Ablation of Dual-Specificity Phosphatase 6 Protects against Nonalcoholic Fatty Liver Disease via Cytochrome P450 4A and Mitogen-Activated Protein Kinase

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Dual-specificity phosphatase 6 (DUSP6) is a specific phosphatase for mitogen-activated protein kinase (MAPK). This study used a high-fat diet (HFD)—induced murine nonalcoholic fatty liver disease model to investigate the role of DUSP6 in this disease. Wild-type (WT) and Dusp6-haploinsufficiency mice developed severe obesity and liver pathology consistent with nonalcoholic fatty liver disease when exposed to HFD. In contrast, Dusp6-knockout (KO) mice completely eliminated these phenotypes. Furthermore, primary hepatocytes isolated from WT mice exposed to palmitic and oleic acids exhibited abundant intracellular lipid accumulation, whereas hepatocytes from Dusp6-KO mice showed minimal lipid accumulation. Transcriptome analysis revealed significant down-regulation of genes encoding cytochrome P450 4A (CYP4A), known to promote ω-hydroxylation of fatty acids and hepatic steatosis, in Dusp6-KO hepatocytes compared with that in WT hepatocytes. Diminished CYP4A expression was observed in the liver of Dusp6-KO mice compared with WT and Dusp6-haploinsufficiency mice. Knockdown of DUSP6 in HepG2, a human liver-lineage cell line, also promoted a reduction of lipid accumulation, down-regulation of CYP4A, and up-regulation of phosphorylated/activated MAPK. Furthermore, inhibition of MAPK activity promoted lipid accumulation in DUSP6-knockdown HepG2 cells without affecting CYP4A expression, indicating that CYP4A expression is independent of MAPK activation. These findings highlight the significant role of DUSP6 in HFD-induced steatohepatitis through two distinct pathways involving CYP4A and MAPK. (Am J Pathol 2023, 193: 1988–2000; https://doi.org/10.1016/j.ajpath.2023.09.003)

Nonalcoholic fatty liver disease (NAFLD) is the most common liver disease, characterized by significant steatosis in individuals without a history of alcohol abuse. NAFLD is often accompanied by systemic metabolic disorders, such as hyperlipidemia and insulin resistance. In developed countries, NAFLD is estimated to affect 30% of the population, with >10% of NAFLD cases progressing to nonalcoholic steatohepatitis (NASH), characterized by histologic features of lobular inflammation and hepatocyte ballooning. The inflammatory process in NASH usually encompasses fibrosis, which can lead to cirrhosis. Although cirrhosis is a precursor to hepatocellular carcinoma, some patients with NASH develop hepatocellular carcinoma without significant fibrosis. Obesity has been identified as a risk factor for NAFLD, as 57% of individuals with NAFLD are overweight. NAFLD is a hepatic manifestation of metabolic syndrome, which includes central obesity, type 2 diabetes mellitus, hypertension, and...
hyperglycemia, and hyperlipidemia. Currently, the primary treatment for NAFLD relies on weight loss and exercise. However, considering its prevalence, challenging diagnosis, complex pathogenesis, and limited effective therapies, research on NAFLD is paramount.

The mechanisms underlying the development and progression of NAFLD are complex and multifactorial. Recently, the mitogen-activated protein kinase (MAPK) signaling pathway, including the extracellular signal-regulated kinase (ERK), p38, and c-Jun NH2-terminal kinase, has emerged as a crucial regulator of metabolic homeostasis.

Dual-specificity phosphatases (DUSPs) are enzymes that dephosphorylate both the serine/threonine and tyrosine residues on their substrates. The cytoplasmic dual-specificity phosphatase 6 (DUSP6), a member of the DUSP family, specifically dephosphorylates activated/phosphorylated ERK and forms a negative feedback loop to regulate ERK activity tightly. Diminished expression of DUSP6 is often observed in human pancreatic cancer, leading to sustained activation of ERK. Over-expressing DUSP6 in pancreatic cancer cells induces dephosphorylation of ERK and apoptosis. In addition, a mouse model with conditional activation of Kras and deletion of Dusp6 exhibited accelerated development of pancreatic cancer, suggesting a suppressive role of DUSP6 in pancreatic cancer development.

For instance, Feng et al demonstrated that Dusp6-knockout (KO) mice with a pure C57Bl/6j background were resistant to diet-induced obesity when a high-fat diet (HFD) was fed. Similarly, Dusp6/8 double-knockout mice exhibited increased ERK activity and lower susceptibility to developing obesity. In contrast, Dusp6-deficient mice (C57Bl/6-SV129j) fed with HFD significantly increased lean muscle mass and body weight. Consequently, the exact role of DUSP6 in regulating obesity-related metabolic disorders remains elusive.

In a preliminary study, Dusp6-KO mice showed resistance to HFD-induced obesity and suppression of hepatic steatosis. On the basis of these findings and the conflicting results from previous studies, the current study investigated the role of DUSP6 in the development of HFD-induced NAFLD and NASH, elucidating the underlying molecular mechanisms. The effect of HFD feeding in Dusp6-KO mice (C57Bl/6-SV129j) was compared with wild-type (WT) and Dusp6-haploinsufficiency (HI) mice. These findings were validated using a human liver-lineage cell line to ensure the relevance of these observations across species.

Materials and Methods

Mice and Diet

Dusp6-knockout mice (C57Bl/6-SV129j hybrid background) were kindly provided by Jeffery Molkentin (Division of Molecular Cardiovascular Biology, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH), which were crossed with C57Bl/6 mice to generate Dusp6-HI mice. Dusp6-HI mice were crossed to obtain Dusp6-WT, Dusp6-HI, and Dusp6-KO mice. The genotypes of mice were confirmed by processing tail genomic DNA using PCR with the following primers: 5’-CAGTCCAT-CAGCAGAAGCGGATAG-3’ (exon 1); 5’-GCTCT ATGGCTTCTGAGGCCG-3’ (3’ neomycin); and 5’-CATT GACCTCGGAGGTGATCGGT-3’ (reverse common primer).

Mice were housed at 23°C ± 2°C in a 12-hour light/dark cycle and supplied with food and water in free access at the animal facilities of the Institute for Animal Experimentation of Tohoku University Graduate School of Medicine (Sendai, Japan). Four-week-old mice were fed either HFD (60.9% fat, 21.8% carbohydrate, and 18.3% protein; D12492; Research Diets Inc., New Brunswick, NJ) or a normal control diet (ND) (Supplemental Table S1) for 6 or 12 months (Supplemental Table S3). To minimize genetic background differences, littermates were randomly assigned to receive either the HFD or the ND. The sample size was determined on the basis of the results of a pilot study conducted before designing the experiment. All animal experiments were approved by the Institute for Animal Experimentation of Tohoku University Graduate School of Medicine and performed following the Regulations for Animal Experiments and Related Activities at Tohoku University (2021-MdA-098).

Lipidomic Analysis

Frozen liver tissues (approximately 25 mg) were homogenized using the Shake Master NEO (Bio Medical Science, Sendai, Japan). Four-week-old mice were fed either HFD or ND for 12 months (Supplemental Table S2). The homogenate (100 μL) was transferred to a new tube. The subsequent steps were performed as previously described. Lipidomic analysis was conducted using supercritical fluid
chromatography coupled with triple quadrupole mass spectrometry, as previously described. Supercritical fluid chromatography coupled with triple quadrupole mass spectrometry experiments were performed using an Agilent 1260 Infinity II supercritical fluid chromatography system equipped with an Agilent 6470A triple quadrupole liquid chromatography—mass spectrometry system and an Agilent jet stream electrospray ionization interface (Agilent Technologies, Santa Clara, CA).

Glucose Tolerance Test and Insulin Measurement

Mice were fasted for 15 hours and then intraperitoneally administered a single dose of glucose (1 g/kg body weight). Tail vein blood samples were collected after 30 and 120 minutes to measure blood glucose concentrations using a glucometer (TERUMO, Tokyo, Japan). Serum insulin levels were quantified using an ultrasensitive mouse insulin enzyme-linked immunosorbent assay kit (M1104; Morinaga Institute of Biological Science Inc., Kanazawa, Japan). The homeostasis model assessment of insulin resistance was calculated using the following formula:

\[
\text{HOMA-IR} = \frac{\text{fasting glucose (mmol/L)} \times \text{fasting insulin (ng/mL)}}{22.4}
\]

Serologic Analysis

After a 15-hour fast, aortic blood was collected using a syringe. The blood samples were allowed to clot at room temperature for 1 hour and then centrifuged for 30 minutes at 1200 × g for serum collection. Aspartate aminotransferase, alanine transaminase, alkaline phosphatase, and lactate dehydrogenase were measured by the Japan Society of Clinical Chemistry metastable method. Total cholesterol and free cholesterol were measured using the cholesterol oxidase/N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline, sodium salt method.

Pathologic Analysis and Immunohistochemistry

Tissues collected from mice were immediately frozen or fixed with 10% buffered formalin and embedded in paraffin. A series of sections (4 μm thick) of the formalin-fixed, paraffin-embedded tissues were stained with hematoxylin-eosin. Liver sections were also stained with Masson trichrome to visualize elastic fibers and fibrosis. Frozen liver sections were stained with oil red O (40491; MUTO PURE CHEMICALS Co, Ltd, Tokyo, Japan) to assess lipid droplet accumulation. Images were acquired using Nikon eclipse Ni (Nikon, Tokyo, Japan).

To measure the islet volume in the pancreas, six hematoxylin-eosin—stained images (×100 magnification) of each individual were selected for analysis. The images were captured by Nikon eclipse Ni.

Immunohistochemical staining of formalin-fixed, paraffin-embedded samples was performed using the following primary antibodies; rabbit anti-DUSP6 (1:100; ab76310; Abcam; Cambridge, UK), mouse anti-cytochrome P450 4A (CYP4A; 1:500; sc-271983; Santa Cruz Biotechnology Inc., Dallas, TX), and rat anti-mouse CD8a (1:100; 4SM15; Thermo Fisher Scientific, Carlsbad, CA) with Histofine Simple Stain MAX-PO (Nichirei Biosciences, Tokyo, Japan), following the manufacturer’s protocol.

Immunohistochemical staining of frozen liver sections was performed using anti-DUSP6 antibody (1:100) and Cy 3—conjugated secondary antibody (1:100; A10520; Thermo Fisher Scientific). The images were acquired by using an all-in-one fluorescence microscope (BX-X800; KEYENCE, Osaka, Japan).

Evaluation of CD8+ T Cells

The five areas in the liver parenchyma with the highest number of CD8+ T cells (hot spots) were selected for analysis in immunohistochemically stained sections. The number of CD8+ T cells was counted at a higher magnification (×400), and the average number of lymphocytes in the five hot spots detected by CD8 was calculated.

NAFLD Activity Score

NASH diagnosis, based on the criteria by Bedossa and FLIP Pathology Consortium,22 uses the scoring of steatosis, activity, and fibrosis, which include hepatic steatosis (0 to 3), hepatocyte ballooning (0 to 2), and lobular inflammation (0 to 3). Hematoxylin-eosin—stained sections were analyzed by two pathologists (Y.S. and T.F.), blinded to the sample identification, following the criteria of the NASH Clinical Research Network.23

Patient Samples

Patient biopsy samples with proven NAFLD were collected at the Tohoku University Hospital between 2010 and 2014. The steatosis, activity, and fibrosis score was calculated as the sum of points for steatosis, lobular inflammation, and hepatocellular ballooning. The patients were classified into three groups: simple steatosis (1 to 2 points), borderline NASH (3 to 4 points), and NASH (5 to 8 points). These specimens were subjected to immunostaining for DUSP6. This study was approved by the Ethics Committee of Tohoku University School of Medicine (2013-1-120)

Western Blot Analysis

Total protein was extracted from tissues or cells using a mixture of lysis buffer (65 mmol/L Tris-HCl and 3% SDS), protease inhibitors (04693124001; Roche, Basel, Switzerland), and phosphatase inhibitors (04906837001; Roche). Protein concentrations were determined using a DC Protein Assay kit (5000113; Bio-Rad Laboratories, Inc., Hercules, CA). The protein samples were loaded onto a 10% SDS-PAGE gel (194 to 15021; FUJIFILM Wako Pure...
Chemical Corp., Osaka, Japan), transferred to a polyvinylidene difluoride membrane (WSE-4053; ATTO, Tokyo, Japan), and incubated with corresponding primary antibodies overnight at 4°C. After incubation with peroxidase-conjugated secondary antibodies, bounded antibodies were visualized in the ImageQuant LAS 4000 (GE Healthcare, Chicago, IL) and quantified using ImageJ software version 1.53 (NIH, Bethesda, MD; https://imagej.nih.gov). The following antibodies were used: mouse anti–β-actin (β-actin; 1:1000; A5441; Sigma-Aldrich, St. Louis, MO), rabbit anti-DUSP6 (1:1000; ab76310; Abcam), rabbit anti-p44/42 MAPK (Erk1/2; 1:1000; number 4695; Cell Signaling Technology, Inc., Danvers, MA), rabbit anti–phosphorylated p44/42 MAPK (Erk1/2; Thr202/Tyr204; 1:1000; number 4370S; Cell Signaling Technology, Inc.), mouse anti-p38 (1:1000; sc-81621; Santa Cruz Biotechnology, Inc.), mouse anti–phosphorylated p38 (1:1000; sc-166182; Santa Cruz Biotechnology, Inc.), mouse anti–jun-amino-terminal kinase (JNK) (1:1000; sc-7345; Santa Cruz Biotechnology, Inc.), mouse anti–phosphorylated JNK (1:1000; sc-6254; Sant Cruz Biotechnology, Inc.), mouse anti-CYP4A (1:1000; sc-4370S; Cell Signaling Technology, Inc.), rabbit anti-4370S; Cell Signaling Technology, Inc.), mouse anti-phosphorylated p44/42 MAPK (Erk1/2; Thr202/Tyr204; 1:1000; number 4370S; Cell Signaling Technology, Inc.).

Primary Mouse Hepatocyte Isolation

Primary hepatocytes were isolated from 6- to 8-week-old mice. Mice were anesthetized with isoflurane by nose cone method, and their vena cavae were perfused with Hank’s solution (H9394; Sigma-Aldrich), 0.5% 0.1 mmol/L EDTA, 1% 1 mol/L HEPES (5630-080; Thermo Fisher Scientific), 1% penicillin and streptomycin (15140 to 148; Thermo Fisher Scientific), 0.1% collagenase type IV (C5138; Sigma-Aldrich), collagenase type IV (C5138; Sigma-Aldrich), 1% 1 mol/L HEPES, 1% Pen Strep, 0.1% glucose, and 0.4% sodium bicarbonate. Subsequently, the liver was dissected, minced, and filtered using a 150-μm cell strainer (43 to 50150-03; plurSelect Life Science, Leipzig, Germany). The primary hepatocytes were separated by centrifugation at 50 × g for 2 minutes and purified in a 50% Percoll solution (17089102; Cytiva). The purified hepatocytes were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a 5% carbon dioxide/water—saturated incubator.

In Vitro Cell Model of Lipid Accumulation

Palmitic acid (PA; P0500; Sigma-Aldrich) and oleic acid (OA; O1008; Sigma-Aldrich) were dissolved in 0.01 mol/L NaOH, diluted in Dulbecco’s modified Eagle’s medium/10% fetal bovine serum with 25% bovine serum albumin (9048-46-8; Nacalai Tesque Inc., Kyoto, Japan), and added to the medium to achieve a final concentration of 0.5 mmol/L PA/1 mmol/L OA. The mitogen-activated protein kinase (MEK) inhibitor U0126 (U120; Sigma-Aldrich) was administrated 12 hours before the PA/OA mixture addition. The study protocol is illustrated in Supplemental Figure S1A.

After incubation for 24 hours, cells were stained with 60% oil red O (40491; MUTO PURE CHEMICALS Co, Ltd) for 30 minutes to visualize lipid accumulation. Ten areas from each slide were randomly selected for lipid content quantification using Halo software version 2.3.2089 (Indica Labs, Albuquerque, NM) after digitalization of the slides using a NanoZoomer scanner (Hamamatsu Photonics, Hamamatsu, Japan).

Transcriptome Sequencing and Analysis

Total RNA was isolated from primary hepatocytes challenged with or without a PA/OA mixture following 24 hours of incubation using the RNeasy Mini Kit (74104; Qiagen, Hilden, Germany) with RNase-Free DNase (79254; Qiagen). RNA purity, concentration, and integrity were evaluated using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). Transcriptome sequencing was performed by Macrogen Japan Corp. (Tokyo, Japan) using the TruSeq Stranded Total RNA Library Prep Gold Kit and NovaSeq 6000 system (Illumina, Inc., San Diego, CA). Fragments per kilobase per million mapped fragments values were calculated to estimate the expression level of genes in each sample. Hierarchical clustering heat maps were obtained using R version 4.2.1 (R Foundation for Statistical Computing, Vienna, Austria). The enrichment test was based on the Gene Ontology resource (http://geneontology.org, last accessed November 7, 2022) and was performed on a significant gene list using g:Profiler (https://biit.cs.ut.ee/gprofiler, last accessed November 7, 2022). Differentially expressed gene analysis was conducted using edgeR.

Cell Line, in Vitro Transfection, and Inhibitor Treatment

HepG2 (TKG 0205), a human liver cancer cell line, was provided by Cell Resource Center for Biomedical Research of the Institute of Development, Aging, and Cancer of Tohoku University (Sendai, Japan). THLE-2 (CRL-2706 TM), an immortalized human hepatocyte cell line, was purchased from ATCC (Manassas, VA). HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Thermo Fisher Scientific). THLE-2 cells were cultured in the BEGM Bullet Kit (CC-3170; Lonza Ltd, Basel, Switzerland) without gentamycin/amphotericin and epinephrine. THLE-2 cells were cultured in the BEGM Bullet Kit without gentamycin/amphotericin and epinephrine, supplemented with 5 ng/mL of human...
recombinant epidermal growth factor (Sigma-Aldrich), 70 ng/mL of phosphoethanolamine (Sigma-Aldrich), and 10% fetal bovine serum. Both cell lines were incubated at 37°C in a 5% carbon dioxide/water–saturated incubator.

HepG2 cells were treated with 1 or 10 mmol/L CYP4A inhibitor HET0016 (SML2416; Sigma-Aldrich) for 3 hours before adding the PA/OA mixture. After an additional incubation for 24 hours, oil red O staining was performed. The study protocol is shown in Supplemental Figure S1B.

To abrogate DUSP6 expression, HepG2 cells were transfected with 10 μmol/L of siRNA using Lipofectamine RNAiMAX (Thermo Fisher Scientific), following the manufacturer’s instructions. The sequence of DUSP6 siRNA oligonucleotides (Integrated DNA Technologies, Inc., Coralville, WI) was as follows: human DUSP6-siRNA sense, 5'-CUCUCUGGAAUCAGUGAACC-3'; and antisense, 5'-GGUCUUCACGAGAUGCGAGAGGUC-3'; human GL2-siRNA (Integrated DNA Technologies, Inc.) sense, 5'-CGUACCGCGAAUCUCCGAAUGTCT-3'; and antisense, 5'-GACAUUUGCGAAGUAUCGCCGACUG-3'.

After transfection with DUSP6 or luciferase (GL2) siRNA for 24 hours and treatment with MAPK inhibitor U0126 (U120; Sigma-Aldrich) for 12 hours, the PA/OA mixture was added to HepG2 cells. After 24 hours, HepG2 cells were stained with oil red O. The study protocol is shown in Supplemental Figure S1C.

Statistical Analysis

All data are presented as means ± SD. Differences between two groups were determined using a two-tailed t-test, and differences among three or more groups were evaluated using a two-way analysis of variance, followed by the Tukey post hoc test. P < 0.05 was considered statistically significant. All statistical analyses were conducted using JASP 0.16.3.0 (JASP Team, https://jasp-stats.org, last accessed October 16, 2022).

Data Deposition

The data presented in this publication have been deposited in National Center for Biotechnology Information’s Gene Expression Omnibus and are accessible through Gene Expression Omnibus Series accession number GSE216881 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE216881).

Results

Dusp6 Ablation Prevents HFD-Induced Obesity, Improves Insulin Sensitivity, and Maintains Glucose Homeostasis

To investigate the potential role of Dusp6 in regulating body weight and glucose homeostasis, HFD was fed to Dusp6-KO mice to mimic NAFLD/NASH in human pathology (Supplemental Table S3). HFD resulted in pronounced obesity in WT and HI mice compared with that in KO mice (Figure 1, A–C). Similarly, WT mice fed with ND exhibited increased weight gain compared with KO mice fed with ND (Figure 1, A–C). Under HFD-fed conditions, organ weight (heart, liver, kidney, and liver) relative to total body weight also increased in WT and HI mice compared with KO mice (Supplemental Figure S2, A–D). Furthermore, male WT mice and HI mice fed with HFD gained significantly more weight than female mice (Supplemental Figure S2E).

Because obesity is associated with insulin resistance, the effect of Dusp6 deficiency on glucose homeostasis was determined. Fasting glucose levels tended to be higher in HFD-fed mice compared with those in ND-fed mice (Figure 1D). Interestingly, irrespective of diet, KO mice displayed significantly lower fasting glycemia compared with WT and HI mice (Figure 1D). Glucose tolerance test revealed a significantly higher glycemia in WT and HI mice compared with KO mice for up to 120 minutes when fed with HFD (Figure 1D). Similarly, in ND-fed WT mice, a significant increase in fasting glucose and insulin levels was observed compared with those in KO mice (Supplemental Figure S2F). These results suggest that KO mice are more resistant to elevated blood glucose levels than WT and HI mice, indicating improved glucose tolerance (Figure 1D).

The HFD-fed mice showed higher homeostasis model assessment of insulin resistance scores compared with ND-fed mice after 12 months of feeding (Figure 1E). Within the ND-fed group, WT mice exhibited considerably higher homeostasis model assessment of insulin resistance scores than KO mice (Figure 1F). Moreover, compared with HFD-fed KO mice, HFD-fed WT and HI mice exhibited a greater islet volume that increased with HFD duration, followed by islet cell hyperplasia (Figure 1, G and H). Therefore, Dusp6 ablation may enhance insulin homeostasis and prevent insulin resistance–related islet cell hyperplasia.

Dusp6 Ablation Prevents NASH Development

Hepatic pathologic changes were assessed in mice fed with ND or HFD for either 6 or 12 months, according to the established NASH scoring system.22 Steatosis and lobular inflammation with hepatocyte ballooning, degeneration, and regeneration were observed in all HFD-fed WT and HI mice without significant differences between these two groups (Supplemental Table S4). HFD-fed WT and HI mice exhibited marked hepatic steatosis from the central zone to the middle zone, as shown in hematoxylin-eosin— and oil red O—stained liver sections, which was more pronounced after 12 months of HFD compared with 6 months of feeding (Figure 2, A–C). In contrast, KO mice did not develop hepatic steatosis even after 12 months of HFD, except for mild lipid accumulation in a few mice on HFD for 6 months (Figure 2, A–C). Lobular inflammation was observed in
most of the HFD-fed mice, with exacerbated inflammation in WT and HI mice after prolonged HFD administration; however, inflammation remained mild in KO mice even after 12 months of HFD (Figure 2D). The aggregation of inflammatory cells was predominantly located around the central veins and contained CD8^+ cells (Supplemental Figure S3). Hepatocyte ballooning was observed in all WT and some HI mice following HFD feeding but not in KO mice (Figure 2E). In addition, sporadic hepatic fibrosis extending from the centrilobular to the perisinusoidal zone was observed in a fraction of WT and HI mice of HFD fed for 12 months, whereas no fibrosis was observed in KO mice (Figure 2F). According to the steatosis, activity, and fibrosis scoring system, most HFD-fed WT and HI mice were diagnosed with NASH, whereas all examined KO mice remained healthy with no evidence of NASH (Figure 2G). Therefore, Dusp6 deletion completely prevented the development of NASH induced by HFD (Supplemental Table S4).

**Dusp6 Ablation Prevents Lipid Accumulation in Vivo**

Hepatic lipidomic analysis revealed a significant increase in the triacylglycerol and cholesterol levels in the liver of HFD-fed WT and HI mice. The baseline lipid levels in the liver tissues of HFD-fed KO mice were comparable to those in the livers of ND-fed WT mice (Figure 3A). Therefore, ablation of Dusp6 prevented HFD-induced lipid accumulation in the liver. Accordingly, diacylglycerol levels were significantly increased in the liver of HFD-fed WT and HI...
mice but not in KO mice (Figure 3B). Elevated diacylglycerol contributes to pancreatic β-cell dysfunction, leading to insulin resistance and/or insulin secretory dysfunction, which is consistent with the improved insulin sensitivity in Dusp6 KO mice. These lipidomic analyses further support the hypothesis that Dusp6 deficiency prevents hepatic steatosis. Moreover, total cholesterol and free cholesterol levels were significantly increased in the serum of HFD-fed WT mice but not in KO mice (Figure 3C). In addition, WT mice displayed a more severe liver injury than KO mice and the KO mice exhibited a better liver function even when fed with HFD, as evidenced by the serum aspartate aminotransferase and lactate dehydrogenase levels (Supplemental Figure S4).

Because DUSP6 is a specific phosphatase for ERK, inhibition of DUSP6 was supposed to result in the activation of ERK. Therefore, expression levels of DUSP6, ERK1/2, and phosphorylated ERK1/2 in the murine liver tissues were examined by immunoblotting. As expected, the liver tissues of KO mice showed marked elevation of ERK1/2 phosphorylation (Figure 3D). However, unexpectedly, ERK1/2 phosphorylation was enhanced by HFD in all groups, with the highest level observed in the KO mice (Figure 3D). Immunostaining revealed that DUSP6 was predominantly found in the cytoplasm of hepatocytes involved in steatosis (Figure 3E and Supplemental Figures S5 and S6). The expression of MAPKs other than ERK (ie, p38 and JNK) was slightly enhanced in the liver of HFD-fed mice (Supplemental Figure S7). Analysis of human liver biopsy specimens from patients with NAFLD also demonstrated increased DUSP6 accompanying disease progression (Supplemental Figure S8).

Figure 2  Deficiency of Dusp6 prevents the progression of nonalcoholic fatty liver disease. A: Representative histologic sections of the liver of mice stained with hematoxylin-eosin (H&E) after high-fat diet (HFD) feeding. B: Representative histologic images of the liver stained with oil red O and H&E from wild-type (WT) and Dusp6-knockout (KO) mice on HFD for 12 months. C–G: Quantification of hepatic steatosis (C), lobular inflammation (arrow; D), hepatocellular ballooning (arrow; E), fibrosis (blue staining; F), and steatosis, activity, and fibrosis (SAF) score (G) in the liver of mice fed with HFD [n = 4 in WT mice fed for 6 months; n = 12 in WT mice fed for 12 months; n = 8 in Dusp6-haploinsufficiency (HI) mice fed for 6 months; n = 13 in HI mice fed for 12 months; n = 7 in KO mice fed for 6 months; n = 8 in KO mice fed for 12 months]. Representative histologic images of the liver stained with H&E of mice fed with HFD for 12 months. Scale bar = 1 mm (A–F). Original magnification: ×200 (A–D and F); ×400 (E). NASH, nonalcoholic steatohepatitis; ND, normal diet.
Dusp6 Ablation Prevents Lipid Accumulation in Primary Hepatocytes in Vitro with Fatty Acids

To determine whether the inhibition of hepatic steatosis in Dusp6-deficient mice is a systemic effect associated with the suppression of obesity and hyperlipidemia, primary hepatocytes isolated from 6- to 8-week-old mice were treated with a PA and OA mixture in vitro. WT primary hepatocytes showed significant accumulation of lipid droplets, whereas KO primary hepatocytes showed minimal lipid accumulation 24 hours after the challenge (Figure 4A). The KO primary hepatocytes exhibited marked elevation of ERK1/2 phosphorylation with no expression of DUSP6 compared with WT and HI primary hepatocytes (Figure 4B). To determine if the activation of the ERK pathway is directly involved in preventing lipid accumulation in these KO hepatocytes, the authors incubated the cells with a specific MEK inhibitor, U0126. This resulted in a significant increase in lipid accumulation in cells challenged with the fatty acids, with no apparent toxic effect of U0126 (Figure 4, C and D). These results suggest that the prevention of lipid accumulation in Dusp6-KO hepatocytes may be mediated by the hyperactivation of ERK.

Down-Regulation of Cyp4a10 and Cyp4a14 in Dusp6-Knockout Hepatocytes

To explore the molecular pathways associated with the effect of Dusp6 in lipid accumulation in hepatocytes, transcriptome sequencing analyses using RNA extracted from primary hepatocytes treated or untreated with PA and OA mixture were conducted. The heat map of genes showing altered expressions (Supplemental Figure S9A) revealed numerous significantly up-regulated or down-regulated genes in Dusp6-KO hepatocytes compared with WT hepatocytes, with or without PA and OA treatment (Supplemental Tables S5 and S6). Gene Ontology enrichment analysis revealed that the altered genes were associated with broad biological processes, including cell-cell adhesion, cytokine production, organic acid production, extracellular structure organization, and lipid localization (Supplemental Figure S9, B and C). Dusp6 was included in the cluster of genes associated with fatty acid metabolism, suggesting a close association between these genes in transcriptional changes (Supplemental Figure S9A). Of particular interest were Cyp4a10 and Cyp4a14, which encode for CYP4A, known to promote ω-hydroxylation of fatty acids and hepatic steatosis. Both Cyp4a10 and Cyp4a14 were markedly down-regulated in Dusp6-KO primary hepatocytes. No changes were observed on fatty acid transporter proteins, such as FATP-1 (Slc27a1), FATP-4 (Slc27a4), and FAT/CD36, which are involved in fatty acid uptake, indicating that fatty acid absorption machinery may not be impaired in KO mice (Figure 5A). The down-regulation of CYP4A protein in primary KO hepatocytes was confirmed by Western blot analysis (Supplemental Figure S9D). Notably, adding PA/OA to the WT hepatocytes increased...
the expression of DUSP6 and CYP4A (Supplemental Figure S10). Consistently, CYP4A expression was significantly down-regulated in the liver tissue of KO mice compared with that of WT and HI mice (Figure 5B).

To confirm the role of CYP4A in lipid accumulation in a human liver-lineage cell, HepG2 was treated with a CYP4A inhibitor, HET0016, which resulted in a significant reduction in lipid accumulation (Figure 5C). These findings suggest that Dusp6 promotes lipid accumulation in hepatocytes through CYP4A.

**Figure 4**  
**Dusp6 deficiency prevents lipid accumulation in primary hepatocytes via extracellular signal-regulated kinase (Erk) activation.**  
**A:** Representative images of oil red O—and hematoxylin-stained primary hepatocytes of wild-type (WT) and Dusp6-knockout (KO) mice treated with palmitic acid (PA) and oleic acid (OA) for 24 hours.  
**B:** Western blot analysis of dual-specificity phosphatase 6 (Dusp6), Erk phosphorylation (p-Erk), and Erk levels in mouse primary hepatocytes.  
**C:** Representative images of oil red O—and hematoxylin-stained primary hepatocytes treated with mitogen-activated protein kinase kinase inhibitor, U0126.  
**D:** Western blot analysis of Erk phosphorylation and total Erk levels in primary hepatocytes treated with or without U0126.  
**A—D:** Statistical analysis was performed using two-tailed t-test. Data are presented as means ± SD (A—D). n = 3 per group (A, C, and D); n = 4 per group (B). *P < 0.05, ***P < 0.001. Scale bars = 1 mm (A and C). Original magnification, × 200 (A and C). HI, Dusp6 haploinsufficiency.

**Figure 5**  
**Dusp6 deficiency prevents lipid accumulation in primary hepatocytes through cytochrome P450 4A (Cyp4a) reduction.**  
**A:** Heat map of RNA sequencing showing the expression of obesity-related genes in primary hepatocytes treated with or without palmitic acid (PA) and oleic acid (OA) obtained from normal diet (ND)—fed wild-type (WT) or Dusp6-knockout (KO) mice.  
**B:** Immunohistochemistry images of Cyp4a in the liver tissues of WT, Dusp6—haploinsufficiency (HI), or KO mice fed with high-fat diet (HFD) or ND.  
**C:** Representative images of oil red O—and hematoxylin-stained cytologic sections of HepG2 cells treated with PA and OA mixture after adding CYP4A inhibitor, HET0016.  
**A—C:** Statistical analysis was performed using two-way analysis of variance. Data are presented as means ± SD (C). n = 3 per group (B). *P < 0.05. Scale bars = 1 mm (B and C). Original magnification: × 200 (B); ×400 (C). FPKM, fragments per kilobase per million mapped fragments.
Knockdown of DUSP6 Down-Regulates CYP4A Expression and Prevents Lipid Accumulation after Fatty Acid Challenge

THLE-2, a normal hepatocyte cell line, and HepG2, a hepatocellular carcinoma cell line, were examined to investigate the role of the DUSP6-CYP4A axis in hepatocyte fat deposition. DUSP6 and CYP4A were highly expressed in HepG2 cells (Figure 6A), whereas they were barely expressed in THLE-2 cells. Furthermore, PA/OA treatment promoted intracellular lipid accumulation in HepG2 cells but not in THLE-2 cells (Figure 6B). These results suggest that the DUSP6-CYP4A axis has a minimal function in THLE-2 cells, partly explaining the absence of lipid accumulation. To confirm the direct effect of DUSP6 on CYP4A expression, HepG2 cells were transfected with siRNAs targeting DUSP6. The knockdown of DUSP6 resulted in a down-regulation of CYP4A expression, suggesting that CYP4A may be a downstream target of DUSP6 (Figure 6C). This knockdown also induced elevation of ERK2 phosphorylation. To investigate whether CYP4A regulation by DUSP6 depends on the activation of ERK, HepG2 cells were treated with an MEK inhibitor, U0126. After 24 hours of U0126 treatment, ERK phosphorylation was down-regulated; however, CYP4A expression was not altered (Figure 6D). Although U0126 effectively reduced the phosphorylation of ERK, it showed minimal effect on p38 or JNK (Supplemental Figure S11). This indicates that DUSP6 maintains CYP4A expression independently of ERK activity.

**Figure 6**  
Dusp6 deficiency prevents lipid accumulation in primary hepatocytes via two distinct pathways: dual-specificity phosphatase 6 (DUSP6)—cytochrome P450 4A (CYP4A) axis and extracellular signal-regulated kinase (ERK) pathways.  
A: Western blot analysis of DUSP6 and CYP4A levels in HepG2 or THLE-2.  
B: Representative images of oil red O— and hematoxylin-stained cytologic sections of HepG2 and THLE-2 cells after 24 hours of exposure to palmitic acid (PA) and oleic acid (OA).  
C: Western blot analysis of DUSP6, ERK phosphorylation (p-ERK), ERK, and CYP4A levels in HepG2 cells transfected with DUSP6 (Si-DUSP6) or luciferase (GL2) siRNAs (Si-GL2).  
D: Western blot analysis of ERK phosphorylation, total ERK, and CYP4A levels in HepG2 cells treated with mitogen-activated protein kinase (MAPK) inhibitor, U0126.  
E: Representative images of oil red O— and hematoxylin-stained cytologic sections of HepG2 cells treated with mitogen-activated protein kinase (MAPK) inhibitor, U0126, after knockdown of DUSP6 or GL2 (control) by siRNA and exposure to PA and OA.  
F: Western blot analysis of DUSP6, ERK phosphorylation, total ERK, and CYP4A levels in HepG2 cells treated with MAPK inhibitor, U0126, after knockdown of DUSP6 or GL2 (control) by siRNA.  
A and C: Statistical analysis was performed using a two-way analysis of variance (C) or two-tailed t-test (A and C). Data are presented as means ± SD (A, C, and E). n = 3 per group (C and D); n = 2 in each experiment (E). *P < 0.05, **P < 0.01, and ***P < 0.001. Scale bars = 1 mm (B and E). Original magnification: ×200 (B, left column, and E); ×400 (B, right column). ACTB, β-actin.
Compared with the control (GL2) siRNA transfection, lipid accumulation was decreased in HepG2 cells transfected with DUSP6 siRNA (Figure 6E). However, after administration of U0126, HepG2 cells transfected with DUSP6 siRNA showed a significant increase in lipid accumulation (Figure 6E). CYP4A expression was decreased by the knockdown of DUSP6 in HepG2 cells (Figure 6F). Although U0126 effectively reduced ERK activity, CYP4A expression remained diminished after treatment (Figure 6F). These results indicate that the inhibition of lipid accumulation due to ablation of DUSP6 depends on ERK activation, independent of CYP4A.

**Discussion**

This study demonstrates that DUSP6 ablation efficiently prevents HFD-induced NAFLD through the down-regulation of CYP4A and activation of the MAPK signaling pathway. The down-regulation of CYP4A was independent of MAPK activity, as shown by the refractoriness of CYP4A expression to ERK inhibition. Therefore, DUSP6 plays a pivotal role in lipid homeostasis through two distinct pathways involving CYP4A and MAPK independently (Figure 7).

CYP4A catalyzes the ω-hydroxylation of saturated, branched-chain, and unsaturated fatty acids, such as arachidonic acid, palmitic acid, and lauric acid.25 In humans, CYP4A11, and in mice, CYP4A10 and CYP4A14, are responsible for the ω-hydroxylation of fatty acids in the liver.27 This study suggests that DUSP6 promotes CYP4A expression to induce hepatic steatosis independently of ERK activity. Wu et al28 reported that DUSP6 (MKP-3) mediates the dephosphorylation of forkhead box O1 (FOXO1) at Ser256 and promotes its nuclear translocation. Several consensus sequences for the FOXO1 binding site are present upstream of the Cyp4a gene. Therefore, it is possible that DUSP6 directly regulates CYP4A expression via FOXO1, which should be further investigated.

As opposed to previous reports,29 this study found no alteration in the expression of the lipid translocase FAT/CD36 between KO and WT mice. The reasons for this discrepancy are currently unknown, although it could be attributed to differences in mouse models, the type and source of HFD, animal housing conditions, laboratory environments, and sample sizes. Nevertheless, this study indicates the crucial role of the DUSP6-CYP4A axis in NAFLD.

HFD-fed Erk1-KO mice gain more fat and liver mass than WT mice, along with higher homeostasis model assessment of insulin resistance values.30 These findings are consistent with the current results, which show that ERK activation mediated by the ablation of DUSP6 prevents hepatic fat accumulation and insulin resistance. However, the downstream targets of activated ERK involved in the inhibition of hepatic steatosis are currently unknown and require further investigation, such as administering MAPK inhibitors to HFD-fed Dusp6-KO mice to observe NAFLD progression.

HFD feeding is a well-established method for developing an NAFLD model.31 In this study, HFD-induced extrahepatic pathologic conditions, including obesity, glucose intolerance, and islet cell hyperplasia, were observed in WT and Dusp6-HI mice but not in Dusp6-KO mice. Maintaining glucose homeostasis in Dusp6-KO mice likely inhibited pancreatic islet cell hyperplasia. Preventing obesity and improved glucose homeostasis may indirectly result in diminished hepatic steatosis. Therefore, suppressing NAFLD and NASH by DUSP6 ablation may involve synergistic, direct, and indirect effects. These results suggest that DUSP6 could be a potential prevention and therapeutic target for NAFLD and NASH, which could contribute to developing a novel treatment strategy.

DUSP6 has multifactorial functions associated with various disease settings, including conflicting roles in tumor development and progression, metabolic homeostasis, immunity, and inflammation.32 However, Dusp6-KO mice do not exhibit obvious phenotypic defects, maintain normal breeding, and live well past 1 year of age in normal conditions.18 These observations suggest that specific attenuation of DUSP6 may not cause severe health problems, but further studies are required to confirm this.

This study has the following limitations. First, it used a single strain of mice, so the results may be strain-specific. In addition, investigating the liver-specific deletion of DUSP6 could provide more specific insights into the role of DUSP6 in HFD-induced NASH, compared with a whole-body knockout. Although HFD-induced hepatic steatosis is a
conventional model for studying NAFLD, phenotypes can vary depending on the experimental design, including the age at which HFD is introduced. Validating the findings using multiple human hepatic lineage cell lines is necessary. Last, studying the therapeutic potential of a pharmacologic inhibitor of DUSP6 in attenuating hepatic steatosis is essential.

In conclusion, this study demonstrated the role of DUSP6 in regulating lipid metabolism and NAFLD progression, primarily through regulating the DUSP6-CYP4A signaling axis and the ERK pathway. This study highlights the potential of DUSP6 to be a specific target for the prevention and treatment of NAFLD, NASH, and metabolic diseases.

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Author Contributions

Y.S. and T.F. conceptualized the study; C.J., Y.S., K.I., X.W., S.H., and Y.I. conducted research; A.H. measured mouse liver lipid content; J.I. and A.M. collected patient samples; C.J., S.H., K.M., and Y.S. analyzed data; C.J. wrote the first draft of the manuscript; Y.S., M.G., T.I., and T.F. reviewed and edited the manuscript; Y.S. acquired funding; and C.J. supervised the different stages of the experiment. All authors have read and agreed to this manuscript.

Disclosure Statement

None declared.

Supplemental Data

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References

22. Bedossa P; FLIP Pathology Consortium: Utility and appropriateness of the fatty liver inhibition of progression (FLIP) algorithm and steatosis, activity, and fibrosis (SAF) score in the evaluation of biopsies of nonalcoholic fatty liver disease. Hepatology 2014, 60:565–575