and lean mass were calculated by time-domain-NMR (TD-NMR) by using a Minispec Live Mouse Analyzer (TESTNIUMAG, MesoMR23-060H-I). The littermate BMP9 WT and BMP9 KO mice were anesthetized and scanned with an NMR

system. After scanning, the proportion of muscle and fat and the distribution of fat in mice were obtained.

ELISA assay

The clinical blood samples were incubated at room temperature for 4 h to separate the serum. Human serum BMP9 levels were detected by using a Human BMP9 ELISA kit (RayBiotech, ELH-BMP9). Similarly, the blood was taken from the eyes of mice, and the mouse serum was isolated by centrifugation. Mouse serum BMP9 levels were detected using a Mouse BMP9 ELISA kit (RayBiotech, ELM-BMP9) and Mouse FGF-21 ELISA Kit (Abcam, ab212160). For all operating procedures, refer to the instructions provided by the manufacturer.

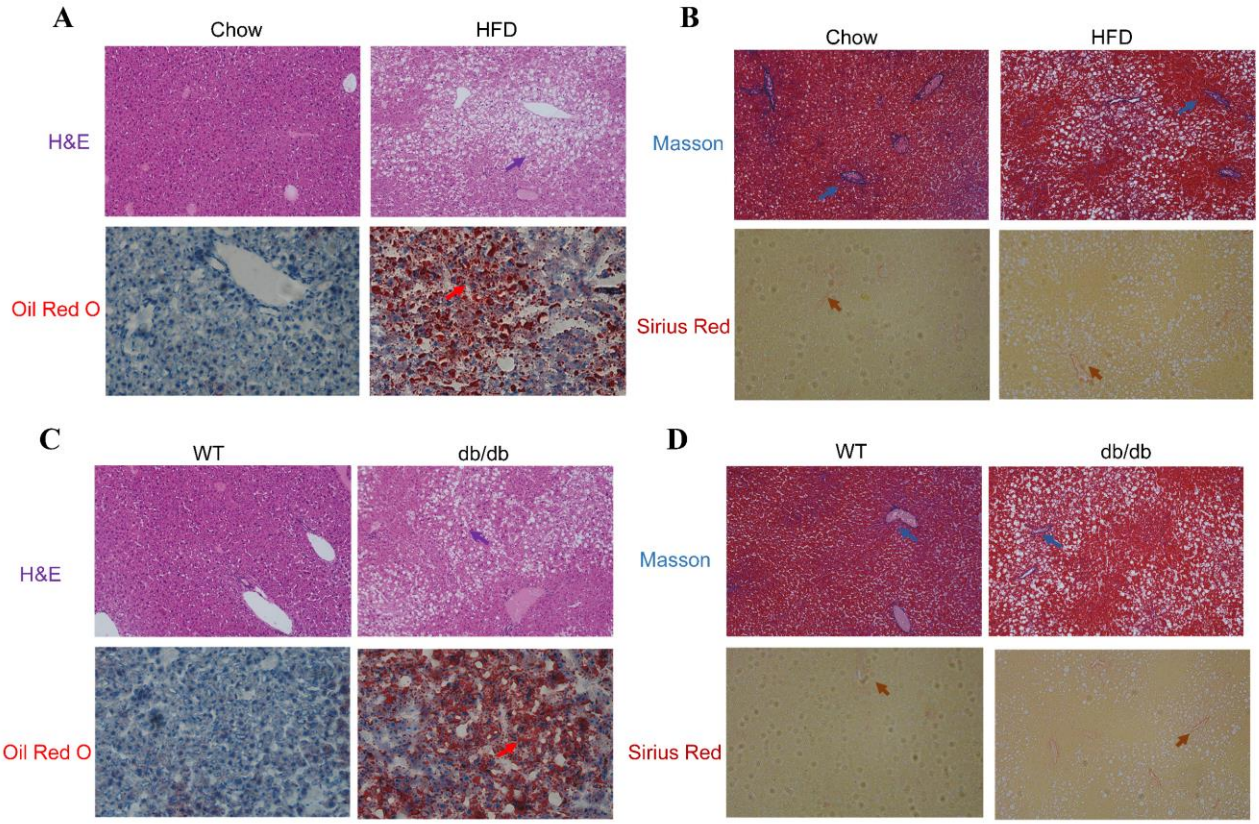
BMP9-overexpressing AAV construction

An AAV8 vector named GPAAV-WPRE (Genomeditech, Shanghai), was used in this study. A mouse BMP9 coding sequence was inserted into this AAV8 vector at Bam HI-EcoR I site. AAV-Vector and AAV-BMP9 vectors were transfected into 293T cells (Cobioer, CBP60439) with Lipofectamine 2000 reagent (Invitrogen, 11668019). The viral supernatants were collected as previously described. Viral genome titers were determined by quantitative PCR.

Administration of the BMP9 overexpression AAV in mice

16-week-old BMP9-KO mice were anesthetized with an intraperitoneal injection of chloral narcotic (Servicebio, WG). Thereafter, the concentrated AAV-Vector and AAV-BMP9 viruses with established titers were diluted in 0.9% sodium chloride injection buffer (Baxter, A6E1323) and injected via the tail vein at 2×10^{12} vg per kg mouse. For short term treatment, the GTT and ITT were performed on these AAV-treated mice after 2 months injection. Whereafter, mice were sacrificed. The blood, livers and other tissues were collected. For long term (4 months) treatment, the changes in mouse body weight were continuously monitored after the injection of AAV.

Fig. S1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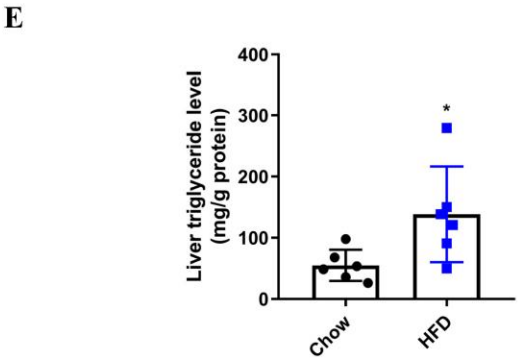
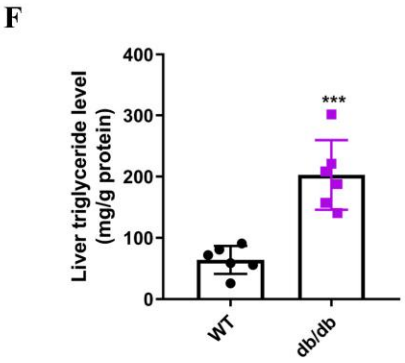
Chow
HFD
H&E
Oil Red O
B
Chow
HFD
Masson
Sirius Red
C
WT
db/db
H&E
Oil Red O
D
WT
db/db
Masson
Sirius Red


E
Liver triglyceride level (mg/g protein)
400
300
200
100
0
*
Chow
HFD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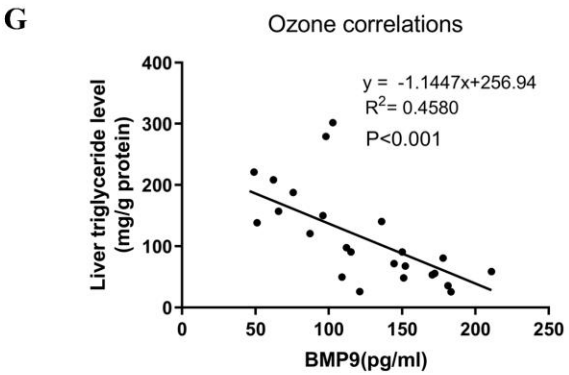
Liver triglyceride level (mg/g protein)
400
300
200
100
0
***
WT
db/db


G
Ozone correlations
y = -1.1447x+256.94
R2= 0.4580
P<0.001
Liver triglyceride level (mg/g protein)
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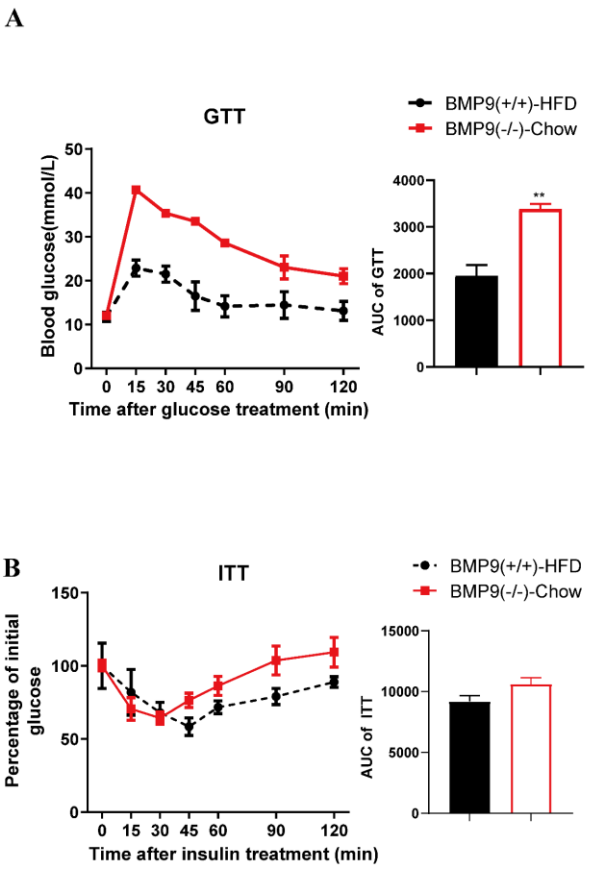
300
200
100
0
0
50
100
150
200
250
BMP9(pg/ml)

Fig. S2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GTT
BMP9(+/+)-HFD
BMP9(-/-)-Chow
Blood glucose(mmol/L)
0
10
20
30
40
50
0 15 30 45 60 90 120
Time after glucose treatment (min)
AUC of GTT
0
1000
2000
3000
4000
**
B
ITT
BMP9(+/+)-HFD
BMP9(-/-)-Chow
Percentage of initial glucose
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50
100
150
0 15 30 45 60 90 120
Time after insulin treatment (min)
AUC of ITT
0
5000
10000
15000

Fig. S3

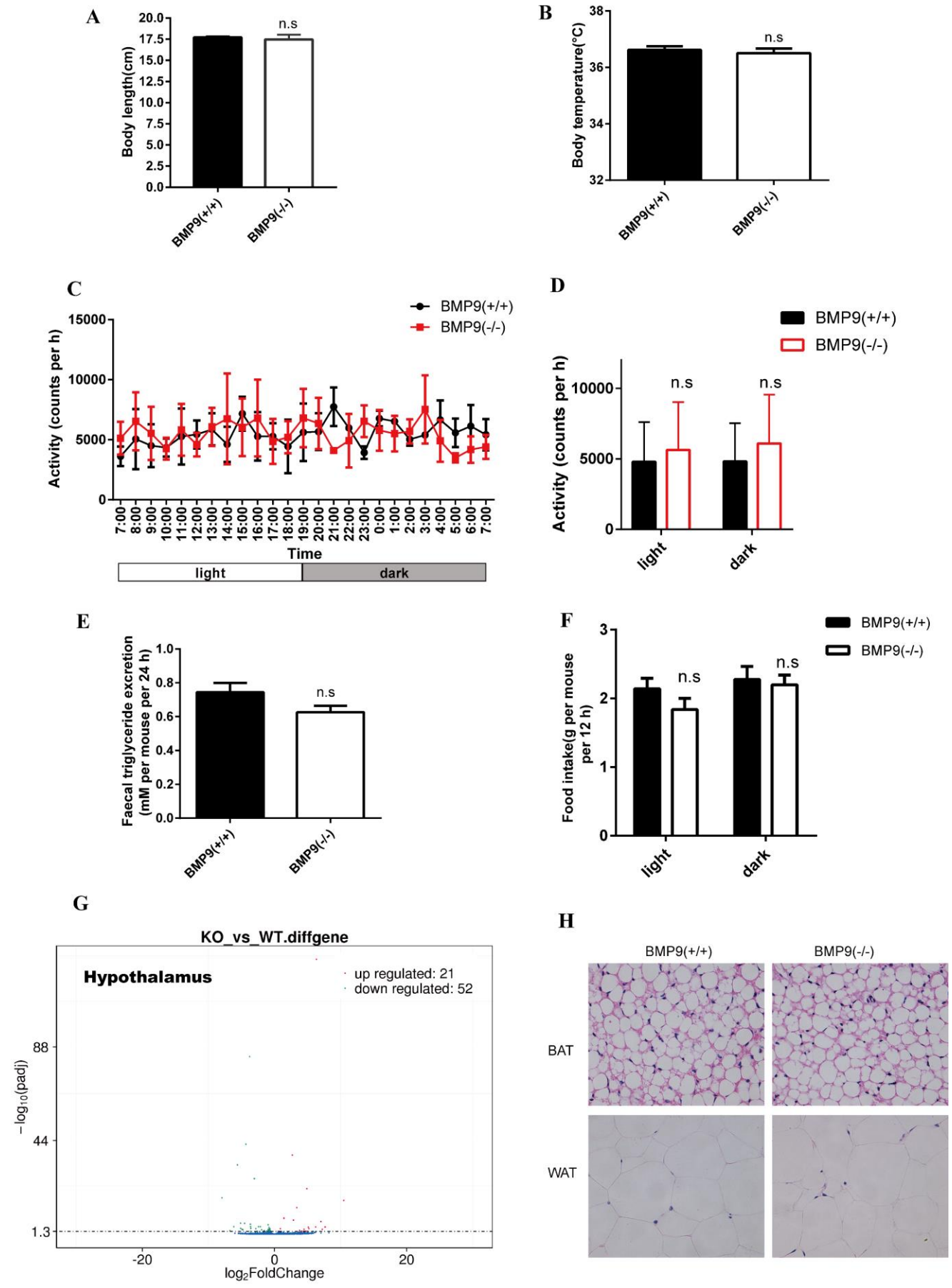
A
Body length(cm)
n.s
BMP9(+/+)
BMP9(-/-)
B
Body temperature(°C)
n.s
C
Activity (counts per h)
BMP9(+/+)
BMP9(-/-)
Time
light
dark
D
Activity (counts per h)
n.s
light
dark
E
Faecal triglyceride excretion (mM per mouse per 24 h)
n.s
F
Food intake(g per mouse per 12 h)
n.s
G
KO_vs_WT.diffgene
Hypothalamus
up regulated: 21
down regulated: 52
log2FoldChange
H
BMP9(+/+)
BMP9(-/-)
BAT
WAT

Fig. S4

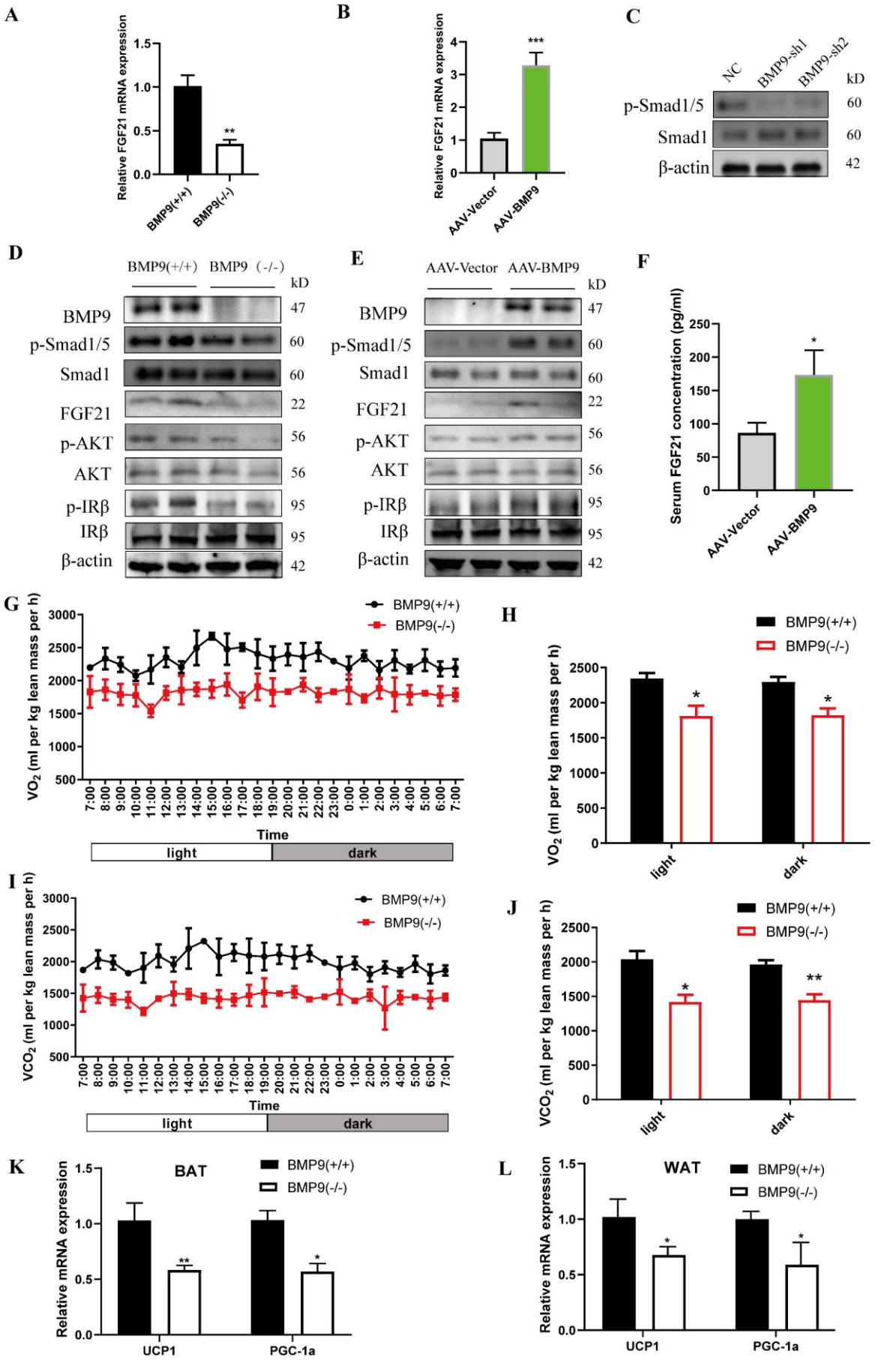
A
Relative FGF21 mRNA expression
BMP9(+/+)
BMP9(-/-)
B
Relative FGF21 mRNA expression
AAV-Vector
AAV-BMP9
C
NC
BMP9-sh1
BMP9-sh2
kD
p-Smad1/5
Smad1
β-actin
D
BMP9(+/+)
BMP9 (-/-)
BMP9
p-Smad1/5
Smad1
FGF21
p-AKT
AKT
p-IRβ
IRβ
β-actin
E
AAV-Vector
AAV-BMP9
F
Serum FGF21 concentration (pg/ml)
G
VO2 (ml per kg lean mass per h)
Time
light
dark
H
I
VCO2 (ml per kg lean mass per h)
J
K
BAT
Relative mRNA expression
UCP1
PGC-1a
L
WAT

Figure legends

Fig. S1. Liver steatosis and fibrosis assessment of HFD and db/db mice. (A) H&E and Oil Red O staining (Arrow heads indicate steatosis feature ballooning and lipid droplet), **(B)** Masson and Sirius Red staining (Arrow heads indicate fibrosis) of HFD mice liver. **(C)** H&E and Oil Red O staining (Arrow heads indicate steatosis feature ballooning and lipid droplet), **(D)** Masson and Sirius Red staining (Arrow heads indicate fibrosis) of db/db mice liver (*n* =6). Original magnification, ×200. **(E)** Fat content of HFD mice measured by liver TG. (n=6). **(F)** Liver TG of db/db mice and control (n=6). **(G)** Pearson's R and P values for serum BMP9 levels versus liver TG levels in NAFLD mice model and control (n=12). (*p <0.05, ** p <0.01, *** p <0.001).

Fig. S2. Comparison of normalized GTT and ITT. (A) GTT, **(B)** ITT comparison between CD-fed 16-week-old BMP9-KO mice and HFD-fed 16-week-old BMP9-WT mice (*n* =6). (*p <0.05, ** p <0.01, *** p <0.001).

Fig. S3. Results to exclude the impact of BMP9 on mouse development and peripheral and central activity. (A) Body length of 16-week-old BMP9 WT and KO mice (n=5~7). **(B)** Body temperature. **(C)** Physical activity monitoring. **(D)** Quantification of physical activity monitoring. **(E)** Fecal triglyceride excretion. **(F)** Food intake **(G)** Volcano plots showing log2-FCs in the expression of BMP9 in WT and KO mouse hypothalami. Blue dots represent genes with downregulated expression. Red dots represent 2genes with upregulated expression (*n* =3). **(H)** BAT and WAT H&E staining; original magnification, ×400. (*p <0.05, ** p <0.01, *** p <0.001).

Fig. S4. Potential role of BMP9-PPARα -FGF21 axis in energy expenditure and insulin signaling pathway. (A) mRNA expression of *FGF21* in BMP9-KO mouse livers (n=3). **(B)** mRNA expression of *FGF21* in BMP9-KO mouse livers (n=3). **(C)** Phosphorylated smad1/5 and total smad1 were determined in Hepa 1-6 cells with *BMP9* knockdown or control. **(D)** Liver of BMP9-KO mice and **(E)** AAV-BMP9 administration mice were detected BMP9, phosphorylated smad1/5, total smad 1, FGF21, phosphorylated Akt, total Akt, phosphorylated IRβ, total IRβ and β-actin by immunoblot analysis (n=3). **(F)** Serum FGF21 protein concentrations after AAV-BMP9 treatment (n=6). **(G)** O_2 consumption (24h period) and **(H)** Quantification (average of light and dark period) in 16-week-old BMP9-KO mice (n=3). **(I)** CO_2 production (24h period) and **(J)** Quantification (average of light and dark period) in 16-week-old BMP9-KO mice (n=3). **(K)** BAT and **(L)** WAT molecular markers UCP1 and PGC-1a were detected in BMP9-KO mice (n=3). (*p <0.05, ** p <0.01, *** p <0.001).

Table S1. Clinical characteristics of subjects with control and NAFLD

Variables	Health control (n=32)	NAFLD (n=32)	p value
Male/Female	18/14	15/17	-
Age (yr)	58.50±2.85	57.72±1.64	-
BMI (kg/m^2)	23.96±0.40	27.03±0.36	<0.001
Fasting plasma glucose (mmol/L)	5.147±0.09	8.853±0.51	<0.001
Triglyceride (mmol/L)	1.41±0.05	2.04±0.06	<0.001
Hepatic triglyceride content (%)	4.36±0.18	10.40±0.54	<0.001
BMP9 (pg/ml)	190.7±19.78	79.43±11.66	<0.001

Data are means ± SEM.

Table S2. Primers for Real time PCR

Gene	Forward primer	Reverse primer
18S	CGGCGACGACCCATTCGAAC	GAATCGAACCCTGATTCCCCGTC
BMP9	CAGAACTGGGAACAAGCATCC	GCCGCTGAGGTTTAGGCTG
PPARα	AGAGCCCCATCTGTCCTCTC	ACTGGTAGTCTGCAAAACCAAA
PPARβ	TCCATCGTCAACAAAGACGGG	ACTTGGGCTCAATGATGTCAC
PPARγ	TCGCTGATGCACTGCCTATG	GAGAGGTCCACAGAGCTGATT
CTP1	CCACAAGAACCTAAATCCTGTGT	AAAGGCTGAGCCCATAAAGTC
CTP2	GTCTGGGGATCATTAAAACAGCG	CACCCTTAAATCCAAAGGTTTGC
ACOX1	TAACTTCCTCACTCGAAGCCA	AGTTCCATGACCCATCTCTGTC
MCAD	AGGGTTTAGTTTTGAGTTGACGG	CCCCGCTTTTGTCATATTCCG
CYP4a12b	GGGGAGATCAGACCCAAAAGC	ATTCGTCGGTGCTGAAACCAT
SCPX	CCTTCTGTCGCTTTGAAATCTCC	GCTTCCTTTGCCATATCAGGAT
EHHADH	ATGGCTGAGTATCTGAGGCTG	GGTCCAAACTAGCTTTCTGGAG
CD36	ATGGGCTGTGATCGGAACTG	GTCTTCCCAATAAGCATGTCTCC
ACBP	GAATTTGACAAAGCCGCTGAG	CCCACAGTAGCTTGTTTGAAGTG
FABP1	ATGAACTTCTCCGGCAAGTACC	CTGACACCCCCTTGATGTCC
FABP3	ACCTGGAAGCTAGTGGACAG	TGATGGTAGTAGGCTTGGTCAT
LPL	GGGAGTTTGGCTCCAGAGTTT	TGTGTCTTCAGGGGTCCTTAG
ACSL5	TCCTGACGTTTGGAACGGC	CTCCCTCAATCCCCACAGAC
FATP1	CGCTTTCTGCGTATCGTCTG	GATGCACGGGATCGTGTCT
OLR1	CAAGATGAAGCCTGCGAATGA	ACCTGGCGTAATTGTGTCCAC
ME1	GTCGTGCATCTCTCACAGAAG	TGAGGGCAGTTGGTTTTATCTTT
SCD1	TTCTTGCGATACACTCTGGTGC	CGGGATTGAATGTTCTTGTCGT
FADS2	TCATCGGACACTATTCGGGAG	GGGCCAGCTCACCAATCAG
FGF21	CTGCTGGGGGTCTACCAAG	CTGCGCCTACCACTGTTCC
UCP1	GTGAACCCGACAACTTCCGAA	TGCCAGGCAAGCTGAAACTC
PGC-1a	GGACATGTGCAGCCAAGACTCT	CACTTCAATCCACCCAGAAAGCT

Table S3. Primers for PPARα promoter ChIP assay

Region	Forward primer	Reverse primer
Region 1	AGAGTCTGGGTGGAAGGAAGT	AGTTCCAAAGATCAGAGTCCACA
Region 2	AGGACCTACAACCAAGCACC	GCACACAAGCACACACACAC
Region 3	TGGTTCTTTCCTTCCACCGT	CCAGACCCTACAGGCACATT
Region 4	TCTCCAGTTCCCAGGGTAAGG	GCTCTGCCAACTGACAGCG
Region 5	CCTGAGCTGGACACAGTCAG	GCTTGTTCCCCGAGTCCC
Region 6	GTGCGATCTAGACCAGCTCA	CAGTGTCTGATTGGCTGCTG
Region 7	TGTTATATACAGGCCAGGGTAGGA	AGACAACATTAGTCCATTTTCCTACC
Region 8	AGGTGGGTTTAAAAGACGTCCC	ACCCTACAGGCACATTCAGG
Region 9	CCCCGAGGCACTAAATGGG	GGGGCAGCTCTCCTCGAT