ORIGINAL RESEARCH

SIRT6 Protects Against Liver Fibrosis by Deacetylation and Suppression of SMAD3 in Hepatic Stellate Cells

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SUMMARY

This work characterized a key function of Sirtuin 6 (SIRT6) in the regulation of liver fibrosis. Mechanistically, SIRT6 deacetylates and suppresses SMAD family member 3 in hepatic stellate cells. Gain- and loss-of-function data show that SIRT6 is a potential therapeutic target for hepatic fibrosis.

BACKGROUND & AIMS: Nonalcoholic steatohepatitis (NASH) is a chronic liver disease that is manifested clinically by an increase in hepatic triglycerides, inflammation, and fibrosis. The pathogenesis of NASH remains incompletely understood. Sirtuin 6 (Sirt6), a nicotinamide adenine dinucleotide–dependent deacetylase, has been implicated in fatty liver disease; however, the underlying molecular mechanisms in the NASH pathogenesis are elusive. The aims of this study were to elucidate the role of hepatic Sirt6 in NASH.

METHODS: Wild-type, liver-specific Sirt6 knockout (KO), hepatic stellate cell (HSC)-specific Sirt6 knockout (HSC-KO), and Sirt6 transgenic mice were subjected to a Western diet for 4 weeks. Hepatic phenotypes were characterized and underlying mechanisms were investigated.

RESULTS: Remarkably, both the liver-KO and HSC-KO mice developed much worse NASH than the wild-type mice, whereas the transgenic mice were protected from the diet-induced NASH. Our cell signaling analysis showed that Sirt6 negatively regulates the transforming growth factor β–Smad family member 3 (Smad3) pathway. Biochemical analysis showed a physical interaction between Sirt6 and Smad3 in hepatic stellate cells. Moreover, our molecular data further showed that Sirt6 deacetylated Smad3 at key lysine residues K333 and K378, and attenuated its transcriptional activity induced by transforming growth factor β in hepatic stellate cells.

CONCLUSIONS: Our data suggest that SIRT6 plays a critical role in the protection against NASH development and it may serve as a potential therapeutic target for NASH. (Cell Mol Gastroenterol Hepatol 2020;10:341–364; https://doi.org/10.1016/j.jcmgh.2020.04.005)

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Nonalcoholic fatty liver disease (NAFLD) is an epidemic chronic liver disease that begins with simple steatosis and gradually progresses to nonalcoholic steatohepatitis (NASH), cirrhosis, and even liver cancer. One of the common risk factors for NAFLD is overweight or obesity. Excessive energy intake is stored as triglycerides not only in the adipose tissue, but also in the liver and other tissues. Hepatic lipid accumulation can cause liver injury and cellular function impairment by an increase in reactive oxygen species, endoplasmic reticulum stress, and other tissue damage responses. Hepatic tissue injury also triggers immune responses including the recruitment of circulated and resident macrophages and neutrophils, which lead to inflammation in the liver. Under the inflammatory conditions, hepatic cells secrete various cytokines and chemokines including transforming growth factor β (TGFβ) and chemokine (C-C motif) ligands 2 and 5. TGFβ is a potent fibrogenic factor that can trigger the activation of quiescent hepatic stellate cells (HSCs). On TGFβ binding to its receptors 1/2, the receptor kinase phosphorylates and activates SMAD2 and SMAD3 transcription factors. Either SMAD family member 2 (SMAD2) or SMAD3 can form heterodimers with SMAD4 and then translocate to the nucleus for activation of numerous target genes including fibrogenesis genes such as ACTA2, COL1A1, and COL3A1. In addition to phosphorylation, SMAD3 also is regulated positively by lysine acetylation, especially at lysine 378.

It is believed that interactions between environmental factors such as diet and epigenetics play an important role in the development of NAFLD. As a key epigenetic regulator, sirtuin 6 (SIRT6) has been implicated in the control of glucose and lipid metabolism, anti-oxidative stress, and anti-inflammation. SIRT6 is a nicotinamide adenine dinucleotide–dependent deacetylase/deacylase. In mice leads to hepatic steatosis and inflammation. In addition, Sirt6 whole-body knockout mice develop fibrosis in the heart, liver, kidneys, and lungs. It has been suggested that regulation of the TGFβ–SMAD2/3 pathway by SIRT6 plays a role in tissue fibrosis. However, the function and mechanism of SIRT6 in hepatic stellate cells remains unclear. In this work, we set out to illustrate the pathophysiological function of Sirt6 in mouse and human HSCs.

Results

Hepatic SIRT6 Is Decreased in NASH Patients and Diet-Induced NASH Mice

To examine the role of SIRT6 in human NASH pathogenesis, we analyzed hepatic SIRT6 protein levels in controls and patients with NASH. Hepatic SIRT6 was decreased by 3-fold in those with NASH compared with controls (Figure 1A), suggesting that SIRT6 might be involved in human NASH pathogenesis. To further examine whether SIRT6 is decreased progressively during fibrogenesis, we stained SIRT6 in liver biopsy specimens from NASH patients with different stages of NASH progression from simple steatosis to different degrees of hepatic fibrosis and cirrhosis. Our data showed that as the disease advanced to fibrosis and cirrhosis, nuclear SIRT6 levels were reduced markedly (Figure 1B), suggesting a potential role of SIRT6 in liver fibrosis. We also determined SIRT6 transcript levels by real-time polymerase chain reaction (PCR) analysis and found a significant decrease in hepatic SIRT6 messenger RNA (mRNA) levels in NASH patients (Figure 1C).

To assess the status of Sirt6 in NASH development in animal models, we fed wild-type (WT) C57BL6/J mice either a control or Western diet (WD) for 4 and 8 weeks and analyzed hepatic Sirt6 and Smad3. Our data showed that a WD induced a significant increase in hepatic steatosis and fibrosis at 4 weeks, and more so at 8 weeks (Figure 2A). Hepatic Sirt6 was decreased significantly and Smad2 and Smad3 phosphorylation levels were increased in the WD-treated mice (Figure 2B). Moreover, hepatic Smad3 acetylation was increased in the livers of the WD-treated mice (Figure 2C). These data suggest that hepatic Sirt6 is down-regulated during NASH development in both human beings and mice. Because HSCs are critical for hepatic fibrogenesis, we also examined Sirt6 gene expression during HSC activation using mouse primary HSCs. Within 5 days of culture, primary HSCs were activated, as shown by an induction of Col1a1 and Acta2 genes. During the same time course, Sirt6 was decreased at both mRNA and protein levels (Figure 3A–D). In addition, Sirt6 mRNA levels also were decreased in the TGFβ-treated primary HSCs (Figure 3E). Taken together, our data suggest that Sirt6 might be involved in HSC activation and liver fibrosis.

Hepatic Sirt6 Protects Against Diet-Induced NASH in Mice

To further investigate the role of Sirt6 in the development of NASH, we generated liver-specific Sirt6 knockout (KO; Alb–Cre-mediated) and whole-body Sirt6 transgenic (Tg) mice. As shown by Western blot analysis, Sirt6 was ablated specifically in the liver of KO mice and over-expressed in both the liver and heart in Tg mice (Figure 4A). To model NASH development, we fed WT, KO, and Tg mice with a WD for 4 weeks. Although body weight did not change significantly, liver weight and the liver-to-body weight ratio were increased remarkably in the KO mice, but completely normalized in the Tg mice (Figure 4B–G). Liver morphology showed a typical fatty liver phenotype in the
KO mice and no abnormal appearance in the Tg mice (Figure 4H). Biochemical analysis of hepatic triglycerides also showed a 2-fold increase in the KO mice and normalization in the Tg mice (Figure 4I). H&E staining and quantification of liver sections also confirmed that hepatic steatosis was much worse in the KO mice than in the WT mice, but normalized in the Tg mice (Figure 5A and B).

To assess hepatic inflammation, we performed immunohistochemistry for F4/80 (a macrophage marker) and myeloperoxidase (a neutrophil marker). Both markers were highly increased in the KO livers and nearly normal in the Tg mice (Figure 5A and B). Real-time PCR analysis showed that inflammatory cytokine genes including Il1b and Tnf were increased significantly in the KO livers and decreased dramatically in the Tg livers compared with the WT livers (Figure 5C and D). Serum alanine aminotransferase (ALT) levels were increased by 50% in the KO mice but nearly normal in the Tg mice compared with the WT mice (Figure 5F).

To examine hepatic fibrosis, we first performed Sirius Red staining of liver sections. Our data showed that KO mice developed much worse fibrosis than the WT mice, whereas the Tg mice were completely protected against fibrosis (Figure 6A and B). This fibrosis phenotype was corroborated by expression of fibrogenesis genes including Col1a1, Acta2, Tgfb1, and Tgfb1 (Figure 6C). To explore potential mechanisms, we analyzed Smad3 phosphorylation and acetylation, indicators of Smad3 activation. Our data showed that both modifications were increased in the KO, but decreased in the Tg, livers (Figure 6D). To examine whether there was any indication of epithelial–mesenchymal transition, we analyzed 2 common markers in the livers of WT and KO mice. Our data showed that E-cadherin was decreased by 75% and vimentin was increased by 4-fold in the KO livers (Figure 6E), suggesting that Sirt6 deficiency promotes epithelial–mesenchymal transition in the liver.

**Sirt6 Deficiency Triggers Inflammation in Hepatocytes and Fibrogenesis in HSCs**

A recent report showed that Alb-Cre is active in both hepatocytes and HSCs, therefore we also analyzed Sirt6 gene deletion in primary hepatocytes and HSCs in our Alb-Cre–mediated Sirt6 KO mice. Indeed, Alb-Cre led to 70% Sirt6 ablation in HSCs, whereas it mediated 90% Sirt6 deletion in hepatocytes (Figure 6A and B). Immunofluorescent microscopy also confirmed the Sirt6 deletion in HSCs and hepatocytes (Figure 7C and D). In addition, we also examined Sirt6 protein in cholangiocytes by staining liver sections. Our data showed that Sirt6 was depleted in only a small fraction of cholangiocytes in the KO mice (Figure 7E).
HSCs showed that a number of cytokine and chemokine genes including Ccl2, Tgfb1, Ccl5, and Tnf were highly increased in the hepatocytes whereas fibrogenesis genes such as Col1a1, Col4a1, Timp1, and Tgfb1 were increased remarkably in the Sirt6-deficient HSCs compared with their WT counterparts (Figure 8A and B). We also performed coculture experiments to test potential interactions between primary hepatocytes and HSCs. Although Sirt6-deficient HSCs did not further induce cytokine and chemokine gene expression in the KO hepatocytes, Sirt6-deficient hepatocytes enhanced fibrogenic gene expression in the KO HSCs (Figure 8C and D).

**Figure 3. Sirt6 is down-regulated during HSC activation.** (A–C) Real-time PCR analysis of Col1a1, Acta2, and Sirt6 mRNAs in mouse primary HSCs after culture for 1–5 days (n = 3). (D) Western blot analysis of Sirt6 in mouse primary HSCs after culture for 1–5 days. (E) Real-time PCR analysis of Col1a1, Acta2, and Sirt6 mRNAs in mouse primary HSCs in the absence or presence of TGFβ1 (5 ng/mL) for 24 hours (n = 4). Data are presented as means ± SEM. **P < .01 and ***P < .001 vs control. Actn, actinin; p-Smad, phospho-SMAD family member 2.

**Figure 2.** (See previous page). Hepatic Sirt6 is down-regulated during NASH development in mice. (A) H&E, Sirius Red staining, and immunofluorescence analysis of myeloperoxidase (MPO) and quantification of liver sections of control and WD-treated mice (4- to 6-month-old males, n = 4). (D) Western blot and quantification analysis of Sirt6, p-Smad2, Smad2, p-Smad3, and Smad3 in the livers of control and WD-treated mice. (C) Western blot and quantification of Smad3 acetylation (Ac-K-Smad3) in the livers of control and WD-treated mice. Data are presented as means ± SEM. *P < .05, **P < .01, and ***P < .001 vs control; #P < .05, and ##P < .01 vs WD_4w.

**Sirt6 Deficiency in HSCs Increases the Susceptibility to Diet-Induced NASH in Mice**

To investigate the in vivo function of Sirt6 in HSCs, we generated Sirt6 HSC-KO mice by crossing the floxed Sirt6 mice with a lecithin retinol acyltransferase (Lrat)-Cre line. Our data showed that Sirt6 was deleted specifically in HSCs, but not hepatocytes or Kupffer cells (Figure 9A–D). Although there was no difference in body weight between WT and HSC-KO mice, both liver weight and liver-to-body weight ratio were increased significantly in the HSC-KO mice after 4 weeks of WD feeding (Figure 9E–G). HSC-KO mice had much worse hepatic inflammation, liver injury,
and fibrosis than WT mice, as indicated by histologic markers, serum ALT, and HSC gene expression (Figure 10A–E). We also analyzed Smad3 phosphorylation in HSC-KO livers and HSCs and observed that phosphorylated Smad3 levels were increased remarkably (Figure 10F). Fluorescent microscopy also confirmed an increase in Smad3 phosphorylation and nuclear translocation in HSC-KO mouse primary HSCs (Figure 10G).

Figure 4. Sirt6 protects the liver from diet-induced hepatic steatosis. (A) Western blot analysis of Sirt6 protein in WT, Sirt6-KO, and Sirt6-Tg mouse liver and heart tissues. (B and C) Body weight measurements for Sirt6-KO vs WT and Sirt6-Tg vs WT mice (n = 5, 4- to 6-month-old males). (D and E) Liver weight measurements (n = 5). (F and G) Liver-to-body weight ratios (n = 5). (H) Gross images of livers of WT, Sirt6-KO, and Sirt6-Tg mice fed with WD for 4 weeks (4- to 6-month-old males). (I) Hepatic triglyceride (TG) measurements (n = 4). Data are presented as means ± SEM. *P < .05, **P < .01, and ***P < .001 for WD vs control for the same genotype; *P < .05, **P < .01, and ***P < .001. Actn, actinin.
Sirt6 Represses the TGFβ-SMAD3 Pathway in HSCs

To further examine the role of SIRT6 in HSCs, we selected the human HSC cell line LX-2, which is used commonly for hepatic fibrosis study. First, we knocked down the SIRT6 gene in the LX-2 cells using short hairpin RNAs (shRNAs) and cultured the cells in the absence or presence of TGFβ. Knockdown of SIRT6 significantly enhanced TGFβ-stimulated SMAD3 phosphorylation (Figure 11A). Second, we performed SIRT6 overexpression in the LX-2 cells. Western blot data showed that overexpression of SIRT6 significantly decreased the TGFβ-stimulated SMAD3 phosphorylation (Figure 11B). Third, we overexpressed catalytically inactive SIRT6 mutant (H133Y) in the LX-2 cells. Our data showed that the TGFβ-stimulated SMAD3 phosphorylation was not decreased by the mutant SIRT6 (Figure 11E), suggesting that the Sirt6 catalytic activity is required for the regulation of SMAD3 phosphorylation. Expression of fibrogenic genes including COL1A1, COL3A1, ACTA2, and TIMP1 in the LX-2 cells also was consistent with the negative regulation of SMAD3 by SIRT6. Wild-type but not mutant SIRT6 overexpression repressed those fibrogenesis genes whereas SIRT6 knockdown enhanced their expression (Figure 11C, D, and F).

To further understand the molecular regulation of SMAD3 by SIRT6, we performed co-immunoprecipitation using either SIRT6 or phosphorylated SMAD3 antibodies. Our data showed that SIRT6 indeed interacted with both SMAD3 and its phosphorylated form (Figure 12A and B). We also performed affinity purification of Sirt6 protein complexes in liver lysates by taking advantage of the HA-tagged Sirt6 transgene in our Tg mice. Indeed, Sirt6-hemaggulitin (HA) co-purified with Smad3 and FoxO1 (a positive control) (Figure 12C). Fluorescent microscopy also showed nuclear co-localization of SIRT6 and SMAD3, especially after TGFβ stimulation (Figure 12D and F).

To analyze the effect of SIRT6 on SMAD3 acetylation, we knocked down or overexpressed wild-type or mutant SIRT6 (H133Y) and immunoprecipitated SMAD3 for acetylation analysis. Our data showed that SIRT6 knockdown increased SMAD3 acetylation whereas overexpression of WT but not mutant SIRT6 decreased SMAD3 acetylation (Figure 13A and B), suggesting that SIRT6 catalytic activity is required for SMAD3 deacetylation. To verify the interaction between SIRT6 and SMAD3 in the chromatin context, we performed chromatin immunoprecipitation (ChIP)–PCR analysis of 2 known SMAD3 target genes: TGFBI and COL1A2 in LX-2 cells. Our data showed that SIRT6 and acetylated histone H3 lysine 9 (a substrate of SIRT6) both were enriched in the SMAD3 binding sites in the TGFBI and COL1A2 gene promoters (Figure 13C). To further characterize which lysine acetylation in SMAD3 is regulated by SIRT6, we mutated 2 previously reported lysine residues, K333 and K378, to arginine that cannot be acetylated. Our data showed that both mutations reduced the SMAD3 acetylation levels and K378 seemed to be a major deacetylation site by SIRT6 (Figure 13D). Real-time PCR analysis further confirmed that acetylation of both K333 and K378 was involved in the SMAD3 transcriptional activity and deacetylation by SIRT6 markedly attenuated the SMAD3 transcriptional activity (Figure 13E).

Discussion

SIRT6 has been implicated in multiple biological processes including glucose and lipid metabolism, inflammation, anti-oxidative stress, DNA repair, and tumor suppression. In this work, we reported that SIRT6 is also a key regulator for liver fibrosis. Because NAFLD is a progressive liver disease, our data suggest that SIRT6 is involved in nearly all stages of NAFLD pathogenesis: from steatosis to inflammation and fibrosis. The major finding of this study is the characterization of the key role of SIRT6 in the regulation of TGFβ–SMAD3 signaling in HSCs. Initially, we set out to investigate the Sirt6 function in hepatocytes. Interestingly, our data have shown that Alb–Cre also leads to the Sirt6 gene deletion in HSCs in addition to hepatocytes, although not as efficiently as in hepatocytes. Thus, we believe that the liver phenotype in our Alb–Cre–mediated Sirt6–KO mice is attributable to Sirt6 deficiency in both hepatocytes and HSCs. Previously, it has been shown that Sirt6 promotes hepatic triglyceride homeostasis through inhibition of de novo lipogenesis and activation of fatty acid oxidation by histone H3 deacetylation; however, whether Sirt6 is deleted or not in HSCs was not examined. Sirt6 also inhibits cholesterol biosynthesis by repression of the master regulator sterol regulatory element-binding protein 2 (SREBP2) and its target genes. Sirt6 also represses the expression of the PCSK9 gene to reduce the levels of low-density lipoprotein cholesterol. Hepatic Sirt6 deficiency leads to increased cholesterol and triglyceride levels in the blood and liver as well. Therefore, hepatic steatosis in Sirt6–KO mice is attributed mainly to Sirt6 deficiency in hepatocytes. With regard to liver fibrosis, Sirt6 function in HSCs plays a major role because the Sirt6 HSC-KO mice develop remarkable hepatic fibrosis after 4 weeks of Western diet feeding. It has been reported that the Alb–Cre–mediated Sirt6 KO mice also developed NASH on a high-fat, high-fructose diet for 16 weeks. Sirt6 expression in HSCs was not examined in that report. The investigators attributed the NASH phenotype in the Sirt6 KO mice to the antioxidative stress function of Sirt6 by regulation of nuclear factor erythroid 2–related factor 2. In this work, we used a different NASH diet that contains 20% kcal from protein, 40% kcal from carbohydrates (mainly corn...
Figure 6. Sirt6 protects against diet-induced hepatic fibrosis. (A and B) Sirius Red staining of liver sections and quantification of the positive staining area in the livers of WT, Sirt6-KO, and Sirt6-Tg mice fed with a WD for 4 weeks (n = 4, 4- to 6-month-old males). (C) Real-time PCR analysis of fibrosis-related genes including Col1a1, Acta2, Tgfb1, and Tgfbr1 in the livers of WT, Sirt6-KO, and Sirt6-Tg male mice fed with a WD for 4 weeks (n = 4). (D) Western blot analysis and quantification of Smad3, p-Smad3, and acetylated-Smad3 (Ac-K-Smad3) in the livers of WT, Sirt6-KO, and Sirt6-Tg mice. (E) Western blot analysis and quantification of E-cadherin and vimentin in the livers of WT and Sirt6-KO mice. Data are presented as means ± SEM. *P < .05, **P < .01, and ***P < .001 for WD vs control for the same genotype; #P < .05, ##P < .01, and ###P < .001. Actn, actinin; p-Smad, phospho-SMAD family member 2.
Figure 7. Alb–Cre leads to Sirt6 gene deletion in both hepatocytes and HSCs in the Sirt6f/f:Alb–Cre mouse liver. (A and B) Western blot and quantification analysis of Sirt6, albumin, and desmin in primary hepatocytes (HEPs) and HSCs from WT and Sirt6 KO mice induced by either Mx1–Cre or Alb–Cre. (C) Immunofluorescence microscopy analysis of Sirt6 and albumin in the WT and the Alb–Cre–mediated Sirt6-KO hepatocytes. (D) Immunofluorescence microscopy analysis of Sirt6 and desmin in the WT and the Alb–Cre–mediated Sirt6-KO HSCs. Data are presented as means ± SEM. *P < .05, **P < .01, and ***P < .001 vs WT; #P < .05, ###P < .01, and ###P < .001 for Alb–Cre KO vs Mx1–Cre KO. Actn, actinin; DAPI, 4’,6-diamidino-2-phenylindole; CK-19, cytokeratin 19.

Figure 8. Gene expression analysis of Sirt6-deficient hepatocytes and HSCs. (A–D) Real-time PCR analysis of selected genes in monocultured or cocultured hepatocytes (HEPs) and HSCs. Data are presented as means ± SEM. *P < .05, **P < .01, and ***P < .001 vs WT or WT/WT; #P < .05, ##P < .01, and ###P < .001.
Figure 9. Characterization of HSC-specific Sirt6 knockout mice. (A) Western blot analysis of Sirt6 protein in primary hepatocytes (HEPs), HSCs, and Kupffer cells (KCs) from WT and HSC-KO mice. (B–D) Immunofluorescence analysis of Sirt6 and cell-type–specific markers in HSCs, hepatocytes, and KCs isolated from WT and Lrat-Cre–mediated HSC-KO mice (n = 5, 3- to 4-month-old males). (E–G) Measurements of body and liver weights of WT and HSC-KO male mice (n = 5). Data are presented as means ± SEM. **P < .01 and ***P < .001.
starch and sucrose), 40% kcal from fat (mainly cocoa butter), 1.25% cholesterol (by weight), and 0.5% sodium cholate (by weight), and treated mice for 4 to 8 weeks. On this diet, Sirt6KO mice developed the NASH phenotype more rapidly than with the high-fat, high-fructose diet used by the Park group. Another reason why we chose to treat the WT and KO mice for 4 to 8 weeks but not much longer is because hepatic Sirt6 gene expression is down-regulated rapidly by the NASH diet in WT mice. This down-regulation of Sirt6 diminishes the gene knockout effect in the long term.

TGFβ is thought to be the most potent cytokine for the induction of hepatic fibrosis. Although the canonical TGFβ–SMAD3 pathway has been well established, the mechanism of epigenetic regulation of SMAD3 activity remains incompletely understood, especially in HSCs. Previous reports have suggested that Sirt6 modulates the TGFβ–SMAD3 pathway23–25; however, HSC-specific Sirt6 function has not been investigated before. Our data suggest that Sirt6 is a key regulator of the SMAD3 transcriptional activity through deacetylation of SMAD3 and histone H3 lysine 9 in HSCs. SMAD3 can be acetylated at multiple lysine residues by p300/CREB binding protein (CBP).5,45 Our data have shown that K333 and K378 of SMAD3 are major substrates of Sirt6. Deacetylation of either of them by Sirt6 has a significant negative effect on the SMAD3 transcriptional activity. Because the SMAD3 acetylation analysis was performed under Sirt6 gain-of-function and loss-of-function conditions, additional physiological conditions may be considered in future studies.

An excessive accumulation of hepatic lipids is believed to cause liver injury. This is also the case in animals with chronic WD feeding. Hepatic injury can trigger inflammation, as manifested by the increased numbers of macrophages and neutrophils in the Sirt6-deficient mouse livers. As a result, a number of inflammatory cytokine and chemokine genes, including TNFα, TGFβ3, IL1β, Ccl2, and Ccl5, are activated. The resultant inflammatory microenvironment activates conversion of quiescent HSCs to activated HSCs (also known as myofibroblasts). The activated myofibroblasts produce excess collagen molecules to cause fibrosis. In addition, Sirt6-deficient myofibroblasts also lose the control of TGFβ gene regulation. This reinforces the vicious cycle of TGFβ → TGFβ receptor → SMAD3 → TGFβ.

The complete reversal of NASH in our Tg mice suggests that Sirt6 is a useful therapeutic target for NASH. Because the Sirt6 Tg mice used in this study were generated using the ubiquitous cytomegalovirus-Cre, additional characterization of other cell types in the liver including liver sinusoidal endothelial cells, cholangiocytes, Kupffer cells, and macrophages is needed to fully understand the cell-type-specific role of Sirt6 in the protection against NASH.

In summary, our data suggest that Sirt6 plays a critical role in HSCs by modulating the SMAD3 activity and histone H3 acetylation. Hepatic Sirt6 deficiency poses a high susceptibility to diet-induced NASH development. Modulation of Sirt6 activity can be a potential therapeutic strategy for NASH treatment.

Materials and Methods

Animals

All animal procedures performed in this work were approved by the Institutional Animal Care and Use Committee of Indiana University School of Medicine in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. Liver-specific Sirt6 KO mice were generated by crossing Sirt6 floxed mice with albumin-Cre Tg mice as previously described. Inducible hepatic Sirt6 gene knockout also was generated by crossing the Sirt6 floxed mice with Mx1-Cre transgenic mice from the Jackson Laboratory (Bar Harbor, ME). We confirmed the efficiency of deletion of the floxed Sirt6 coding sequence in the Sirt6fl/fl:Mx1-Cre−/− mouse liver after 3 intraperitoneal injections of polyinosinic-polycytidylic acid every other day, which induces an interferon response to activate expression of the Cre recombinase under the Mx1 gene promoter. The induced ablation of hepatic Sirt6 was nearly complete 10 days after the last polyinosinic-polycytidylic acid injection. Sirt6 HSC-KO mice were generated by crossing the Sirt6 floxed mice with Lrat-Cre mice. Sirt6 global Tg mice were generated by crossing an inducible Sirt6 Tg mouse carrying a floxed transcriptional/translational stop (STOP) cassette at the Rosa 26 locus with a cytomegalovirus-Cre transgenic mouse from the Jackson Laboratory. Animals were fed either a control diet (Teklad Diets 2018SX: 24% calories from protein, 18% calories from fat, and 58% calories from carbohydrate, Indianapolis, IN), or a WD (Research Diets D12109C, 20% calories from protein, 40% calories from fat, 40% calories from carbohydrate, and 1.25% cholesterol and 0.5% sodium cholate by weight, New Brunswick, NJ). Both males and females were used in the experiments and they were on the mixed background (C57BL/6J and 129/sv). At the end of the experiments, the animals were killed for blood and tissue collection.

Human Liver Specimens

Human liver samples were obtained from diagnosed NASH patients and control subjects. Paraffin-embedded

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**Figure 10.** (See previous page). HSC-specific Sirt6 KO mice develop NASH. (A and B) H&E, myeloperoxidase (MPO), F4/80, Sirius Red, and CoI1 staining and quantification of liver sections of WT and HSC-KO mice (n = 5, 3- to 4-month-old males) fed with a WD for 4 weeks. (C) Serum ALT measurements of WT and HSC-KO male mice fed with a WD for 4 weeks (n = 5). (D) Real-time PCR analysis of selected genes in primary HSCs from WT and HSC-KO mice fed with a WD for 10 days (n = 3, 3-month-old males). (E) Real-time PCR analysis of selected genes in livers from WT and HSC-KO mice fed with a WD for 4 weeks. (F and G) Smad3 and p-Smad3 analyses (immunoblotting or fluorescence microscopy) in the livers of WT and HSC-KO male mice fed with a WD for 4 weeks and primary HSCs from WT and HSC-KO mice fed with a WD for 10 days. Data are presented as means ± SEM. *P < .05, **P < .01, and ***P < .001 vs WT. DAPI, 4',6-diamidino-2-phenylindole; p-Smad3, phospho-SMAD family member 3.
Figure 11. (See previous page). SIRT6 suppresses the TGFβ–SMAD3 pathway in HSCs. (A) Western blot analysis and quantification of SMAD3 and p-SMAD3 in LX-2 cells that were transduced with adenoviral GFP or SIRT6 shRNA (sh-GFP or sh-SIRT6) in the absence or presence of TGFβ1 (5 ng/mL) for 24 hours. (B) Western blot analysis and quantification of SMAD3 and p-SMAD3 in LX-2 cells that were transduced with adenoviral GFP or SIRT6 (AdGFP or AdSIRT6) in the absence or presence of TGFβ1 (5 ng/mL) for 24 hours. (C) Real-time PCR analysis of selected fibrosis genes in LX-2 cells that were transduced with adenoviral sh-GFP, sh-SIRT6, AdGFP, or AdSIRT6 in the absence or presence of TGFβ1 (5 ng/mL) for 24 hours. Data are presented as means ± SEM. *P < .05, **P < .01, and ***P < .001 for TGFβ1 treatment vs control for the same protein or gene; #P < .05, ##P < .01, and ###P < .001 for TGFβ1 treatment vs control for the same protein or gene. ACTN, actinin; AdGFP, adenoviral green fluorescent protein; GFP, green fluorescent protein; p-SMAD3, phospho-SMAD family member 3.

Figure 12. SIRT6 interacts with SMAD3 in hepatic stellate cells. (A and B) Co-immunoprecipitation analysis of interactions between the endogenous SIRT6 and SMAD3 or p-SMAD3 in LX-2 cells in the presence of TGFβ1 (5 ng/mL) for 24 hours. (C) Co-immunoprecipitation analysis of interactions between Sirt6 and Smad3/p-Smad3 in the livers of Sirt6 transgenic mice fed with a WD for 4 weeks. (D) Immunofluorescence microscopy of SIRT6 and SMAD3 in LX-2 cells in the absence or presence of TGFβ1 (5 ng/mL) for 3 hours. (E) Immunofluorescence microscopy of SIRT6 and p-SMAD3 in LX-2 cells in the presence of TGFβ1 (5 ng/mL) for 3 hours. Ab, antibody; DAPI, 4',6-diamidino-2-phenylindole; Flag, FLAG tag; HA, hemagglutinin; IP, immunoprecipitation; PCNA, proliferating cell nuclear antigen; p-SMAD3, phospho-SMAD family member 3.
liver sections from patients with simple steatosis and different stages of fibrosis by histopathologic diagnosis of NASH were obtained under the institutional review board–approved protocol at the Indiana University–Purdue University Indianapolis. General characteristics of the NASH patients are described in Table 1.

### Plasmid Constructs and Adenoviral Vectors

Human SIRT6 and SIRT6 H133Y mutant (catalytically inactive) and green fluorescent protein (GFP) coding sequences were cloned into a pcDNA3 plasmid vector (Invitrogen, Carlsbad, CA) with a FLAG epitope or hemagglutinin (HA) epitope tag. SIRT6 and GFP overexpression adenoviruses were prepared in an AdEasy adenoviral vector system (Agilent, Santa Clara, CA) following the manufacturer’s manual. SIRT6 and GFP shRNA adenoviruses were made using a BLOCK-iT system (Invitrogen). Human SMAD3 lysine acetylation sites were mutated using a Q5 site-directed mutagenesis kit (New England Biolabs, Ipswich, MA). DNA oligonucleotides used in this work are described in Table 2. The recombinant DNA work was approved by the Indiana University Institutional Biosafety Committee.

### Cell Culture, Plasmid Transfection, and Adenoviral Transduction

Human hepatic stellate cell line LX-2 (MilliporeSigma, Burlington, MA) was cultured in Dulbecco’s modified Eagle medium (DMEM) (Thermo Fisher Scientific, Waltham, MA) supplemented with 2% fetal bovine serum and penicillin/streptomycin (Thermo Fisher Scientific). For adenoviral transduction, we used a multiplicity of infection of 20 for overexpression and a multiplicity of infection of 100 for shRNA knockdown experiments. Mouse primary cells were isolated from WT, Sirt6-KO (Alb-Cre), Sirt6-KO (Mx1-Cre), and HSC-KO (Lrat-Cre) mice. Hepatocytes, Kupffer cells, and HSCs were isolated as previously described.50 Experiments were performed from 3–4 mice per genotype. To set up coculture of hepatocytes and HSCs, freshly isolated primary mouse hepatocytes were seeded in a Transwell insert at 0.5×10^6/well with DMEM plus 10% fetal bovine serum, and primary mouse HSCs were seeded in a well of a Transwell plate at 0.5×10^6/well with DMEM plus 10% fetal bovine serum.

### Real-Time PCR Analysis

Total RNAs were isolated from cells or tissues using the TRI reagent (MilliporeSigma) and converted into complementary DNA using a complementary DNA synthesis kit (Applied Biosystems, Foster City, CA). Real-time PCR analysis was performed using SYBR Green Master Mix (Promega, Madison, WI) in an Realplex PCR system (Eppendorf North America, Hauppauge, NY). Transcript levels were analyzed with the 2^-delta delta cycle threshold method, and quantification was normalized to the internal

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Table 1. Demographic and Pathologic Characteristics of Patients With NASH

| ID | Age, y | Sex | Steatosis | Ballooning | Lobular inflammation | NAS score |
|----|--------|-----|-----------|------------|---------------------|-----------|
| 1  | 68     | F   | 1         | 2          | 2                   | 5         |
| 2  | 49     | F   | 1         | 2          | 1                   | 4         |
| 3  | 56     | M   | 1         | 2          | 1                   | 4         |
| 4  | 51     | F   | 2         | 1          | 1                   | 4         |
| 5  | 58     | F   | 3         | 1          | 1                   | 5         |
| 6  | 56     | M   | 2         | 1          | 1                   | 4         |
| 7  | 48     | F   | 3         | 1          | 1                   | 5         |
| 8  | 57     | F   | 2         | 1          | 1                   | 4         |
| 9  | 62     | F   | 1         | 2          | 1                   | 5         |
| 10 | 53     | F   | 2         | 1          | 1                   | 5         |
| 11 | 44     | F   | 1         | 0          | 1                   | 2         |
| 12 | 64     | M   | 1         | 0          | 1                   | 2         |

NAS, NAFLD activity score.

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Figure 13. (See previous page). SIRT6 attenuates the SMAD3 transcriptional activity via deacetylation. (A and B) SMAD3 acetylation and phosphorylation and quantification analysis in LX-2 cells either transduced with adenoviral Sirt6 (AdSirt6), Sirt6 shRNA (sh-Sirt6), sh-Sirt6, or green fluorescent protein shRNA (shGFP) adenoviruses, or transfected with SIRT6 WT, Sirt6-H133Y mutant, or GFP plasmids. (C) ChIP-PCR analysis of chromatin binding of SIRT6 and SMAD3 in the indicated gene regions in LX-2 cells in the presence of TGFβ1 (5 ng/mL) for 24 hours using specific antibodies against SIRT6, SMAD3, and H3K9Ac, or IgG control. The chromatin binding activity of SIRT6, SMAD3 and H3K9Ac is presented as the percentage of the input DNA amount. (D and E) Western blot analysis of deacetylation of WT or mutant SMAD3 by SIRT6 and real-time PCR analysis of the deacetylation effect on fibrosis genes in LX-2 cells. Data are presented as means ± SEM. *P < .05, **P < .01, and ***P < .001 vs (A) control or GFP, (C) PPIA-non-specific (NS), or (E) vector for the same SMAD3 genotype; ##P < .01 and ###P < .001. Ad, adenoviral; Flag, FLAG tag; IP, immunoprecipitation; Pro, promoter; p-SMAD3, phospho-SMAD family member 3.
Table 2. DNA Oligonucleotide Sequences

| Name            | Sequence                                                                 |
|-----------------|--------------------------------------------------------------------------|
| **Mouse Sirt6 qPCR** | Forward: 5'-ACGTCAAGAGACACGGTGGTG-3'  
                      Reverse: 5'-CTCTACAGGCCACCTTTTGA-3'  
| **Mouse Tnf qPCR** | Forward: 5'-GGCCCTCCTCTCATAGTTG-3'  
                      Reverse: 5'-CTTGAAATGCCACCTTTTGA-3'  
| **Mouse Il1b qPCR** | Forward: 5'-TGTGAAATGCCACCTTTTGA-3'  
                      Reverse: 5'-GGTCAAAGGTTTGGAAGCAG-3'  
| **Mouse Col1a1 qPCR** | Forward: 5'-CACCTGGTCCACAAGGTTTC-3'  
                      Reverse: 5'-CCCATCATCTCCATTCTTGC-3'  
| **Mouse Col3a1 qPCR** | Forward: 5'-TGCTGGAAAGGATGGAGAGT-3'  
                      Reverse: 5'-TGGGCCTTTGATACCTGGAG-3'  
| **Mouse Col4a1 qPCR** | Forward: 5'-TTCGCCTCCAGGAACGACTA-3'  
                      Reverse: 5'-AAACCGCACACCTGCTAATG-3'  
| **Mouse Acta2 qPCR** | Forward: 5'-AGGCACCACTGAACCCTAAG-3'  
                      Reverse: 5'-GACAGCACAGCCTGAATAGC-3'  
| **Mouse Tgfb1 qPCR** | Forward: 5'-CGCAACAACGCCATCTATGA-3'  
                      Reverse: 5'-ACTGCTTCCCGAATGTCTGA-3'  
| **Mouse Timp1 qPCR** | Forward: 5'-CATGGAAAGGCTCCTTTCAG-3'  
                      Reverse: 5'-TGAGTTCCGAAGCAAGCCAA-3'  
| **Mouse Timp1 qPCR** | Forward: 5'-CCAATGAGTAGGCTGGAGA-3'  
                      Reverse: 5'-TCTGGACCCATTCCTTCTTG-3'  
| **Mouse Timp1 qPCR** | Forward: 5'-GTGCCCACGTCAAGGAGTAT-3'  
                      Reverse: 5'-CTCTGGGTTGGCACACACTT-3'  
| **Mouse Timp1 qPCR** | Forward: 5'-CAGAGCCACATGCTCCTAGA-3'  
                      Reverse: 5'-GCTTGGCAACCCAAGTAACC-3'  
| **Mouse Timp1 qPCR** | Forward: 5'-ACCGTCTGCAGGATCCCACCAGG-3'  
                      Reverse: 5'-GGCCGGGTGCCAGCCATA-3'  
| **Mouse Timp1 qPCR** | Forward: 5'-AGCTTCGTCAGAGGCTGGGGA-3'  
                      Reverse: 5'-CATGCGGATGGTGCACATTC-3'  
| **Mouse Timp1 qPCR** | Forward: 5'-ACGTCAGAGACACGGTGGTG-3'  
                      Reverse: 5'-AGGGTGTCAGTGGGAGGA-3'  
| **Mouse Timp1 qPCR** | Forward: 5'-ACTCCGACGTGTCCCATAGTG-3'  
                      Reverse: 5'-GGCTGGCTTCTTAAATTGGTTCCA-3'  
| **Mouse Timp1 qPCR** | Forward: 5'-CTAAACGCGCAGGTAGTGAAGCA-3'  
                      Reverse: 5'-GGCAATTTCTTCAGGCAAAG-3'  
| **Mouse Timp1 qPCR** | Forward: 5'-ACTCCGACGTGTCCCATAGTG-3'  
                      Reverse: 5'-GGCTGGCTTCTTAAATTGGTTCCA-3'  
| **Mouse Timp1 qPCR** | Forward: 5'-CTTCCGATATCGCCACCATGTCGGTGAATTACGCGGC-3'  
                      Reverse: 5'-AAGGAACTCGAGGCTGGGGACCGCCTTG-3'  
| **GFP control shRNA** | Forward: 5'-GCTACGTAGGCTAGGCTAGGA-3'  
                      Reverse: 5'-GCATCAAGGTGAAACTCAAG-3'  

qPCR, quantitative polymerase chain reaction.
control gene *PPIA*. Primer sequences of the genes used in this work are described in Table 2.

**Blood Chemistry and Metabolic Analysis**

Lipids were extracted from tissues using a chloroform–methanol extraction protocol as previously described. Triglyceride measurements were performed using an L-type triglyceride assay kit (FUJIFILM Wako Diagnostics, Richmond, VA). Serum ALT analysis was performed using an assay kit from Thermo Fisher Scientific.

**Immunocytochemistry and Immunohistochemistry Analysis**

Cells were grown in a glass-bottom dish and then fixed with 4% paraformaldehyde for 15 minutes at room temperature, followed by washing 3 times with phosphate-
buffered saline (PBS), and incubating overnight with primary antibodies as described in Table 3. After washing 3 times with PBS, cells were incubated with Alexa Fluor-conjugated secondary antibodies (1:250; Invitrogen) for 1.5 hours, followed by counterstaining and mounting with Prolong Gold antifade mountant with 4',6-diamidino-2-phenylindole (Invitrogen). Images were taken with a Zeiss Axio Observer Z1 fluorescent microscope (Zeiss USA, Thornwood, NY). Liver tissue samples were fixed in 10% formalin solution and stored in 70% ethanol before they were processed for embedding and sectioning at the Histology Core of Indiana University School of Medicine. Liver sections were stained with H&E or Sirius Red stain (Milli-poreSigma). Immunohistochemistry analysis was performed for myeloperoxidase, F4/80, and other proteins using the following procedures. Liver sections were deparaffinized and hydrated in 1 mmol/L EDTA buffer for antigen retrieval at 100°C for 5 minutes, and the liver sections then were incubated with normal horse serum for 1 hour. Next, the liver sections were incubated with primary antibodies described in Table 3 at 4°C overnight. After they were washed with PBS containing 0.05% Tween-20, the liver sections were incubated with either a biotinylated universal pan-specific antibody included in a Vectastain ABC kit from Vector Laboratories (Burlingame, CA) or fluorophore-conjugated secondary antibody for 2 hours. The sections incubated with the biotinylated antibody subsequently were incubated with avidin–biotin peroxidase complexes (Vector Laboratories) for 1 hour. Peroxidase activity was detected using diaminobenzidine solution (Vector Laboratories). Images were taken using a regular microscope (original magnification, 100× or 200×; Leica, Buffalo Grove, IL) or a fluorescent microscope (original magnification, 200×, 400×, or 630×; Zeiss USA). Quantification of the images was performed from randomly selected sections of at least 5 fields of each sample using ImageJ software (version 1.52; National Institutes of Health, Bethesda, MD).

**Western Blot and Immunoprecipitation Analyses**

Immunoblotting and immunoprecipitation analyses were performed as previously described. Tissue samples were homogenized in the lysis buffer (50 mmol/L HEPES, pH 7.5, 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1.5 mmol/L MgCl2, 1 mmol/L EDTA, 10 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, and freshly added 100 µmol/L sodium vanadate, 1 mmol/L phenylmethylsulfonyl fluoride, and 1× Complete protease inhibitor cocktail [Roche, Indianapolis, IN]). Cell lysates were prepared in the NP-40 lysis buffer (1% NP-40, 20 mmol/L Tris, pH 7.4, 137 mmol/L NaCl, 2 mmol/L EDTA, 10% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride, and 1× Complete protease inhibitor cocktail). Protein samples were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane for immunoblotting analysis using specific antibodies. Enhanced chemiluminescence signals on Western blot were analyzed by Gelpro32 Software (Media Cybernetics, Marlow, UK) for quantitative analysis. For immunoprecipitations, equal amounts of protein extracts were incubated with 1 µg of specific antibodies for 16 hours at 4°C. Protein A/G plus agarose beads (Santa Cruz Biotechnology, Dallas, TX) then were added for a 3-hour incubation at 4°C. Normal rabbit or mouse IgG was used as a negative control. Immunoprecipitated proteins were analyzed by immunoblotting. Antibodies used in this work are described in Table 3.

**ChIP**

Chromatin association analysis was performed in LX-2 cells by chromatin preparation as previously described, immunoprecipitation with antibodies against Sirt6 (MilliporeSigma, St. Louis, MO), Smad3 (Cell Signaling Technology, Danvers, MA), and H3K9Ac (Cell Signaling Technology), and real-time PCR analysis. ChIP DNA amounts for gene promoters of interest were normalized to the input DNA for the same gene sequence. Primers used in the ChIP PCR reactions are described in Table 2.

**Statistical Analysis**

All statistical data are expressed as means ± SEM. Statistical analysis was performed using Prism 8 software from GraphPad (La Jolla, CA). Comparisons between 2 groups were performed using a 2-tailed Student t test and comparisons for more than 2 groups were performed using 1-way analysis of variance followed by the Tukey post hoc test.

All authors had access to the study data and reviewed and approved the final manuscript.

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Xiaolin Zhong, Menghao Huang, and Hyeong-Geug Kim were responsible for the acquisition of data, analysis and interpretation of data, and drafting of the manuscript; Yang Zhang, Kushan Chowdhury, and Wenjie Cai were responsible for the acquisition of data; Romil Saxena and Robert F. Schwabe provided material support; Suthat Liangpunsakul critically revised the manuscript and provided material support; and X. Charlie Dong was responsible for the study concept and design, analysis and interpretation of data, drafting of the manuscript, statistical analysis, obtaining funding, and study supervision.

Conflicts of interest
The authors disclose no conflicts.

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