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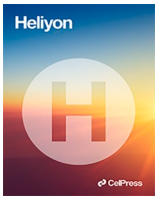

Research article

# Overexpression of METTL14 mediates steatohepatitis and insulin resistance in mice

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ABSTRACT

*Background:* Lipid accumulation and redox imbalance, resulting from dysregulation of hepatic fatty acids oxidation, contribute to the development of steatohepatitis and insulin resistance. Recently, dysregulated RNA $N^6$-methyladenosine ($m^6A$) methylation modification has been found involving fatty liver. However, the role of methyltransferase-like 14 (METTL14), the core component of $m^6A$ methylation, in the development of steatohepatitis is unknown. Herein, we aimed to explore the role of METTL14 on steatohepatitis and insulin resistance in mice with metabolic dysfunction-associated steatotic liver disease (MASLD).

*Methods:* The liver tissues of mice and patients with MASLD were collected to detect the expression of METTL14. METTL14 overexpression and METTL14 silence were used to investigate the effect of METTL14 on lipid metabolism disorder *in vivo* and *in vitro*. Knockout of METTL14 in primary hepatocytes was used to investigate the role of Sirtuin 1 (SIRT1) on lipid accumulation induced by METTL14.

*Results:* METTL14 was dramatically up-regulated in the livers of db/db mice, high-fat diet (HFD)-fed mice, and patients with MASLD. METTL14 overexpression exacerbated MASLD and promoted lipid metabolism disorder and insulin resistance in mice. Conversely, METTL14 knockout ameliorated lipid deposition and insulin resistance in HFD-fed mice. Furthermore, METTL14 overexpression facilitated lipid accumulation, while METTL14 knockout reduced lipid accumulation in HepG2 cells and primary hepatocytes. In addition, METTL14 lost up-regulated SIRT1 expression in hepatocytes. SIRT1 deficiency abrogated the ameliorating effects of METTL14 downregulation in MASLD mice.

*Conclusions:* These findings suggest that dysfunction of the METTL14-SIRT1 pathway might promote hepatic steatosis and insulin resistance.

## 1. Introduction

The obesity rate, characterized by excessive accumulation and abnormal distribution of lipids, continues to rise [1,2].

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Accumulating evidence reveals that obesity contributes to many pathological conditions, such as metabolic dysfunction-associated steatotic liver disease (MASLD), type 2 diabetes, and cardiovascular diseases [3,4]. Because of the high-fat diet (HFD) in modern life, more than 15 % of individuals display early manifestations of hepatic steatosis [5,6]. The liver is the critical organ responsible for lipid metabolism. Dysfunction in the liver is a nonnegligible pathogenesis in the development of steatosis [7]. Understanding the complex molecular mechanisms underlying the development of hepatic steatosis may provide a new strategy for the therapy of hepatic steatosis.

Mitochondrial fatty acid β-oxidation (FAO) is chronically disrupted in hepatic steatosis onset and progression [8,9]. Increased glucose metabolism raises the level of malonyl-CoA, which inhibits carnitine palmitoyltransferase 1 (CPT1) and blocks FAO, resulting in excessive lipids accumulation [10–12]. It has been reported that promoting FAO could reduce lipid accumulation and suppress the development of obesity-associated metabolic disorders [11–13]. However, the underlying causative mechanisms of hepatic FAO disorder remain incompletely understood.

Recently, $N^6$-methyladenosine ($m^6A$) methylation has attracted a lot of attention as the most abundant and conserved internal RNA modification on mRNA in eukaryotes [14]. RNA $m^6A$ methylation is precisely regulated by $m^6A$ methyltransferase complex and demethylases [15–17]. Dysregulated RNA $m^6A$ modification is involved in various diseases, including MALFD [18–20]. The methyltransferase-like (METTL3) and METTL14 are well-documented methyltransferase complexes. Notably, it has been reported that METTL14 is closely associated with liver diseases, such as liver fibrosis and liver cancer [21,22]. However, the role of METTL14 in hepatic steatosis remains unknown. Therefore, this study aimed to investigate the role and underlying mechanisms of METTL14 in steatohepatitis and insulin resistance.

## 2. Methods

### 2.1. Human sample studies

Human sample studies were carried out in accordance with the tenets of the Declaration of Helsinki. Hepatic steatotic samples were obtained from subjects with fatty livers who had undergone a biopsy ($n = 5$). Control liver samples were acquired from patients undergoing hepatic resection for intrahepatic bile duct stones ($n = 5$). All patients provided informed consent.

**Table 1**
Sequences of specific primers used in this study.

| Gene | Species | Forward primer (5′–3′) | Reverse primer (5′–3′) |
|---|---|---|---|
| *Mettl14* | mouse | TTCTGGGGAAGGATTGGACC | ACGGTTCCTTTGATCCCCAT |
| *Mgll* | mouse | GTGGCCGTTATGATGAGCTG | ACCATCCTCTCTCCCTCACT |
| *Hsl* | mouse | AGTTCCCTCTTTACCGGTGG | ACGACAGCACCTCAATCTCA |
| *Atgl* | mouse | CAACGCCACTCACATCTACG | ACCAGGTTGAAGGAGGGATG |
| *Srebp1c* | mouse | GTTACTCGAGCCTGCCTTCAGG | CAAGCTTTGGACCTGGGTGTG |
| *Fas* | mouse | GCTGCGGAAACTTCAGGAAAT | AGAGACGTGTCACTCCTGGACTT |
| *Acc* | mouse | GGACAGACTGATCGCAGAGAAAG | TGGAGAGCCCCACACACA |
| *Pparα* | mouse | ATCCAGGGTTCAGTCCAGTG | GCTTAGGGACAGTGACAGGT |
| *Acadvl* | mouse | GGATCCGAGAAAACATGGCC | GGCATCAGAGAAGGCACATG |
| *Acadl* | mouse | CTTGGGAAGAGCAAGCGTAC | TAAGTCACTTCCAGCCCCAG |
| *Cpt2* | mouse | CGCCCAGCTTCCATCTTTAC | AACAAGTGTCGGTCAAAGCC |
| *Cpt1a* | mouse | AGTGGCCTCACAGACTCCAG | GCCCATGTTGTACAGCTTCC |
| *Acox1* | mouse | CCTGATTCAGCAAGGTAGGG | TCGCAGACCCTGAAGAAATC |
| *Lcad* | mouse | GCATCAACATCGCAGAGAAA | TCGCAATATAGGGCATGACA |
| *Hmgcs2* | mouse | ATACCACCAACGCCTGTTATG | CAATGTCACCACAGACCACCA |
| *Cyp4a10* | mouse | AAGGGTCAAACACCTCTGGA | GATGGACGCTCTTTACCCAA |
| *Cox7a1* | mouse | GTCTCCCAGGCTCTGGTCCG | CTGTACAGGACGTTGTCCATTC |
| *Etfdh* | mouse | GTCTTGATCCAGCTGCCTTC | ACCTGGAAGAATTGGCACAG |
| *Mcad* | mouse | CCAGAGAGGAGATTATCCCCG | TACACCCATACGCCAACTCTT |
| *Pgc1α* | mouse | TCCTCCTCATAAAGCCAACC | GCCTTGGGTACCAGAACACT |
| *Chrebp* | mouse | CCTCACTTCACTGTGCCTCA | ACAGGGGTTGTTGTCTCTGG |
| *Pparγ* | mouse | GCTGTTATGGGTGAAACTCT | TGGCATCTCTGTGTCAACCA |
| *Sirt1* | mouse | GGATGATATGACGCTGTGGC | ACAGGAGACAGAAACCCCAG |
| *β-actin* | mouse | TTCCAGCCTTCCTTCTTG | GGAGCCAGAGCAGTAATC |
| *METTL14* | human | TTTCCCACTGACCTTCCTCC | ACCTGCCCTCTTCTTTCTCC |
| *LCAD* | human | AAGCGAAACGTTTGGACTCC | CCCACATGTATCCCCAACCT |
| *ETFDH* | human | GCTCTTGGTCTTGTGGTTGG | AGCCACCTTCATTGAGAGCT |
| *ACOX1* | human | GGGACCCATAAGCCTTTGCC | CTTGTTACTACGCGGTTTCACG |
| *CPT1A* | human | ATCAATCGGACTCTGGAAACGG | ATCTTGGTGGCACGACTCATCT |
| *HMGCS2* | human | TCTGTCCCCTTGCAATTCCA | TAGAACAGGGAAGTGGGCTG |
| *COX7A1* | human | GTACCGAGTGACAATGACGC | GGCCAGCGTTTATTGACACT |
| *PGC1A* | human | CACCAGCCAACACTCAGCTAAG | AGGGTCATCGTTTGTGGTCAG |
| *MCAD* | human | TGTGGAAGCAGATACCCCAG | ACCAGCTCCGTCACCAATTA |
| *GAPDH* | human | CCAAGGAGTAAGACCCCTGG | AGGGGAGATTCAGTGTGGTG |

## 3. Animals

Male db/db mice and wild-type C57BL/6J mice (6–8 weeks old) were purchased from Hunan SJA Laboratory Animal Co., LTD (Hunan, China). To establish the MASLD model, we fed C57BL/6 mice with an HFD (Rodent Diet with 60 kcal% fat) ad libitum for 16 weeks. For the METTL14 overexpression model, C57BL/6J mice received an injection of adeno-associated virus expressing METTL14 (AAV8-METTL14, $8.45 \times 10^{12}$ gc/mice) *via* the tail vein. After four weeks of injection, experiments were conducted. For the METTL14 silenced model, mice were administered with 100 μL of lentiviral carrying short-hairpin (sh) RNA for Mettl4 (shMettl4, $1 \times 10^9$ TU/mL) or control shRNA ($1 \times 10^9$ TU/mL) through tail veins. In the study, mice were fed an HFD diet for 16 weeks, and the lentivirus shMettl4 was injected 8 weeks before the sacrifice. The body weight of the mice was recorded weekly during all experimental phases.

### 3.1. Histology

Liver tissue was fixed in 10 % neutral-buffered formalin, embedded in paraffin, and cut into 4-μm sections. H&E staining was performed according to the standard protocols.

### 3.2. Real-time PCR

Total RNA was isolated from liver tissue or cells using Trizol reagent (Invitrogen, Carlsbad, USA) and reverse transcribed by RevertAid reverse transcription (Thermo Fisher Scientific, USA). For PCR amplifications, the LightCycler 480 SYBR Green I Master kit (Roche Diagnostics, Rotkreuz, Switzerland) was used and measured with the Roche LightCycler 480 Real-Time PCR System. We used the expression of β-actin as the reference. The primers for the targeted genes are detailed in Table 1.

### 3.3. Western blot

Protein from cells and liver tissue was extracted with RIPA buffer containing protease inhibitor cocktail (Roche). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membranes were incubated with primary antibodies at 4 °C overnight after the blockade with 5 % bovine serum albumin. Primary antibodies used in this study were listed as follows: anti-METTL14 (HPA038002, Sigma-Aldrich), anti-PPARα (Servicebio, Wuhan, China), anti-CPT1a (Servicebio, Wuhan, China), anti-SIRT1 (ab110304, Abcam, USA), and anti-β-actin (3700, Cell Signaling Technology, USA). Blots were visualized using a chemiluminescence kit (Millipore, Billerica, MA, USA). The densities of bands were quantified using the Quantity One analysis software (Bio-Rad, Hercules, CA, USA).

### 3.4. Glucose tolerance test (GTT)

Mice were fasted for 12 h and intraperitoneally injected with glucose (2 g/kg). Then, blood glucose concentrations were measured using a Glucometer Elite (Bayer, Germany) 15, 30, 60, and 120 min after glucose injection.

### 3.5. Insulin tolerance test (ITT)

Insulin (0.75 unit/kg, Sigma-Aldrich, USA) was injected intraperitoneally. Blood samples were collected *via* tail vein to measure glucose concentrations using a Glucometer Elite (Bayer) at 0, 15, 30, 60, and 120 min.

### 3.6. Serum biochemical analysis

Plasma triacylglyceride (TAG), β-hydroxybutyrate, total cholesterol (TCHO), low-density lipoprotein cholesterol (LDL CHOL), high-density lipoprotein cholesterol (HDL CHOL), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were determined enzymatically using a COBAS 6000 autoanalyzer (Roche Diagnostics).

### 3.7. Liver TAG content analysis

The liver TAG content analysis was conducted according to a report [23]. Briefly, liver tissue was homogenized in chloroform-methanol for 3 min and then extracted by shaking at room temperature for 3 h. After centrifuging, the organic phase was collected. The organic solvent was evaporated to dryness under a vacuum at room temperature. The content of TAG in each sample was then quantified in duplicate by spectrophotometry (505 nm).

### 3.8. Cell culture

Primary hepatocytes were isolated from the liver tissue of C57BL/6J mice. After anesthesia, liver cells were isolated by injection of 0.5 mg/mL type collagenase (Sigma-Aldrich) through the inferior vena cava in mice. Primary hepatocytes were cultured in RPMI-1640 Medium containing 10 % fetal bovine serum (FBS, Cellsera, NSW, Australia), 100 units/mL penicillin, and 0.1 mg/mL streptomycin. HepG2 cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, MA, USA) supplemented with 10 % FBS and 100 U/

mL penicillin, 100 mg/mL streptomycin. For interference treatment, hepatocytes were transfected with SiMettl14 or SiControl using lipofectamine 3000 (Lipo3000, Invitrogen, Carlsbad, CA, USA). The primer sequence of SiMettl4 was AGCATTGGTGCCGTGTTAAAT.

### 3.9. Oxygen consumption rate (OCR) measurement

The OCR of HepG2 cells and primary hepatocytes were measured. The supernatant of cells was removed and replaced with pre-warmed assay medium, composed of Seahorse XF DMEM medium (Seahorse Bioscience, Agilent Technologies, USA) containing 1 mM sodium pyruvate, 2 mM L-glutamine, and 10 mM glucose. After cultured at 37 °C for 1 h, five basal measurements of OCR were performed. Then, the modulators of respiration oligomycin (Oligo, 1.5 μM), carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP, 1 μM), and rotenone/antimycin (Rot/AA, 0.5 μM) were added sequentially. The basal respiration, ATP-linked respiration, proton leak, maximal respiration, spare capacity, and non-mitochondrial oxygen consumption were calculated according to the manufacturer's user guide (Seahorse Bioscience, Agilent Technologies). The protein content of each well was quantified by a BCA Protein Assay Kit.

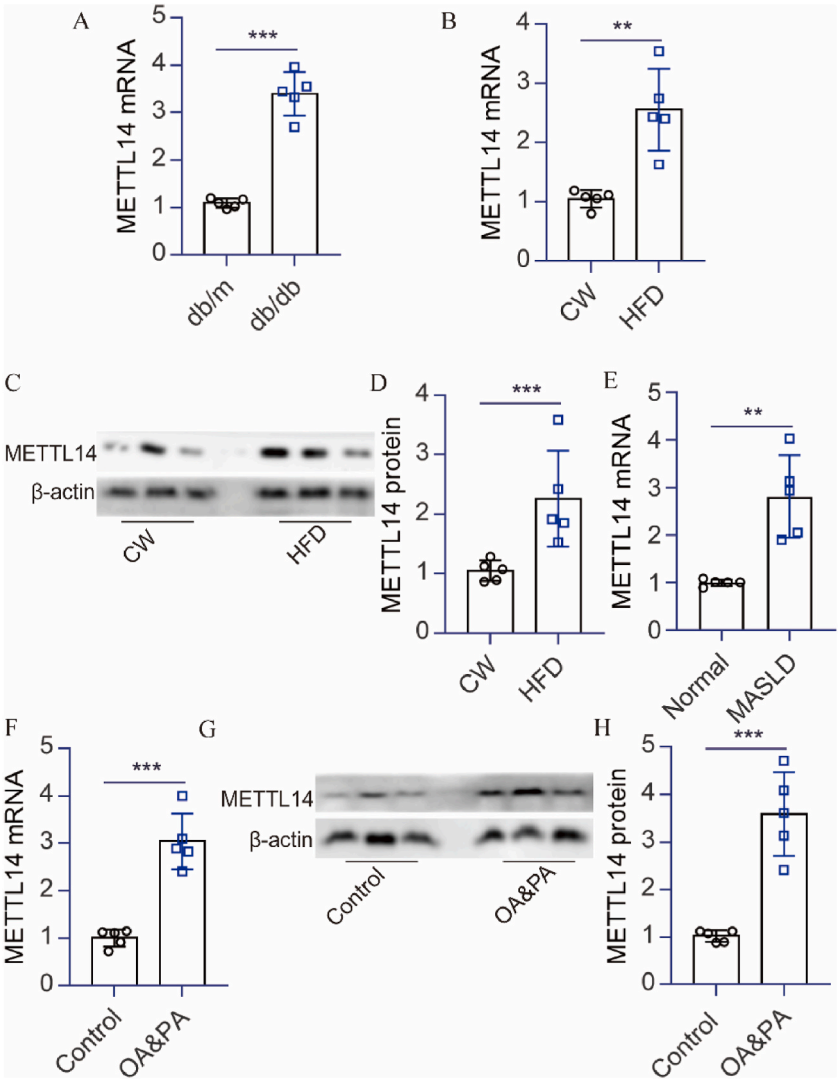


**Fig. 1. Hepatic METTL14 expression is up-regulated in hepatic steatosis.** The hepatic *Mettl14* mRNA expression in db/db (A) and HFD-fed mice (B) was detected by RT-PCR. (C–D) The hepatic METTL14 protein expression in HFD-fed mice was detected by Western blot (n = 5). (E) *METTL14* mRNA expression in patients with MASLD was detected by RT-PCR (n = 5). (F–H) The METTL14 expression in HepG2 cells treated with OA and PA was detected by RT-PCR and Western blot (n = 5). Data are expressed as the mean ± SD. Differences between the two groups were measured using an unpaired Student's t-test. $^{**}P < 0.01$ and $^{***}P < 0.001$. CW: chow diet, HFD: high-fat diet, MASLD: metabolic dysfunction-associated steatotic liver disease, OA: oleic acid, PA: palmitic acid. Uncropped images in C are available in Supplementary Fig. S1.

### 3.10. Statistical analysis

Animals were randomly grouped in this study. Histology was performed and analyzed in a double-blinded way. Data are expressed as the mean ± SEM. Statistical analysis was performed with SPSS19.0. Differences between the two groups were measured using an unpaired Student's *t*-test. Differences were considered significant when *P*-value <0.05.

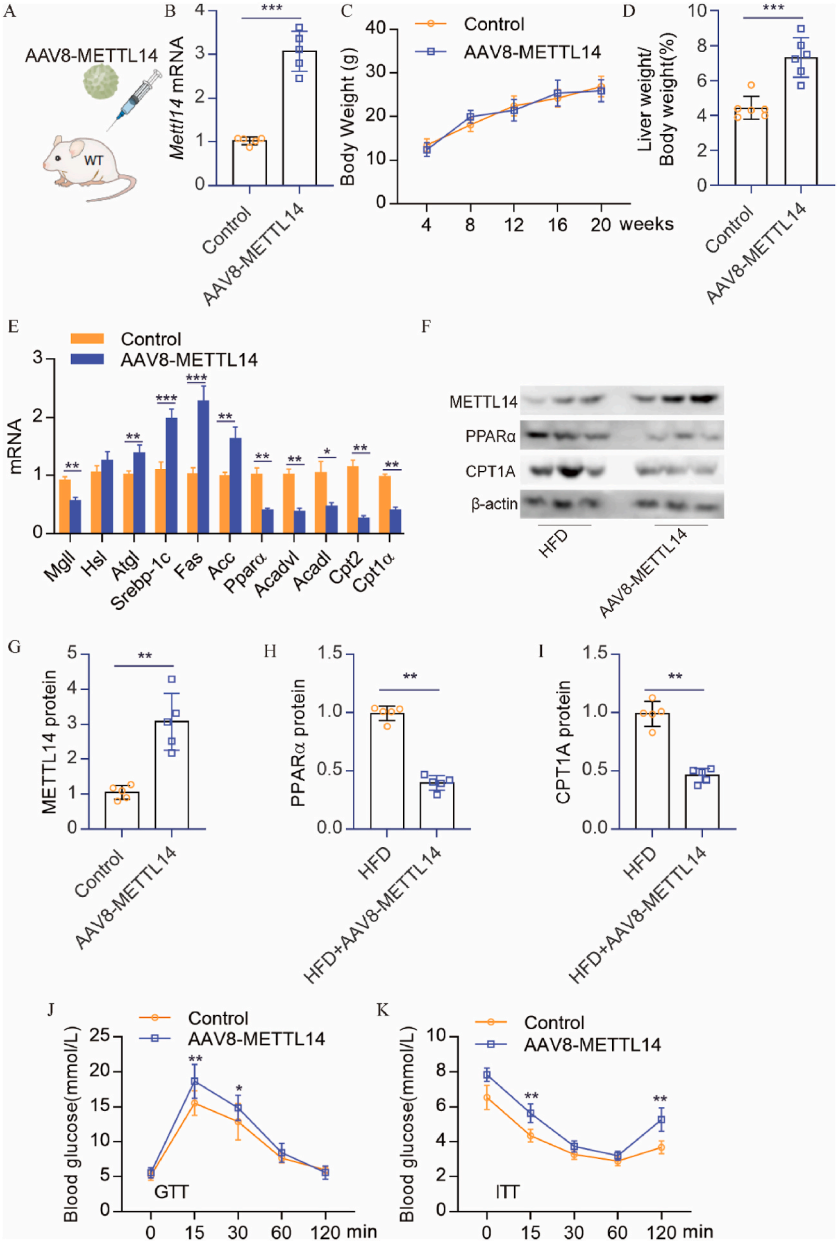


**Fig. 2. METTL14 overexpression impairs lipid catabolism and insulin sensitivity in mice fed with the standard chow diet.** (A) AAV8-METTL14 was injected *via* the tail vein to overexpress the METTL14 in the livers. (B) The *Mettl14* mRNA expression in the livers was detected by RT-PCR (n = 5). (C) The body weight of mice was recorded for 16 weeks (n = 15). (D) The ratio of liver/body weight (n = 5). (E) The expression of genes involved in fatty acid synthesis, lipolysis, and FAO was detected by RT-PCR (n = 5). (F–I) The protein expression of METTL14, PPARα, and CPT1A was detected by Western blot (n = 5). (J–K) GTT and ITT were performed (n = 8). Data are expressed as the mean ± SD. Differences between the two groups were measured using an unpaired Student's *t*-test. $^{**}P < 0.01$ and $^{***}P < 0.001$. HFD: high-fat diet. Uncropped images in F are available in Supplementary Fig. S1.

## 4. Results

### 4.1. Hepatic METTL14 expression is up-regulated in hepatic steatosis

Firstly, we investigated the expression pattern of METTL14 during the development of hepatic steatosis. Interestingly, the levels of METTL14 dramatically increased in db/db and HFD-fed mice (Fig. 1A–D). Consistent with this finding, METTL14 expression levels were higher in patients with MASLD than in controls (Fig. 1E). Besides, the treatment of oleic acid (OA) and palmitic acid (PA) significantly up-regulated the expression of METTL14 in HepG2 cells (Fig. 1F–H). Taken together, these data indicate that METTL14 plays an essential role in hepatic steatosis.

METTL14 overexpression impairs lipid catabolism and insulin sensitivity in mice fed with the standard chow diet.

Subsequently, we investigated the effects of METTL14 on hepatic lipid catabolism and metabolic disorders in normal mice. We set up METTL14-overexpressing mice models through tail vein injections of AAV8-METTL14 (Fig. 2A). We found that *Mettl14* mRNA expression was significantly increased in the liver tissue of mice (Fig. 2B). METTL14 overexpression did not alter the body weight of mice receiving the chow diet, while the ratio value of liver weight/body weight was increased (Fig. 2C and D). Moreover, we found that METTL14 overexpression significantly increased the expression of genes involved in fatty acid synthesis while suppressed the genes

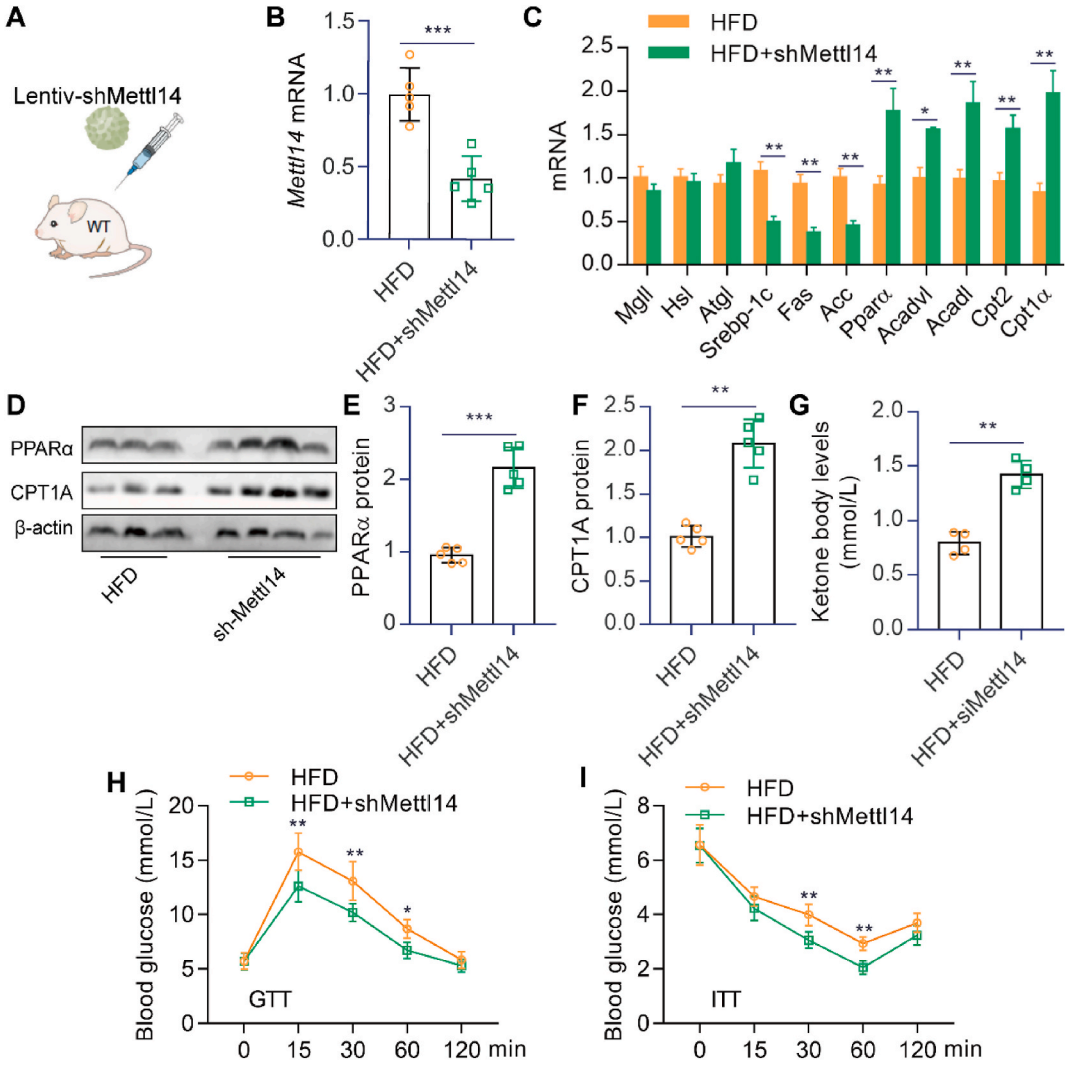


**Fig. 3. METTL14 deficiency alleviates hepatic steatosis in HFD-fed mice.** (A) Mice were injected with a lentivirus encoding for a scrambled shRNA or shRNA against Mettl14 *via* the tail vein. (B) Hepatic *Mettl14* mRNA was detected by RT-PCR (n = 5). (C) The expression of genes involved in fatty acid synthesis, lipolysis, and FAO was detected by RT-PCR (n = 5). (D–F) The protein expression of PPARα and CPT1A was detected by Western blot (n = 5). (G) The circulating ketone bodies in the mice receiving shMettl14 or not were detected (n = 5). (H–I) GTT and ITT were performed (n = 8). Data are expressed as the mean ± SD. Differences between the two groups were measured using an unpaired Student's *t*-test. $^{**}P < 0.01$ and $^{***}P < 0.001$. HFD: high-fat diet. Uncropped images in D are available in Supplementary Fig. S1.

involved in lipolysis and FAO, especially *PPARα* and its downstream gene *CPT1A* (Fig. 2E–I). METTL14 overexpression impaired glucose tolerance and went along with impaired insulin sensitivity (Fig. 2J and K). Together, these results demonstrate that METTL14 overexpression impairs lipid catabolism and insulin sensitivity in mice fed with the standard chow diet.

### 4.2. METTL14 deficiency alleviates hepatic steatosis in mice fed with HFD

Next, we investigated the effects of METTL14 deficiency on hepatic steatosis in HFD-fed mice. Mice were injected with shMettl4 or control shRNA lentivirus *via* the tail vein. Hepatic METTL14 was decreased after the lentiviral injection (Fig. 3A and B). Liver TAG and total CHOL content of HFD mice with METTL14-knockdown were lower than in HFD control mice (Table 2). Hepatic injury was attenuated in METTL14-deficiency mice than in HFD control mice, as indicated by lower serum levels of AST and ALT enzymes (Table 2). Besides, mice in the HFD-shMettl14 group showed higher serum levels of ketone bodies such as β-hydroxybutyric acid (BHB) than those in the HFD group (Table 2). METTL14-knockdown up-regulated the expression of genes involved in fatty acid oxidation, while lipogenic gene expression did not change (Fig. 3C–F). Moreover, METTL14-knockdown mice displayed a higher level of fatty acid oxidation, characterized by elevated levels of circulating ketone bodies (Fig. 3G). METTL14-knockdown also improved glucose tolerance and insulin sensitivity in HFD-fed mice (Fig. 3I and J). Collectively, these data indicate that the METTL14 knockdown reduces the HFD-induced dysregulation of lipid metabolism and glucose metabolism in mice.

### 4.3. METTL14 regulates lipid accumulation in hepatic cells in vitro

Next, we assessed the effects of METTL14 on hepatocellular lipid metabolism *in vitro*. The results showed that METTL14 overexpression significantly increased the lipid accumulation induced by PA in human hepatic cell HepG2 (Fig. 4A) and primary mice hepatocytes (Fig. 4B). Furthermore, METTL14 overexpression significantly reduced the expression of genes involved in FAO in HepG2 and primary mice hepatocytes. At the same time, it did not affect the expression of genes related to fatty acid synthesis (Fig. 4C–F). While the PA-induced lipid accumulation was blunted when METTL14 was silenced by siRNA in HepG2 (Fig. 4 G). Importantly, the expression of genes involved in FAO was augmented when METTL14 was silenced (Fig. 4H and I). Overall, these data indicate that METTL14 might regulate lipid metabolism in hepatocytes.

### 4.4. METTL14 modulates mitochondrial function in hepatocytes

The overexpression of METTL14 in HepG2 cells decreased oxygen consumption rate (Fig. 5A). More specifically, METTL14 overexpression decreased basal respiration, ATP-linked respiration, maximal respiration, spare capacity, and 24 h non-mitochondrial oxygen consumption (Fig. 5B). PPARα and Sirtuin 1 (SIRT1) promote mitochondrial function and fatty acid oxidation [24,25]. We found that PPARα and SIRT1 were decreased after METTL14 overexpression in HepG2 cells (Fig. 5C–F). In contrast, METTL14 knockdown increased the oxygen consumption rate, with significant stimulation of basal respiration, ATP-linked respiration, proton leak, maximal respiration, and spare capacity in HepG2 cells (Fig. 5G and H). METTL14 knockdown also increased protein expression of SIRT1 in HepG2 cells (Fig. 5I and J). In summary, the above results indicate that METTL14 might modulate mitochondrial function in hepatocytes.

### 4.5. Inhibition of SIRT1 blunts the effect of downregulation of METTL14 in hepatic steatosis

We next assessed the functional relevance of SIRT1 as a mediator of the METTL14 effects. For this, mice fed with a standard chow diet were injected with AAV8-METTL14. At week 4, a second lentiviral vector with shRNA-scrambled or shRNA-SIRT1 was injected (Fig. 6A). The expression of *Mettl14* mRNA was increased in the livers of mice, while the expression of *Sirt1* mRNA was decreased (Fig. 6B and C). Body weight was unchanged (Fig. 6D). AST and ALT were reduced after SIRT1 knockdown (Fig. 6E and F). SIRT1 knockdown caused a significant increase of FAO-related genes in the livers of mice (Fig. 6G). On the other hand, the expression of FAS-related genes in the livers of mice was unchanged (Fig. 6H). Overall, these data indicate that METTL14 might require SIRT1 to modulate lipid metabolism in hepatic steatosis.

**Table 2**
Effects of siMettl14 on lipid metabolism-related indicators in HFD-fed mice.

| | CW | HFD | HFD + siMettl14 |
|---|---|---|---|
| TCHO (mg/dL) | 109.4 ± 8.64 | 197.1 ± 6.13$^{\#}$ | 119.7 ± 8.98* |
| TAG (mg/dL) | 35.4 ± 2.82 | 37.2 ± 1.93 | 34.8 ± 1.68 |
| LDL CHOL (mg/dL) | 30.3 ± 4.97 | 110.4 ± 6.64$^{\#}$ | 85.5 ± 7.68* |
| HDL CHOL (md/dL) | 67.6 ± 3.35 | 73.0 ± 0.96 | 80.0 ± 1.14 |
| BHB (mM) | 1.1 ± 0.08 | 1.4 ± 0.09$^{\#}$ | 1.9 ± 0.11* |
| ALT (U/L) | 15.2 ± 1.98 | 104.7 ± 13.88$^{\#}$ | 67.7 ± 14.25* |
| AST (U/L) | 46.5 ± 6.21 | 108.4 ± 12.94$^{\#}$ | 82.3 ± 10.98* |
| Liver weight (g) | 1.2 ± 0.04 | 2.7 ± 0.12$^{\#}$ | 1.6 ± 0.15* |
| Liver TAG (mg/g liver) | 4.2 ± 0.39 | 44.2 ± 0.86$^{\#}$ | 26.3 ± 0.99* |

Compared to the HFD group, $^{*}P < 0.05$ *vs.* HFD, $^{\#}P < 0.05$ *vs.* CW. CW: chow diet, HFD: high-fat diet.

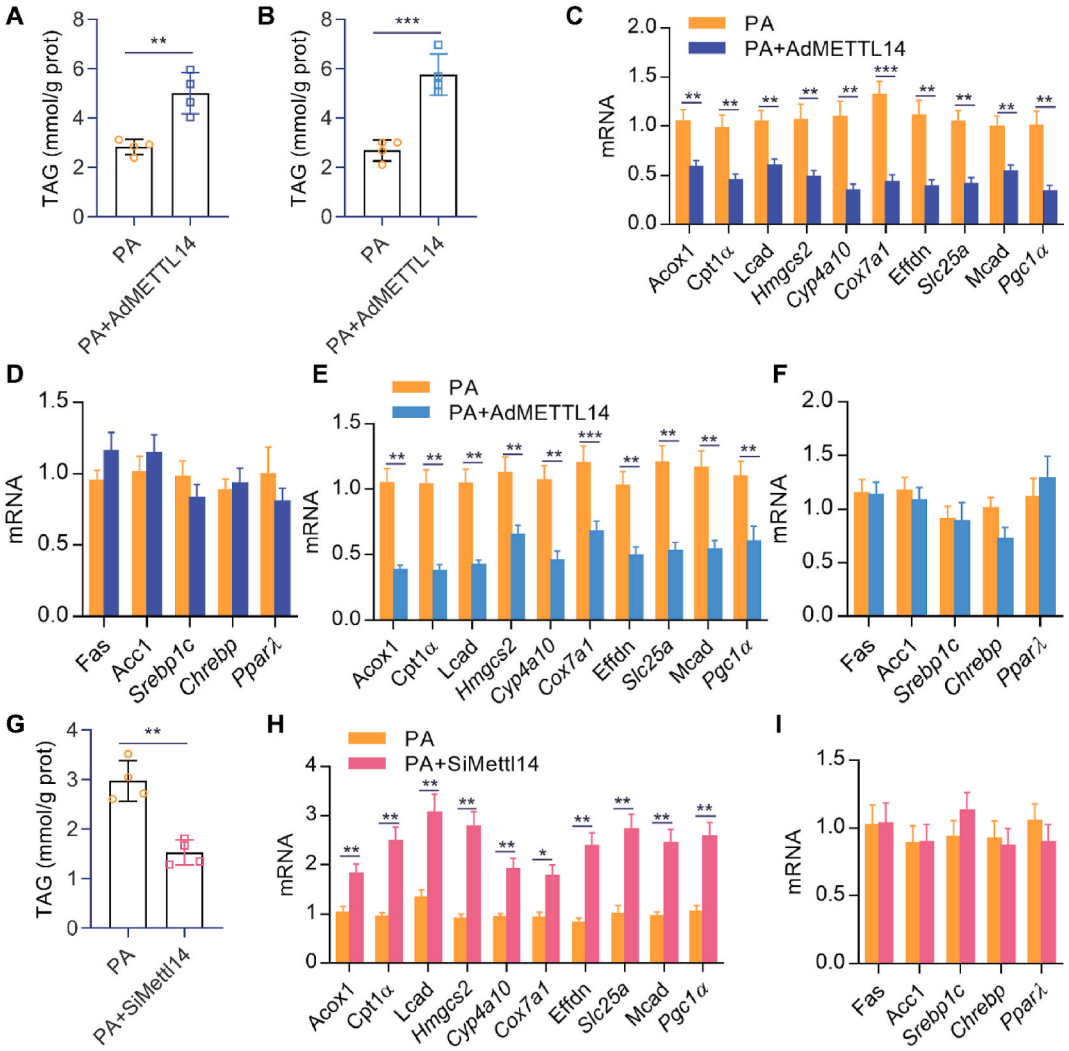


**Fig. 4. METTL14 regulates lipid accumulation in hepatic cells *in vitro*.** (A) TAG content in the human hepatic cell HepG2 was detected (n = 4). (B) TAG content in primary mice hepatocytes was detected (n = 4). (C–F) The expression of genes involved in fatty acid synthesis, lipolysis, and FAO was detected by RT-PCR(n = 4). (G) TAG content in the human hepatic cell HepG2 was detected (n = 4). (H–I) The expression of genes involved in fatty acid synthesis, lipolysis, and FAO was detected by RT-PCR (n = 4). Data are expressed as the mean ± SD. Differences between the two groups were measured using an unpaired Student's *t*-test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. PA: palmitic acid.

## 5. Discussion

This study evaluated the pathophysiology role of METTL14 in hepatic steatosis and insulin resistance. Using specific METTL14-overexpressing mice through tail vein injections of AAV8-METTL14, we found that METTL14 overexpression had a remarkable effect on impairing hepatic lipid metabolism, leading to hepatic steatosis and insulin resistance. METTL14 knockdown ameliorated the HFD-induced dysregulation of lipid metabolism and glucose metabolism and exerted a prominent therapeutic effect on steatosis in mice. Consistent with this, METTL14 knockdown treatment of either mouse or HepG2 suppressed the lipid accumulation *in vitro*. Here, we provide evidence that METTL14 may be a potential therapeutic target for hepatic steatosis and insulin resistance.

The RNA $m^6A$ methylation is one of the most widespread gene regulatory mechanisms. METTL14, which belongs to methyltransferases, mediates $m^6A$ modification of mRNA and plays an essential role in the development and progression of various liver diseases [20,26–29]. In a previous study, the upregulation of METTL3/14 expression promotes the $m^6A$ level of *ATP citrate lyase* (*ACLY*) and *stearoyl-CoA desaturase 1* (*SCD1*) mRNA in the livers, increasing the TAG production and lipid accumulation in MASLD mice [20]. In pancreatic β-cells, *Mettl14* plays a key role in β-cell survival and glucose homeostasis. While β-cell deletion of Mettl14 increase fat accumulation in livers by up-regulating acetyl-CoA carboxylase and fatty acid synthase expression [30]. Here, we found that METTL14 was present at a low level in normal liver tissue, but markedly increased in the liver of MASLD patients. Consistent with this phenotype in human MASLD, METTL14 was up-regulated in db/db and HFD-induced steatohepatitis mouse models. Further, we

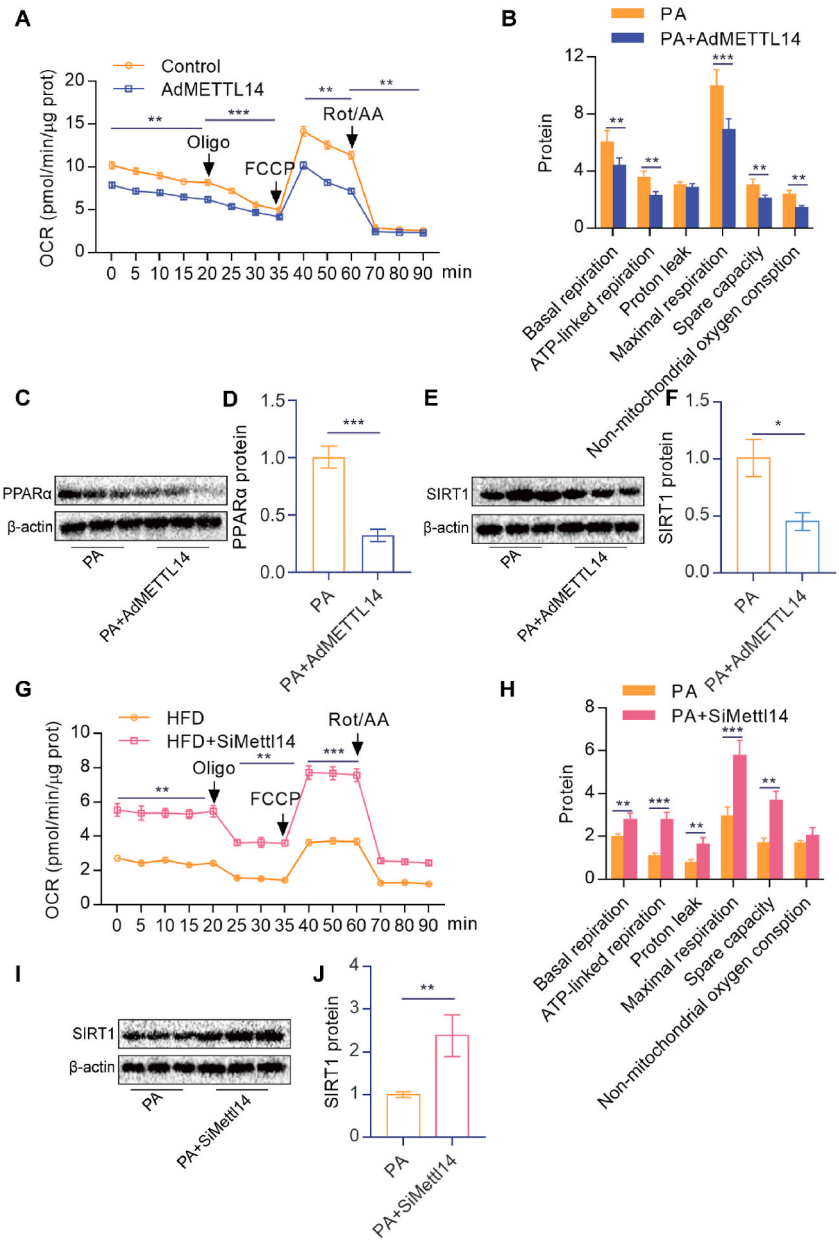


**Fig. 5. METTL14 modulates mitochondrial function in hepatocytes.** (A–B) The OCR in HepG2 cells receiving adMETTL14 was detected (n = 4). (C–F) The protein expression of PPARα and SIRT1 was detected by Western blot (n = 4). (G–H) The OCR in HepG2 cells receiving siMettl14 was detected (n = 4). (I–J) The protein expression of SIRT1 was detected by Western blot (n = 4). Data are expressed as the mean ± SD. Differences between the two groups were measured using an unpaired Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. PA: palmitic acid. Uncropped images in C, E, and I are available in Supplementary Fig. S1.

also found that PA treatment triggered METTL14 upregulation in hepatocytes *in vitro*. In this study, we examined the consequences of METTL14 upregulation or downregulation in the pathogenesis of MASLD. METTL14 overexpression promoted lipid accumulation in PA-treated hepatocytes and accelerated the susceptibility of mice to MASLD. On the contrary, METTL14 knockdown reduced the HFD-induced dysregulation of lipid metabolism and glucose metabolism. These observations suggest that targeting METTL14 might be a promising strategy for MASLD therapy.

It has been reported that hepatic lipid homeostasis is tightly regulated by fatty acid synthesis, fatty acid oxidation, and lipoprotein uptake and secretion [31–33]. The increase of fatty acid synthesis or decrease of fatty acid oxidation could induce MASLD, and any treatment which represses the fatty acid synthesis and increases fatty acid oxidation might effectively alleviate MASLD [12,34,35]. A previous study has reported that abnormal $m^6A$ modification is involved in regulating fatty acids synthase expression, which may contribute to lipogenesis and, ultimately, MASLD [20]. Overexpression of METTL14 led to increased levels of ACLY and SCD1, which

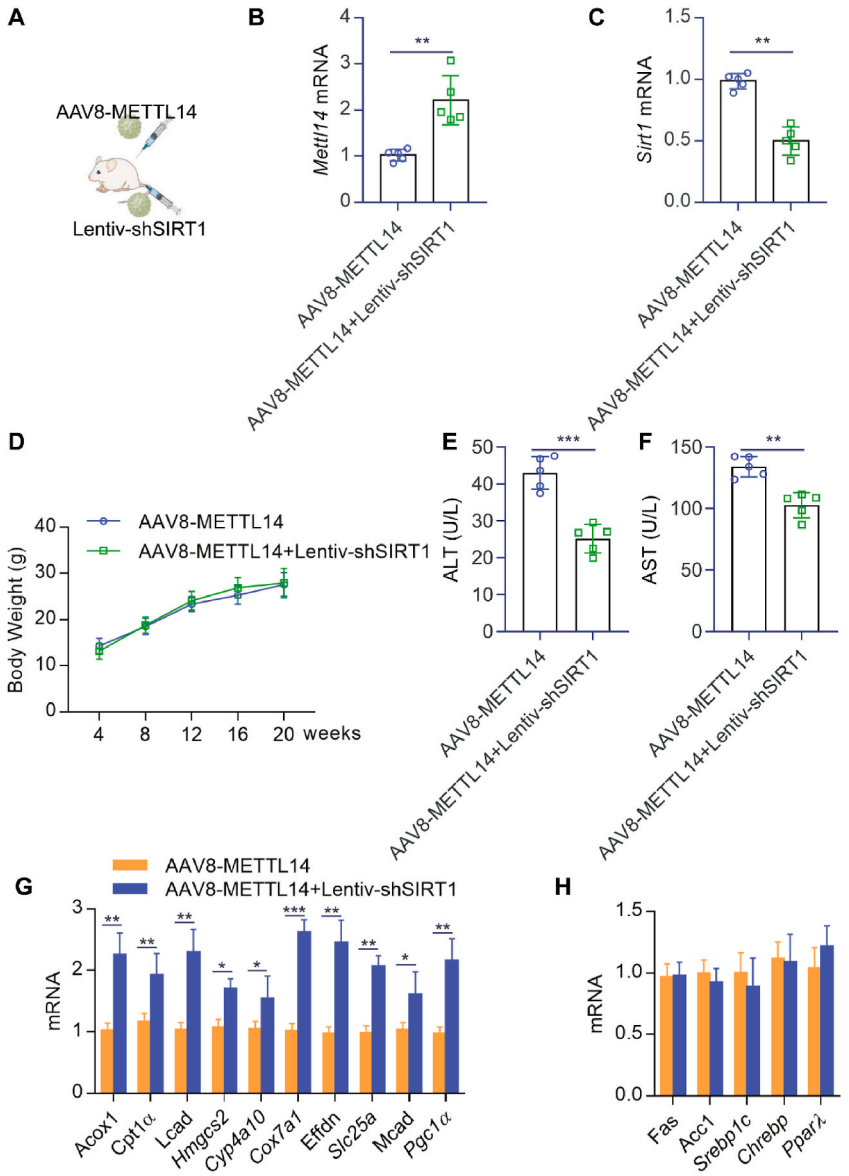


**Fig. 6. Inhibition of SIRT1 blunts the effect of liver-specific downregulation of METTL14 in hepatic steatosis.** (A) Wild-type mice received AAV8-METTL14 and shRNA-SIRT1 treatment. (B–C) The expression of *Mettl1*4 mRNA and *Sirt1* mRNA in the livers was detected by RT-PCR (n = 5). (D) The body weight of mice was recorded for 16 weeks (n = 10). (E–F) AST and ALT activities of mice were detected (n = 5). (G–H) The expression of genes involved in fatty acid synthesis, lipolysis, and FAO was detected by RT-PCR (n = 5). Data are expressed as the mean ± SD. Differences between the two groups were measured using an unpaired Student's *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

are pivotal for fatty acid synthesis [20]. However, in this study, we found that overexpression of METTL14 led to decreased gene expression of fatty acid oxidation. Furthermore, the downregulation of METTL14 decreased the expression levels of fatty acid oxidation. There was no difference in the expression levels of genes involved in fatty acid synthesis between control and METTL14-overexpression or METTL14-knockout mice. These data suggest that METTL14 might play a vital role in regulating lipid accumulation by targeting fatty acid oxidation directly.

SIRT1 is an $NAD^{+}$-dependent deacetylase, regulating a variety of cellular functions, including mitochondrial biogenesis, inflammation, metabolism homeostasis, autophagy, and apoptosis [36–38]. SIRT1-deficient mice exhibited exacerbated HFD-induced hepatic steatosis and insulin resistance [39,40]. Furthermore, it has been elucidated that METTL14-mediated $m^6A$ modification epigenetically promoted *Sirt1* mRNA degradation in podocytopathies [36]. In our study, we fouond the expression of SIRT1 was dramatically decreased by METTL14 overexpression in hepatocytes, suggesting SIRT1 as an essential downstream effector of the METTL14 actions in hepatocytes. Results from our *in vivo* studies clearly showed that inhibition of SIRT1 by shRNA blunts the effect of liver-specific downregulation of METTL14 in hepatic steatosis. These results suggest that METTL14 insufficiency protects against fatty

liver primarily by targeting SIRT1 in hepatocytes.

In conclusion, our work reveals an increased METTL14 expression in the liver with MASLD, which results in hepatic steatosis. Inhibition of METTL14 in the liver could ameliorate HFD-induced liver steatosis in a SIRT1-dependent manner. Overall, our results point towards METTL14 as a novel molecule implicated in the development of steatosis.

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**Data availability statement**

Data will be made available on request.

**Ethics approval and consent to participate**

All methods were carried out in accordance with relevant guidelines and regulations. The Ethics Committee of the Center for Scientific Research with Animal Models at Central South University (Changsha, China) approved the experiments, which were performed in accordance with the guidelines of the National Institutes of Health.

**CRediT authorship contribution statement**

**Ji-Xiang Zhou:** Writing – original draft, Investigation, Data curation, Conceptualization. **Man-Yi Yang:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Deng-Gao Zhai:** Writing – review & editing, Investigation, Formal analysis. **Qin Jiang:** Writing – review & editing, Formal analysis, Data curation. **Qi Zhang:** Writing – review & editing, Writing – original draft, Project administration, Investigation, Conceptualization.

**Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Not Applicable.

**Abbreviations**

| | |
|---|---|
| AAV | adeno-associated virus |
| ACLY | ATP citrate lyase |
| ALT | alanine aminotransferase |
| AST | aspartate aminotransferase |
| BHB | β-hydroxybutyric acid |
| CPT1 | carnitine palmitoyltransferase 1 |
| CW | chow diet |
| Epi | epididymal fat pad |
| FAO | fatty acid β-oxidation |
| GTT | glucose tolerance test |
| HDL | high-density lipoprotein |
| HFD | high-fat diet |
| ITT | insulin tolerance test |
| LDL | low-density lipoprotein |
| MASLD | metabolic dysfunction-associated steatotic liver disease |
| METTL14 | methyltransferase-like 14 |
| OA | oleic acid |
| OCR | oxygen consumption rate |
| PA | palmitic acid |
| PPARα | peroxisome proliferators-activated receptor α |
| SCD1 | stearoyl-CoA desaturase 1 |
| SIRT1 | Sirtuin 1 |
| TAG | triacylglyceride |
| TCHO | total cholesterol |

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e35467.

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