Supplementary data

Fatty Acids Promotes Fatty Liver Disease via the Dysregulation of 3-Mercaptopyruvate Sulfurtransferase/ Hydrogen Sulfide Pathway

Meng Li, Chengfu Xu, Junping Shi, Jiexia Ding, Xingyong Wan, Dahua Chen, Jianguo Gao, Chunxiao Li, Jie Zhang, Yiming Lin, Zhenhua Tu, Xiaoni Kong, Youming Li, Chaohui Yu

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Supplementary materials and methods

Human samples

Liver biopsies from patients with NAFLD (*n*=37) who underwent liver biopsies for suspected NAFLD or NASH, or during liver surgeries were randomly selected from the liver biopsy samples recruited at the First Affiliated Hospital, College of Medicine, Zhejiang University, The Affiliated Hospital of Hangzhou Normal University, Renji Hospital, School of Medicine, Shanghai Jiao Tong University, Hangzhou Sixth People's Hospital, and Ningbo Medical Treatment Center Lihuili Hospital. The inclusion criteria for patients with NAFLD were based on the absence of significant alcohol consumption, the presence of biopsy-proven steatosis with or without necroinflammation and/or fibrosis, and tested negative for viral hepatitis. Patients with other potential causes of chronic liver disease were excluded. Healthy control liver samples (n=19) were obtained from parts of donor livers that were not used for liver transplantation. All of these samples were histological normal without evidence of viral infections (hepatitis B virus, hepatitis C virus and human immunodeficiency virus). Moreover, none of these patients consumed alcohol >20 g for males and >10 g for females per day or used drugs for any chronic medical disorder. Clinical examinations including standing height, body weight, waist and hip circumference, systolic and diastolic blood pressure, biochemical variables recorded for all patients were performed according to procedures described previously,[1] summarized in Supplementary Table 1. Experiments for all human liver tissue samples were approved by the Ethics Committee of the First Affiliated Hospital, College of

Medicine, Zhejiang University, and all patients provided their written informed consent.

Immunohistochemistry

Liver sections were deparaffinised with xylene and rehydrated. Slides were incubated in 3% hydrogen peroxide. Heat epitope retrieval was done for 20 minutes in target-retrieval solution at pH7.5. The sections were preblocked with 10% normal goat serum (ZSGB-BIO, Beijing, China) and then incubated with Anti-MPST (GeneTex) at dilution of 1:100 for overnight at 4^{o}C. Tissue sections were stained with HPR secondary antibody (dilution: 1:1000, ZSGB-BIO, Beijing, China) for 1 h at 37^{o}C in an incubator. Immunoreactivity was detected using a DAB kit (ZSGB-BIO, Beijing, China) and visualized as brown staining. Slides were counterstained with hematoxylin. Five fields with a final magnification of ×400 were randomly selected for each sample, and the Integral Optical Density (IOD) of all the positive staining in each photograph was measured by Image Pro Plus 6.0 software.

Animal experiments and procedures

Male C57BL/6 mice, 6 weeks of age, weighing 18-20 g, were purchased from B&K Laboratory Animal Corp. Ltd. (Shanghai, China). Before experiment, mice were allowed to acclimate for 1-2 weeks with *ad libitum* access to food and water. NAFLD mice model was induced by HFD feeding for 8 weeks as we previously described.[1] Adenovirus plasmids containing green fluorescent protein (Ad-GFP) coding sequence

and MPST short hairpin RNA (Ad-shMPST) were constructed by Life Technologies (Shanghai, China). The *i.v.* hydrodynamic injection of adenovirus is an effective method to silence specific liver gene expression *in vivo*.[2-4] The recombinant viruses were named Ad-shMPST or Ad-GFP, and were used for inhibiting liver MPST expression or served as negative controls, respectively. The mice received *i.v.* hydrodynamic injection of 1×10^9 plaque-forming units (PFU) of Ad-GFP or Ad-shMPST via the tail vein every two weeks from the beginning of the experiments, and were sacrificed 8 weeks after HFD feeding. Mice were fasted overnight prior to sacrifice.

Heterozygous MPST-deficient (MPST +/-) mice on a C57BL/6 background were generated through frameshift mutation by TAL-effector nuclease (TALEN) system by Beijing ViewSolid Biotechnology, China. Male 7-8 weeks old MPST $^{+/-}$ and their wild-type (WT, MPST $^{+/+}$) littermates were fed with an HFD for 8 weeks. Detailed explanation of generation strategy was described in the next part of methods. All of the animal experiments were performed according to the guidelines approved by the Animal Care and Use Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University.

Generation of heterozygous MPST deficient mice

Heterozygous MPST-deficient (MPST +/-) mice on a C57BL/6 background were generated through frameshift mutation by TAL-effector nuclease (TALEN) system by Beijing ViewSolid Biotechnology, China. The TALEN constructs were designed to

target exon 2 of the mouse MPST gene, leading to inactivation of the MPST gene. All constructs were validated by DNA sequencing. The activity of TALEN was detected by SSA Luciferase assay in 293T cells. To generate the mice, in vitro-transcribed TALEN mRNAs were injected into the cytoplasm of mouse pronuclear-stage embryos to produce mutant founders (F0). Founders carrying frameshift mutations on one allele were intercrossed to produce the F1 generation. To confirm the frameshift mutations, the tail-derived DNA from 3-week-old newborn mice was genotyped by sequencing the PCR products (Supplementary Figure 1A) amplified by the primers: MPST-sense (GAGTTCTTGGACTCTGCTCTGC) and MPST-anti (CTGATTCAGCCAGTGGCGGAAG). The mutant mice were mated with wildtype C57BL/6 mice to obtain heterozygous MPST +/- mice. Western blotting analysis was then performed to confirm the MPST expression (Supplementary Figure 1B). Mice were kept in ventilated rooms, in a pathogen-free facility under conditions of controlled temperature (23℃), humidity (50%) and illumination (12-hour light/12-hour dark cycle).

Cell culture and treatment

Human normal hepatocyte cell line (L02 cells and 7701 cells) was purchased from Chinese Academy of Sciences (Shanghai, China). The cells were plated on 6-well plates, cultured with dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS at 37 ℃ and 5% CO_2 in air. To establish a cellular model of NAFLD, the L02 cells were treated for 24 h with a 1 mM concentration mixture of FFAs including oleate and palmitate in a ratio of 2:1, as we previously described.[1] L02 cells were

transfected with MPST siRNA (sense 5' GCCAUCUGUUCCAGGAGAATT 3'; anti-sense 5' UUCUCCUGGAACAGAUGGCTT 3'), CSE siRNA (sense 5' CCCAGUUCCUGGAAUCUAATT 3'; anti-sense 5' UUAGAUUCCAGGAACUGGGTT 3') or corresponding scramble siRNA as negative control (sense 5' UUCUCCGAACGUGUCACGUTT 3'; anti-sense 5' ACGUGACACGUUCGGAGAATT 3') using Lipofectamine 2000 (Invitrogen, Shanghai, China). Overexpression plasmid containing full length MPST DNA was transfected into L02 cells using Lipofectamine 3000 (Invitrogen, Shanghai, China) according to the manufacturer's instructions. The cells were transfected with siRNA or plasmid DNA for 24 h, and then incubated for another 24 h with FFAs before harvesting. The Lenti-Flag-MPST vector system (Ubi-MCS-3FLAG-SV40-EGFP-IRES-puromycin) was constructed, packed, and purified by GeneChem (Shanghai, China), and was manipulated according to the protocol provided by the manufacturer. L02 cells stably overexpressing Flag-MPST were generated using lentiviral constructs and maintained with 2 μg/mL of puromycin (Sigma-Aldrich, St. Louis, MO). The NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC) was added 1h before addition of FFAs. For NF-κB/p65 overexpression, pENTER-p65 plasmid was constructed and transfected into L02 cells 24h before FFAs stimulation. H_2S supplement experiments were performed by incubation of FFA-treated cells with NaHS (Sigma-Aldrich, St. Louis, Mo).

Measurement of H_2S level

The fresh supernatants of L02 cells were collected and measured immediately for H_2S levels using a kit from AMEKO (Shanghai Lianshuo Biological Technology Co., Ltd, China). Fresh homogenates of mouse livers were prepared for instant determination of intrahepatic H_2S contents using the Hydrogen Sulfide BioAssay™ Kit (United States Biological, Swampscott, MA). The concentrations of H_2S were calculated by using a standard curve of NaHS, a source of H_2S.

Hepatic and cellular TG and cholesterol measurement

Intracellular and intrahepatic triglyceride (TG) and total cholesterol (TC) contents were assayed using kits purchased from Applygen Technologies Inc. (Beijing, China) according to the manufacturer's recommended protocols. The protein concentration in the resulting lysates was determined using the bicinchoninic acid protein assay kit (Applygen Technologies Inc.).

Measurement of hepatic FFA

Intrahepatic FFA levels were measured with a commercial kit from BioVision (Milpitas, CA). Samples were prepared and measured according to the manufacturer's instructions.

Hematoxylin–Eosin (HE) and Oil Red O staining

The liver samples were fixed in 10% neutral formalin, embedded in paraffin, sectioned and then stained with H&E for histological examination. To determine

hepatic fat accumulation, frozen liver sections (8 μm) were stained with freshly diluted Oil Red O solution for 10 min, washed, and counterstained with hematoxylin for 5 min. Cells in 6-well plates were washed twice with PBS, fixed with 10% neutral formalin for 15min, washed and stained with freshly diluted Oil Red O solution for 10 min, followed by another two washes with PBS and stained with hematoxylin for 5 min. Representative photomicrographs were captured at 400 ×magnification using a microscope (BX53F, Olympus, Japan).

Analysis of MPST-centered protein interaction prediction network using STRING database

STRING analysis was done to determine and validate functional protein-interaction networks (available at http://www.string-db.org/).[5] We analyzed protein-protein interaction network center on MPST, which is based on experimental findings, text mining, and in silico predictions either alone or mixed together. Colored lines between the proteins indicate various types of interaction evidence.[6]

Biochemical analysis and metabolic measurements

Plasma concentrations of transaminases, triglyceride (TG), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-c), low-density lipoprotein-cholesterol (LDL-c), very low density lipoprotein (VLDL-c), uric acid and total bilirubin were determined with a Hitachi 7600 autoanalyzer (Hitachi, Tokyo, Japan). Plasma monocyte chemoattractant protein (MCP)-1, interleukin (IL)-1β, TNF-α, IL-6, IL-17A,

IL-18 concentrations were measured by ELISAs (MCP-1, TNF-α, IL-6, IL-17A, Biolegend, San Diego, CA; IL-1β, eBiosciences, San Diego, CA; IL-18, Abcam, USA) following the manufacturer's instructions. MDA contents and SOD activity were measured colorimetrically with kits from BioVision (Milpitas, CA). The final results of hepatic biochemical indexes were normalized to total protein concentration determined by the bicinchoninic acid protein assay kit (Applygen Technologies Inc.). All procedures were performed according to the instructions of the assay kits.

Quantitative Real-time PCR

Total RNA was isolated from hepatic tissues or cell lysates using the standard TRIzol method according to the manufacturer's instructions (Invitrogen, Shanghai, China). The RNA were reversely transcribed into cDNA using a PrimeScript® RT reagent Kit (Takara, Japan). Quantitative real-time PCR was performed using an ABI 7500 real-time PCR System (Applied Biosystems, Carlsbad, CA) with SYBR Green (Takara, Japan). The relative expression levels of target genes were normalized using GAPDH as an internal control, and calculated with formula $2^{-\Delta\Delta CT}$.

Western bolt analysis

Proteins were extracted using RIPA buffer (Applygen Technologies Inc.) added with protein and phosphatase inhibitor (Sigma), separated by SDS-PAGE, and electrophoretically transferred to PVDF membranes (Millipore). After blocking with 5% nonfat milk in TBST, the membranes were incubated overnight with primary

antibodies (detailed antibody information described in supplemental data) at dilutions specified by the manufacturers. The membranes were then incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody at a 1:5000 dilution for 1 h. After three washes with TBST, the signals were detected using a chemiluminescence ECL kit (Multisciences, Hangzhou, China) and quantitated using Quantity One software.

Antibodies

MPST (Abcam ab85211 and Novus NBP1-82617 for human and mouse, respectively), CSE (Proteintech 12217-1-AP), Abcam: SREBP-1 (ab3259), FAS (ab128856), ACC (ab45174), GAPDH (ab8245), Tubulin (ab6160), CPT1A (ab128568), PPARα (ab), ACOX1 (ab184032), MCAD (ab110296), LCAD (ab196655), FGF21 (ab64857), Flag (Sigma F1804), Cell Signaling Technology: phosphorylated JNK (4671), JNK (3708).

Co-immunoprecipitation

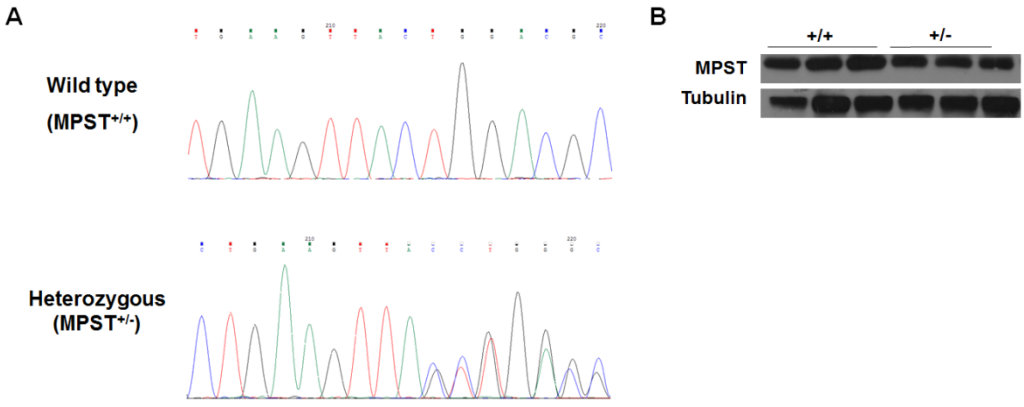
Dynabeads® Co-Immunoprecipitation Kit (Life technologies, Catalog number 14321D) was used to perform co-immunoprecipitation (CoIP). For every antibody, 1.5mg beads and 10 μg antibody were incubated at 37°C (200rpm/min shaking) for 24 hours. To extract protein, FFA-treated L02 cells with or without Flag-MPST stabling overexpression was resuspended in Extraction Buffer on ice for 15 min. The cell lysate was centrifuged (2600 g, 5 min) at 4 ℃ and supernatant was collected. After a series of buffer washing, the beads were then resuspended in Elution Buffer and

incubated at room temperature for 5 min. The supernatant of elute liquid containing the purified protein complex was collected. Then Western blot was performed to analyze the proteins in the cell lysate and elute liquid.

Statistical analysis

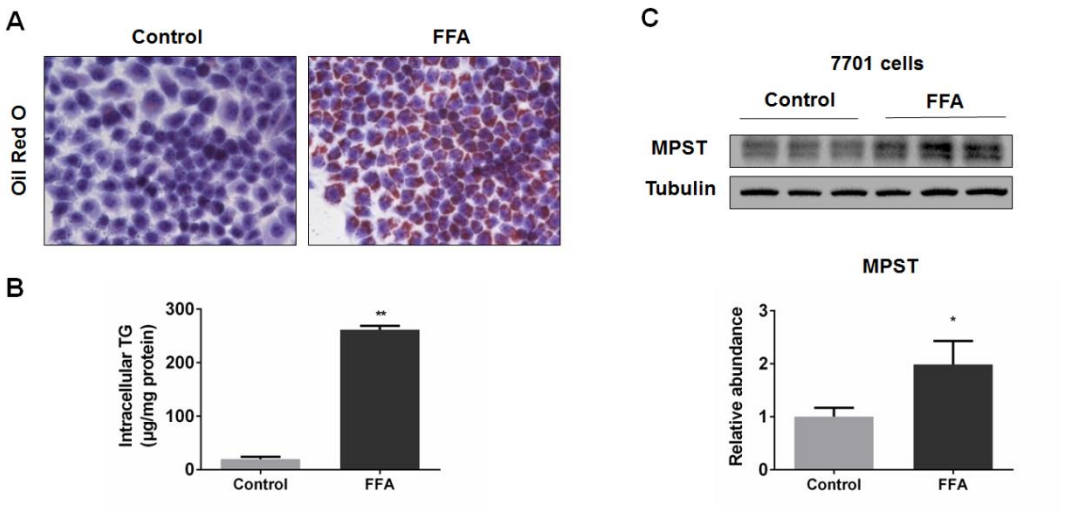
Statistical analysis was performed using SPSS version 22 (SPSS Inc.). IHC data were compared between groups using Mann-Whitney *U* tests. Values were shown as mean ± SD. Statistical differences were determined by Student's *t*-test or Mann-Whitney *U* tests. Statistical significance was displayed as $*P<0.05$, $**P<0.01$, $***P<0.001$. $P<0.05$ was considered statistically significant.

Supplementary Figures

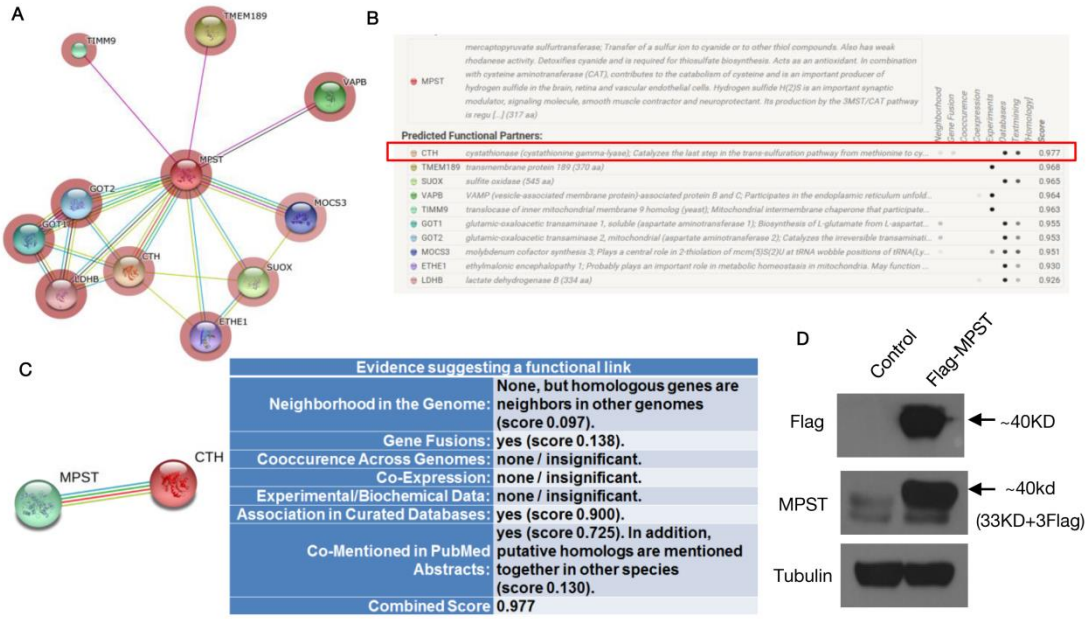


Supplementary Figure 1. TALEN-mediated MPST gene mutation in mice

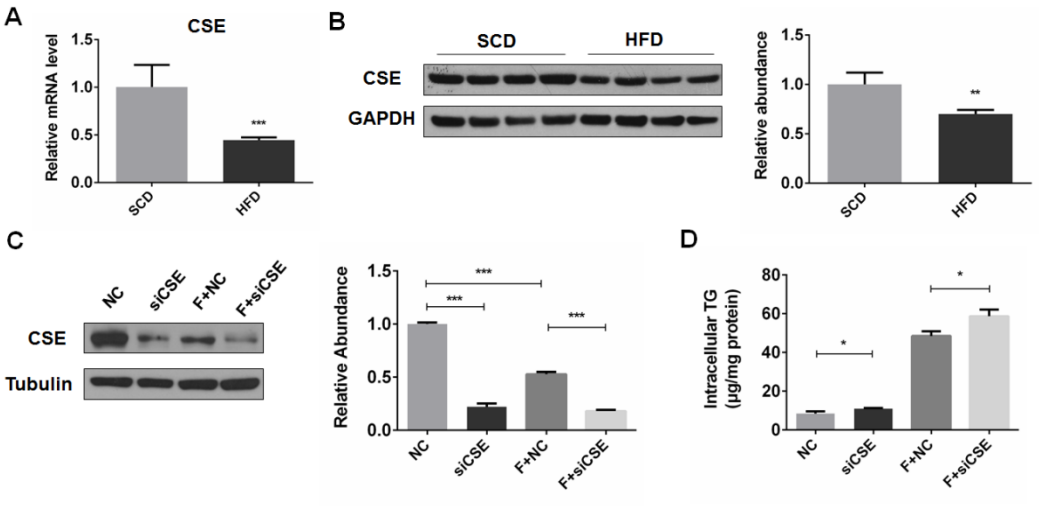
(A) The representative images of sequencing traits of MPST gene mutations in heterozygous MPST deficient mice detected through sequencing the PCR products (The tail-derived DNA from 3-week-old newborn mice). (B) Western blot confirmed decreased MPST expression heterozygous MPST deficient mice.



Supplementary Figure 2. The establishment of NAFLD cell models and increased protein expression of MPST in FFA-treated 7701 cells. (A-B) L02 cells were treated with FFAs (1mM) for 24 h. Representative images of Oil Red O staining of L02 cells is shown (panel A). Original magnification ×400. Intracellular TG contents were determined (panel B). (C) 7701 cells were treated with FFAs (1mM) for 24 h and then subjected to Western blot analysis. The results are expressed as mean ± SD of 3 independent experiments. **P* < 0.05, ***P* <0.01. (2-tailed Student's *t* test).

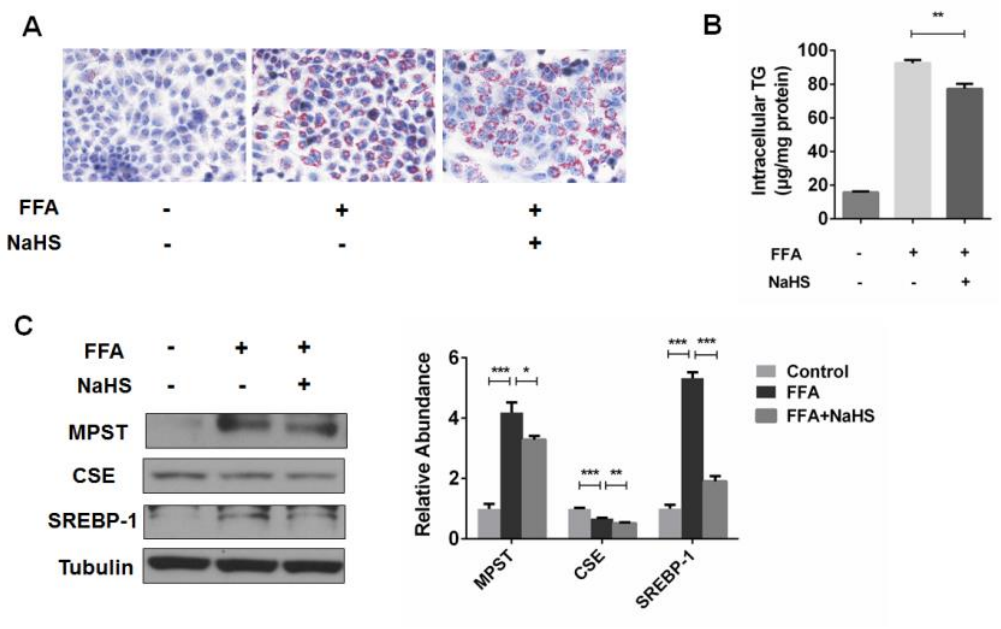


Supplementary Figure 3. Interaction networks of MPST-regulated proteins from STRING analysis. (A) MPST-centered protein interaction prediction network. (B) Among the top ten MPST-regulated proteins, CSE (CTH) ranked first. (C) MPST-CSE interaction analysis. (D) Vector for Flag-tagged MPST was generated by inserting the full-length human MPST coding sequence (NM_001130517) into the Lenti-Flag vector (GV358) system. The efficiencies of the MPST-overexpression constructs were tested by stable transfection into L02 cells, followed by western blot analysis.

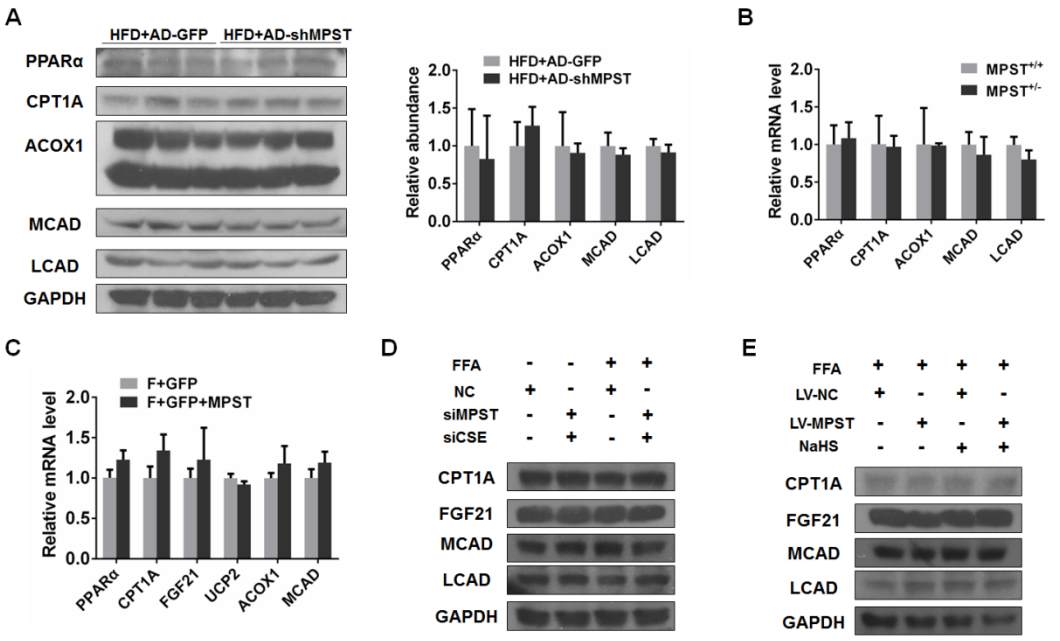


Supplementary Figure 4. Hepatic CSE expression is down-regulated in NAFLD.

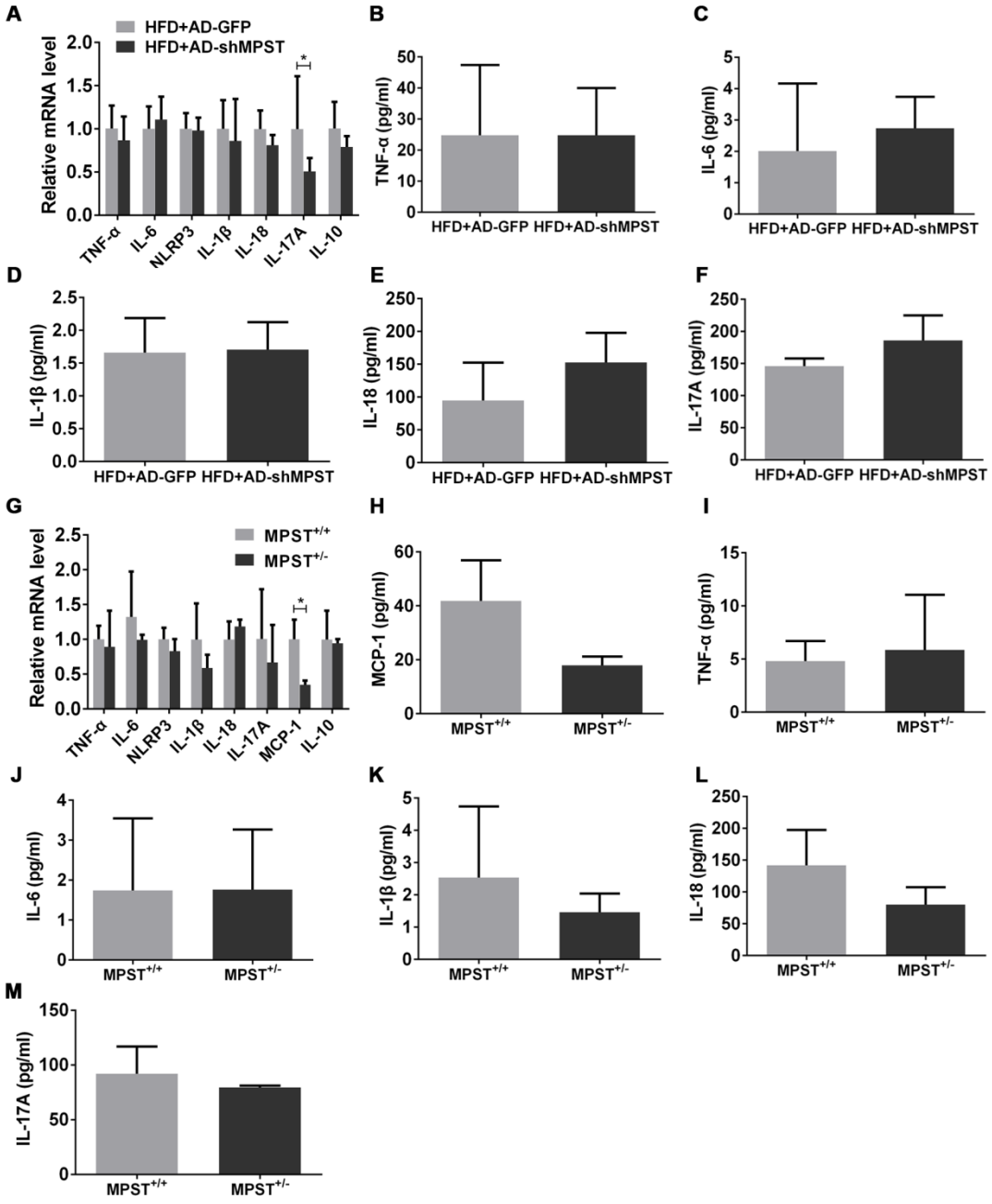
(A, B) Hepatic mRNA (panel A) and protein (panel B) levels of CSE were determined and relative CSE protein level quantified in HFD-fed mice. (C-D) After transfection with scrambled siRNA (Negative Control) and CSE siRNA for 24 h, L02 cells were treated with or without FFAs for 24 h. (C) Western blot analyses were performed to determine CSE expression. (D) Intracellular TG contents were determined. The results are expressed as mean ± SD of 5 mice per group or 3 independent experiments. **P* < 0.05, ***P* <0.01, ****P* <0.001 (2-tailed Student's *t* test).



Supplementary Figure 5. Effects of NaHS treatment on hepatocyte steatosis in FFA-stimulated L02 cells. L02 cells were pretreated with NaHS (1 mM) for 2 h and then treated by FFAs (1 mM) with or without NaHS for 24 h. (A) Representative images of Oil Red O staining was shown. (B) Intracellular TG contents were determined. (C) Western blot analysis of MPST, CSE and SREBP-1. The results are expressed as the mean ± SD of 3 independent experiments. **P* < 0.05, ***P* <0.01, ****P* <0.001 (2-tailed Student's *t* test).



Supplementary Figure 6. Fatty acid oxidation pathway is not involved in MPST-regulated hepatocyte steatosis. (A) Adenovirus mediated partial knockdown of hepatic MPST did not affect the protein expression of PPARα signaling pathway in HFD-fed mice. (B) The expression of PPARα and its target genes in the livers of HFD-fed $MPST^{+/+}$ and $MPST^{+/-}$ mice. (C) Over-expression of MPST did not affect the transcriptional level of PPARα signaling pathway in FFAs-treated L02 cells. (D) An additional inhibition of CSE in MPST knockdown cells did not affect the protein level of genes involved in fatty acid oxidation. (E) Western blot analysis of CPT1A, FGF21, MCAD and LCAD in FFAs-treated L02 cells transfected with lentivirus-NC or lentivirus-MPST (LV-MPST) incubation with or without NaHS. The results are expressed as the mean ± SD of 5 or 3 mice per group or 3 independent experiments.



Supplementary Figure 7. Effects of partial knockdown of MPST on hepatic inflammatory gene profiles and circulating inflammatory biomarkers in HFD-fed mice. (A) Hepatic inflammatory gene profiles in HFD-fed mice infected with AD-GFP and AD-shMPST, respectively. (B-F) Plasma TNF-α, IL-6, IL-1β, IL-18, and IL-17A concentrations in HFD-fed mice infected with AD-GFP and AD-shMPST, respectively. (G) Hepatic inflammatory gene profiles in HFD-fed $MPST^{+/+}$ and

$MPST^{+/-}$ mice. (H-M) Plasma MCP-1, TNF-α, IL-6, IL-1β, IL-18, and IL-17A concentrations in HFD-fed $MPST^{+/+}$ and $MPST^{+/-}$ mice. The results are expressed as the mean ± SD of 5 or 3 mice per group. *$P < 0.05$ (2-tailed Student's *t* test).

Table S1 Clinical and Biochemical Characteristics of Patients with NAFLD and Controls

	Control	NAFLD	*t* value	*p* value
N (male/female)	19 (14/5)	37 (27/10)	0.003[a]	0.955
Age (years)	42.9 (10.7)	42.7 (12.9)	0.039	0.969
Body mass index (kg/m^2)	23.50 (2.06)	27.57 (4.28)	4.441	<0.001
Waist circumference (cm)	82.4 (4.3)	97.1 (9.9)	6.36	<0.001
Hip circumference (cm)	86.4 (6.3)	100.5 (7.2)	5.677	<0.001
Systolic blood pressure (mmHg)	117.4 (18.4)	131.6 (16.8)	2.346	0.031
Diastolic blood pressure (mmHg)	73.7 (13.2)	84.9 (11.1)	2.883	0.006
Alanine aminotransferase (U/L)	32 (20-51)	65 (39-100)	3.149[b]	0.002
Aspartate aminotransferase (U/L)	39 (25-66)	39 (28-57)	0[b]	1
Gamma-glutamyl transpeptidase (U/L)	54 (22-84)	65 (41-133)	1.247[b]	0.212
Free fatty acid (mmol/L)	0.55 (0.43-0.68)	0.79 (0.63-0.86)	2.808[b]	0.005
Triglyceride (mmol/L)	1.16 (0.78-1.74)	2.05 (1.45-2.84)	3.539[b]	<0.001
Total cholesterol (mmol/L)	2.66 (0.92)	5.10 (1.03)	8.694	<0.001
HDL-cholesterol (mmol/L)	1.49 (0.91-2.05)	1.13 (1.03-1.33)	1.143[b]	0.253
LDL-cholesterol (mmol/L)	0.56 (0.25)	3.04 (1.06)	13.52	<0.001
VLDL-cholesterol (mmol/L)	0.58 (0.38-0.75)	0.73 (0.65-0.79)	2.702[b]	0.007
Uric acid (μmol/L)	203.5 (159.3-241.5)	427 (352.0-491.5)	5.292[b]	<0.001
Total bilirubin (μmol/L)	10.5 (6.50-19.00)	16.3 (12.75-23.25)	2.619[b]	0.009

Supplementary Table 1

Clinical and Biochemical Characteristics of Patients with NAFLD and Controls. Data are expressed as means (SD) or medians (IQR) depending on the data distribution. Statistical differences were determined by Student's *t*-test or Mann-Whitney *U* test. HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low density lipoprotein. [a]χ2 value; [b]Z value.

Table S2 Physiologic data and biochemical parameters of HFD-fed mice

	HFD + AD-GFP	HFD + AD-shMPST
liver wt/body wt (%)	4.5±0.3	4.6±0.2
Plasma TG(mM)	0.8±0.1	1.1±0.3
Plasma TC (mM)	4.2±0.5	3.5±0.2*
Plasma ALT (U/L)	92±32.9	69.7±16.1
Plasma AST (U/L)	150.8±21.3	144±29.3
Liver HDL (ug/mg protein)	12.4±2.4	14.5±2.4
Liver VLDL (ug/mg protein)	1.4±1.3	0.4±0.3

Supplementary Table 2

Physiologic data and biochemical parameters of HFD-fed mice given AD-GFP or adenovirus-mediated knockdown of MPST (shMPST) via tail vein. Data are the mean ± SD. *P < 0.05 HFD+AD-shMPST versus HFD+AD-GFP group (2-tailed Student's *t* test). ALT, alanine aminotransferase; AST, aspartate aminotransferase; HDL, high density lipoprotein; TC, total cholesterol; TG, triglyceride; VLDL, very low density lipoprotein

Table S3 Physiologic data and biochemical parameters of HFD-fed MPST heterogeneous deficient mice

	$MPST^{+/+}$	$MPST^{+/-}$
liver wt/body wt (%)	3.66±0.9	2.82±0.13
Plasma TG(mM)	0.64±0.06	0.6±0.06
Plasma TC (mM)	4.86±0.42	3.49±0.12**
Plasma ALT (U/L)	62±36.66	27±3
Plasma AST (U/L)	114±45.4	101±18.08
Plasma HDL-c (mM)	4.27±0.35	3.06±0.2**
Plasma VLDL-c (mM)	0.68±0.03	0.34±0.07**

Supplementary Table 3

Physiologic data and biochemical parameters of HFD-fed $MPST^{+/+}$ and $MPST^{+/-}$ mice. Data are the mean ± SD. **P < 0.01 HFD-fed $MPST^{+/-}$ mice versus HFD-fed WT ($MPST^{+/+}$) group (2-tailed Student's *t* test). ALT, alanine aminotransferase; AST, aspartate aminotransferase; HDL, high density lipoprotein; TC, total cholesterol; TG, triglyceride; VLDL, very low density lipoprotein

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