Sirtuin 2 Prevents Liver Steatosis and Metabolic Disorders by Deacetylation of Hepatocyte Nuclear Factor 4α

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BACKGROUND AND AIMS: Sirtuin 2 (SIRT2), an NAD⁺-dependent deacetylase, is involved in various cellular processes regulating metabolic homeostasis and inflammatory responses; however, its role in hepatic steatosis and related metabolic disorders is unknown.

APPROACH AND RESULTS: Integrating the published genomic data on NAFLD samples from humans and rodents available in the Gene Expression Omnibus, we found that SIRT2 was significantly down-regulated in livers from patients with advanced NAFLD and high-fat diet (HFD)–induced NAFLD mice. This study further revealed that SIRT2 was markedly decreased in obese (ob/ob) mice and in palmitate-treated HepG2 cells. Restoration of hepatic SIRT2 expression in ob/ob or HFD-fed mice largely alleviated insulin resistance, hepatic steatosis, and systematic inflammation, whereas SIRT2 liver-specific ablation exacerbated these metabolic dysfunctions in HFD-fed C57BL/6J mice. Mechanistically, SIRT2 stabilized the hepatocyte nuclear factor 4α (HNF4α) protein by binding to and deacetylating HNF4α on lysine 458. Furthermore, HNF4α was sufficient to mediate SIRT2 function, and SIRT2-HNF4α interaction was required for SIRT2 function both *in vivo* and *in vitro*.

CONCLUSIONS: Collectively, the present study provided evidence that SIRT2 functions as a crucial negative regulator in NAFLD and related metabolic disorders and that targeting the SIRT2-HNF4α pathway may be a promising strategy for NAFLD treatment. (HEPATOLOGY 2021;74:723-740).

NAFLD has become a major health issue due to its high prevalence worldwide; it continues to rise along with the growing obesity and diabetes epidemic.[1,2] NAFLD is characterized by excess fat accumulation in the liver and is closely associated with insulin resistance, inflammation, hepatic glucose, and lipid metabolic disorders.[3] Moreover, NAFLD not only predisposes patients to a cascade of liver disorders, including hepatosteatosis, steatohepatitis, liver cirrhosis, and eventually HCC, but also confers a high risk for cardiometabolic diseases.[4,5] Morbidity and all-cause mortality are substantially higher in people with NAFLD than in the general population.[6] So far, the mechanism underlying NAFLD pathogenesis remains elusive. Despite this challenge, there are no approved NAFLD pharmacological therapies, and it remains a markedly unmet medical need.

Sirtuins (SIRTs) are highly conserved NAD⁺-dependent histone deacetylases.[7] Seven members (SIRT1-7) were identified in mammals, among

which cytosolic member SIRT2 is less recognized. SIRT2 is highly enriched in metabolically active tissues, including the liver, heart, brain, and adipose tissue. SIRT2 is a major metabolic regulator that deacetylates multiple protein targets.[8] Studies have indicated that SIRT2 deacetylates glucokinase regulatory protein and facilitates impaired hepatic glucose.[9] Additionally, SIRT2 regulates adipocyte differentiation,[10] lipolysis,[11] lipid synthesis,[12] and fatty acid oxidation,[13] suggesting its potential role in lipid metabolism. Moreover, the NAD^+–SIRT2 pathway is involved in the silybin inhibitory effect on NLR family pyrin domain containing 3 inflammasome assembly in mice with NAFLD.[14] SIRT2 hepatic overexpression ameliorates insulin sensitivity, oxidative stress, and mitochondrial dysfunction in obese mice.[15] These observations suggest that SIRT2 maintains metabolic homeostasis through glucose and lipid metabolism, insulin sensitivity, and inflammation regulation, which are critical pathological processes implicated in NAFLD development. However, a link between SIRT2 and NAFLD has not yet been established.

The present study sought to provide evidence that SIRT2 is involved in NAFLD and related metabolic disorder regulation. SIRT2 level was reduced in the obese mouse liver, and a SIRT2 downstream regulator, liver-enriched nuclear hormone receptor hepatocyte nuclear factor 4α (HNF4α), was identified. Additionally, liver-specific SIRT2 deficiency promoted insulin resistance, hepatic steatosis, and inflammation, whereas liver-specific SIRT2 overexpression reversed metabolic dysfunction. More specifically, SIRT2 regulated hepatic steatosis and related metabolic disorders by HNF4α deacetylation.

Materials and Methods

ANIMAL MODEL AND EXPERIMENTAL PROTOCOL

All animal protocols were reviewed by the Animal Care Committee of Tongji Hospital, Huazhong University of Science and Technology. Animals were fed *ad libitum* and housed in a temperature-controlled environment (23 ± 2°C) with a 12/12-hour light/dark cycle. Male C57BL/6 and obese (ob/ob) mice aged 7-8 weeks were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). Mice were maintained with either a normal chow diet (NCD; 10 kcal% fat, 70 kcal% carbohydrate, and 20 kcal% protein; Beijing Huafukang Bioscience Co., Ltd.) or a high-fat diet (HFD; D12492; Research Diets; 60 kcal% fat, 20 kcal% carbohydrate, and 20 kcal% protein; Beijing Huafukang Bioscience Co., Ltd.) for 12 weeks.

HISTOLOGICAL ANALYSIS

Hematoxylin and eosin (H&E) staining was performed to visualize lipid accumulation patterns in liver tissues. Lipid droplet accumulation in the liver was analyzed by oil red O (ORO) staining of frozen liver sections. Periodic acid-Schiff (PAS) staining was performed to determine the glycogen content of liver tissues using paraffin-prepared liver sections.

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METABOLIC ASSAYS

Glucose tolerance tests (GTTs) and insulin tolerance tests (ITTs) were performed by i.p. injection of 1 g/kg glucose (Sigma-Aldrich Co., St. Louis, MO) and 0.75 U/kg insulin (Novolin R; Novo Nordisk Co., Bagsvaerd, Denmark) into mice after 6-hour fasting. Blood glucose levels were detected at 0, 15, 30, 60, and 120 minutes after injection. Fasting blood glucose (FBG) and fasting serum insulin (FINS) levels were examined in tail blood every 2 weeks using a glucometer (One Touch Ultra Easy; Life Scan) and ELISA (Millipore, Billerica, MA), respectively. Homeostasis model of assessment of the insulin resistance index (HOMA-IR) was calculated using the following equation: [fasting blood glucose (mmol/L) × fasting serum insulin (mIU/L)]/22.5. AUCs were calculated to reflect glucose and insulin tolerance levels.

BIOCHEMISTRY ASSAY

Liver and serum lipid contents were determined using commercial kits for triglyceride (TG), total cholesterol (TC), and nonesterified fatty acid (NEFA). Liver function was evaluated in the animals by measuring the serum concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) using commercial kits.

CELL CULTURE, TREATMENT, AND TRANSFECTION

HCC HepG2 and human embryonic kidney (HEK) 293T cells were purchased from the American Type Culture Collection (Manassas, VA), and cultured in DMEM (Gibco; 10569044) supplemented with 10% fetal bovine serum (Gibco; 16140071), 100 IU/mL penicillin, and 100 μg/mL streptomycin. Cells maintained in a 37°C incubator with 5% CO_2 and grown to 70%-90% confluence were collected for analyses unless otherwise noted.

To establish an *in vitro* model of lipid accumulation in HepG2 cells, they were treated with 0.5 mM palmitate (PA) for 24 hours. For SIRT2 inhibitor treatments, HepG2 cells were supplemented with 10 μM acylglycerol kinase 2 for 24 hours or with DMSO as a control. Transient transfections were performed using Lipofectamine 2000 (Invitrogen/Life Technologies; 11668019) according to the manufacturer's instructions. All small interfering RNAs (siRNAs) were produced by RiboBio (Guangdong, China). siRNA target sequences were as follows: SIRT2, 5′-CCTGCTCATCAACAAGGAGAA-3′ and 5′-CCTGCTCATCAACAAGGAGAA-3′; HNF4α, 5′-GATCAGCACTCGAAGGTCAA-3′. Endogenous or exogenous HNF4α protein degradation was analyzed using a protein stability assay. Briefly, HepG2 cells transfected with si-Ctrl (control) or si-SIRT2 for 48 hours were incubated with 20 μM MG132 or 100 μg/mL cycloheximide (CHX) for the indicated time points. HNF4α protein levels were analyzed by western blotting.

PROTEIN INTERACTION ANALYSIS

Protein interactions associated with biological processes related to lipid metabolism were identified using the online STRING database (https://string-db.org/).[16]

Details of real-time quantitative PCR, western blotting, plasmid constructs, immunoprecipitation (IP), and immunofluorescence are shown in the Supporting Information.

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS software (version 19.0), and all data are presented as means ± standard deviations. A two-tailed Student t test was performed to compare data of the two groups with a normal distribution. For multiple comparisons, one-way ANOVA with Tukey's *post hoc* analysis was applied. Nonparametric statistical analysis was performed using the Kruskal-Wallis test, followed by Dunn's test for multiple comparisons. P values are represented as follows: [#]$P < 0.05$, [*]$P < 0.05$, [**]$P < 0.01$, [***]$P < 0.0001$.

Results

HEPATIC SIRT2 EXPRESSION REDUCTION IN PATIENTS WITH NAFLD AND ANIMAL MODELS

To identify whether SIRT2 is potentially involved in NAFLD pathogenesis, an analysis of the published

microarray data generated from the liver tissue of diabetic obese mice (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE83596) and 72 patients with NAFLD (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE49541) in the Gene Expression Omnibus (GEO) database was previously performed. The study defined mild NAFLD as fibrosis stage 0-1 and advanced NAFLD as fibrosis stage 3-4. SIRT2 expression was decreased in the liver of mice fed the HFD compared with mice fed the NCD (Fig. 1A). Moreover, hepatic SIRT2 was significantly decreased in patients with advanced NAFLD compared to patients with mild disease ($P < 0.05$) (Fig. 1B).

Hepatic SIRT2 expression in genetically obese ob/ob and HFD-fed mice and PA-induced HepG2 cells was examined. In both ob/ob mice (Fig. 1C) and HFD-fed mice (Fig. 1D), SIRT2 protein levels gradually decreased in the livers from 4 to 12 weeks. Additionally, reduced SIRT2 was confirmed in HepG2 cells treated with PA in a time-dependent and dose-dependent manner (Fig. 1E,F). These data indicated that hepatic SIRT2 expression significantly decreased in the context of NAFLD, suggesting its crucial role in the pathogenesis of this disease.

HEPATIC SIRT2 DEFICIENCY EXACERBATED HFD-INDUCED INSULIN RESISTANCE

To investigate the pathophysiological role of SIRT2 in the liver, hepatocyte-specific SIRT2 knockout mice were generated using commercially available adeno-associated virus (AAV) containing short hairpin RNA (shRNA) targeting SIRT2 (Supporting Fig. S2A), with these mice challenged with the HFD or NCD for 12 weeks. SIRT2 expression decreased prominently after SIRT2-AAV transfection (Fig. 2A). As expected, HFD treatment significantly increased body weights of both short hairpin normal control (shNC) HFD and shSIRT2 HFD mice compared with those of shNC NCD and shSIRT2 NCD, respectively ($P < 0.05$) (Fig. 2B). Noticeably, no significant difference was observed between shNC HFD and shSIRT2 HFD mice (Fig. 2B).

Insulin resistance is closely associated with hepatic steatosis. Evidently, shSIRT2 HFD mice exhibited higher FBG and FINS levels and higher HOMA-IR values than shNC HFD mice (Fig. 2C,D; Supporting Fig. S3A). Furthermore, SIRT2 deficiency negatively
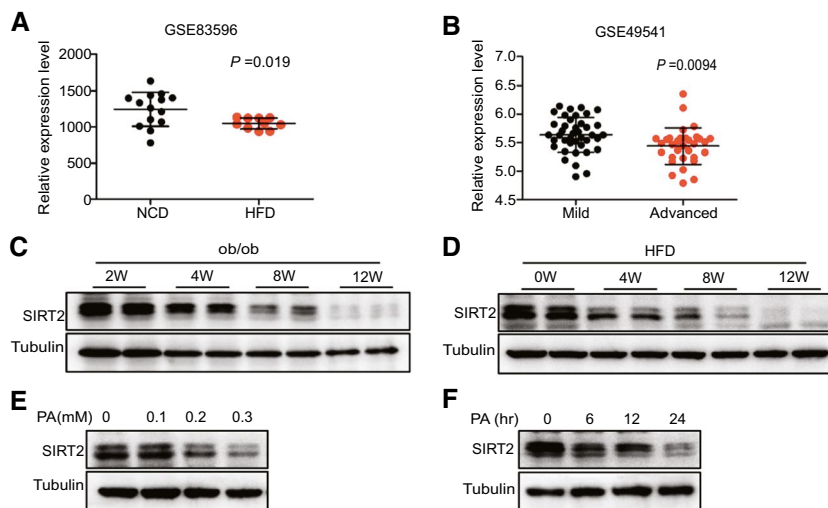

FIG. 1. Decreased hepatic SIRT2 expression in fatty livers. (A) Scatter diagram indicating differentially expressed genes in the HFD group relative to the NCD controls from the GEO database in a microarray assay. (B) Scatter diagram indicating differentially expressed genes in patients with mild NAFLD relative to advanced controls from the GEO database in microarray assay. (C) Liver tissue lysates from ob/ob mice fed the NCD for 12 weeks; SIRT2 protein levels determined with western blot analysis (n = 5/group). (D) Liver tissue lysates from mice fed the HFD for 12 weeks; SIRT2 protein levels determined with western blot analysis (n = 5/group). (E) HepG2 cells treated with PA at 0.1, 0.2, and 0.3 mM concentrations for 24 hours. SIRT2 protein expression was measured by western blotting (n = 3). (F) HepG2 cells treated with 0.3 mM PA for the indicated time. SIRT2 protein expression was measured by western blotting (n = 3). For (A) and (B), data were analyzed by a two-tailed Student t test. All results are representative of three independent experiments.
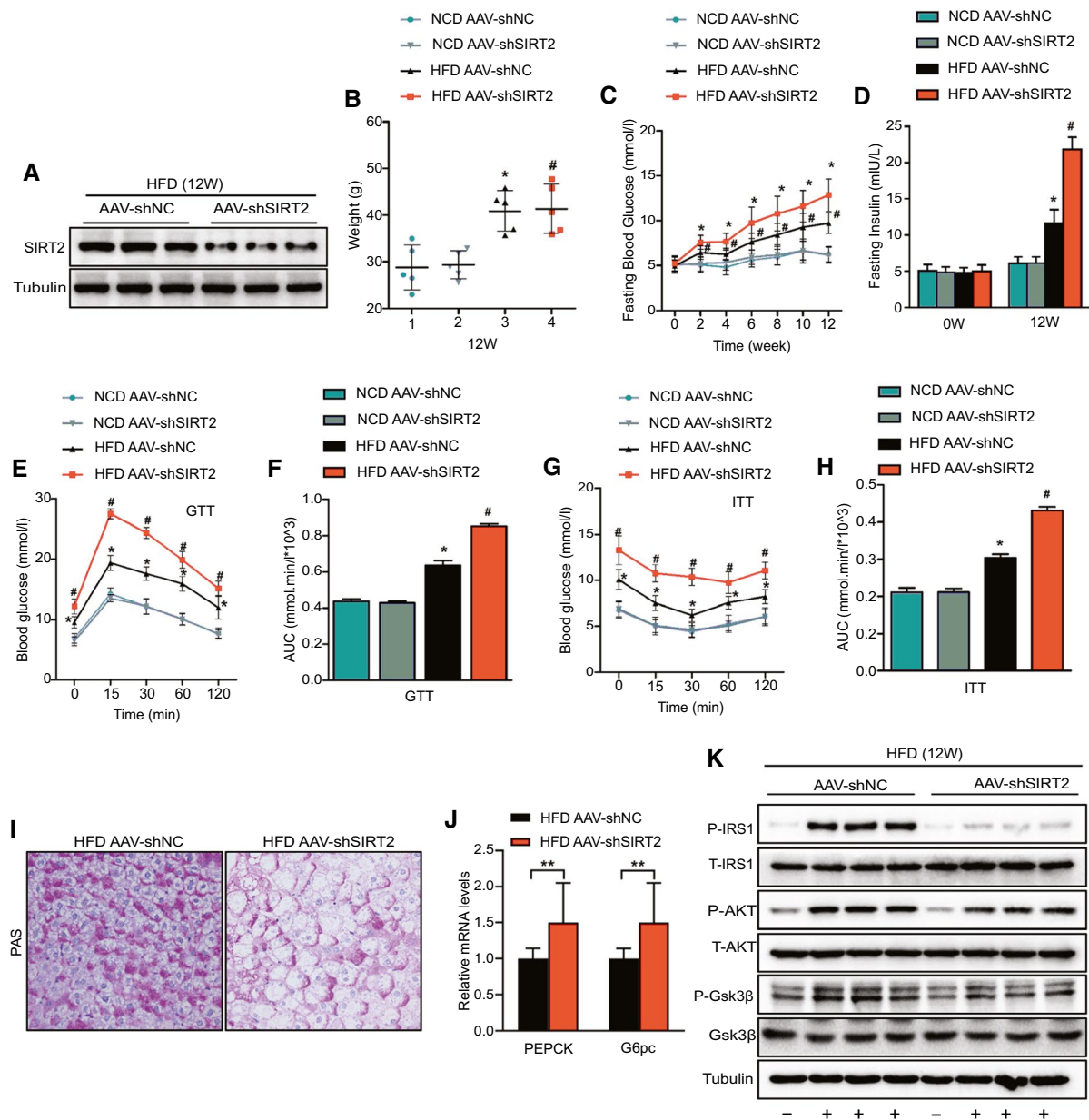
FIG. 2. Hepatic SIRT2 deficiency (AAV-shSIRT2) aggravates HFD-induced insulin resistance. (A) Immunoblotting of SIRT2 protein expression in livers of mice with AAV-shSIRT2 transfection. Data are representative of three samples for each protein. (B) Body weights of AAV-shSIRT2 mice or their controls (AAV-shNC) after 12 weeks of NCD or HFD feeding (n = 5/group). FBG levels (C) and FINS levels (D) in NCD-fed or HFD-fed AAV-shNC or AAV-shSIRT2 mice at indicated time points (n = 5/group). (E,G) Blood glucose levels after 12 weeks of NCD or HFD feeding in AAV-shNC or AAV-shSIRT2 mice during GTTs and ITTs. (F,H) AUC of GTTs (F) and ITTs (H) were calculated. (I) Representative images of PAS in liver sections from HFD-fed AAV-shNC or AAV-shSIRT2 mice (n = 5/group). (J) mRNA expression of PEPCK and G6pc was measured in AAV-shNC or AAV-shSIRT2 mouse liver with HFD feeding for 12 weeks by real-time quantitative PCR (n = 5/group). (K) Mice were injected i.p. with insulin after overnight fasting. Activation of insulin signaling (IRS1/AKT/Gsk3β activities) in livers from HFD-fed AAV-shNC or AAV-shSIRT2 mice was assayed by western blotting (n = 5/group). All results are representative of three independent experiments. Values are presented as mean ± SD. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparisons test. For (A-H), *$P < 0.05$, AAV-shNC HFD group versus AAV-shNC NCD group; #$P < 0.05$, AAV-shSIRT2 HFD group versus AAV-shNC HFD group. For other data, *$P < 0.05$, **$P < 0.01$.

affected glucose tolerance and insulin sensitivity according to GTTs and ITTs (Fig. 2E-H), reduced gluconeogenesis as revealed by PAS staining, and increased phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase catalytic subunit (G6pc) mRNA (Fig. 2I,J). Consistently, insulin signaling was impaired in shSIRT2 HFD mouse livers, as reflected by decreased insulin receptor substrate 1 (IRS1), AKT, and glycogen synthase kinase 3β (Gsk3β) phosphorylation levels compared to those in shNC HFD mice (Fig. 2K; Supporting Fig. S3B).

HEPATIC SIRT2 OVEREXPRESSION ALLEVIATED HFD-INDUCED INSULIN RESISTANCE

Opposite phenotypes were observed in AAV-mediated, liver-specific SIRT2 overexpressed mice (Supporting Fig. S2B; Fig. 3A). SIRT2 overexpression in HFD-fed mice had no effect on body weight (Fig. 3B) but reduced FBG and FINS levels and HOMA-IR values (Fig. 3C,D; Supporting Fig. S3C) and improved insulin sensitivity and glucose tolerance (Fig. 3E-H). Additionally, AAV-SIRT2 HFD mice showed enhanced gluconeogenesis and insulin signaling compared with AAV-NC HFD mice (Fig. 3I-K; Supporting Fig. S3D).

SIRT2 PROTECTED AGAINST HFD-INDUCED HEPATIC STEATOSIS AND INFLAMMATORY RESPONSES

Studies have reported that sustained HFD exposure causes prominent lipid accumulation in the liver. SIRT2 deficiency further promoted hepatic steatosis. shSIRT2 mice exhibited increased liver weights; increased liver-to-body weight ratios (Fig. 4A); and elevated TG, TC, and NEFA levels in the liver (Fig. 4C). Histological analysis (H&E and ORO staining) revealed greater lipid accumulation in livers of shSIRT2 mice than in shNC mice (Fig. 4E). Moreover, shSIRT2 mice showed worse liver dysfunction with higher ALT, AST, and ALP levels in the serum than shNC mice (Fig. 4G). Lastly, SIRT2 deficiency led to more severe inflammation compared with that in shNC mice, as indicated by increased proinflammatory factor levels (IL-1β, ILl-6, and monocyte chemoattractant protein 1 [MCP-1]), decreased anti-inflammatory factor IL-10 level

in the liver, and greater P65 signaling activation in livers (Fig. 4I-K). In contrast, hepatic SIRT2 overexpression reversed these phenotypes, ameliorated hepatic steatosis, and reduced inflammation and liver damage (Fig. 4B,D,F,H,J,L). Taken together, these observations suggested that SIRT2 protects against HFD-induced insulin resistance, hepatic steatosis, inflammatory response, and liver damage.

Similarly, SIRT2 silencing in human HepG2 cells using an siRNA increased cellular lipid content, as indicated by ORO staining, and increased cellular TG and TC levels (Supporting Fig. S4A-C), while over-expressing SIRT2 by transfecting a plasmid carrying the SIRT2 gene reduced lipid accumulation in HepG2 cells (Supporting Fig. S4D-F). To further confirm the observed effects of SIRT2 on hepatic steatosis, two deacetylation-null SIRT2 mutants that lacked deacetylase activity (SIRT2-H187Y, SIRT2-N168A) were constructed. HepG2 cells were transfected with either wild-type (WT) or catalytic SIRT2 (SIRT2-H187Y or SIRT2-N168A) mutants. These catalytic SIRT2 mutants failed to reverse lipid accumulation in hepatoma cells (Supporting Fig. S5A-D). These results indicate that SIRT2 regulates cellular lipid content at least in part through its deacetylation activity.

SIRT2 INTERACTED WITH HNF4α AND SUPPRESSED ITS DEGRADATION

To understand the molecular mechanism underlying the positive effects of SIRT2 on NAFLD and related metabolic disorders, a bioinformatics tool was used to screen for SIRT2-interacting proteins. The STRING database, a predictive web interface for gene functions, generated a possible interaction between SIRT2 and partner proteins, including 52-kDa HNF4A, SMAD family member 3 (SMAD3), SMAD4, histone deacetylase 1 (HDAC1), HDAC2, and catenin beta 1 (Fig. 5A). Among the targeted proteins, HNF4α was a master regulator of gene expression for bile acid, lipid, glucose, and drug metabolism. Thus, it was hypothesized that SIRT2 directly controls HNF4α. To test this hypothesis, SIRT2 or HNF4α was immunoprecipitated from cultured HepG2 cells, with its binding between SIRT2 and HNF4α tested by western blotting. The results suggested that SIRT2 physically interacted with HNF4α (Fig. 5B). Furthermore, coimmunoprecipitation (co-IP) with total proteins from
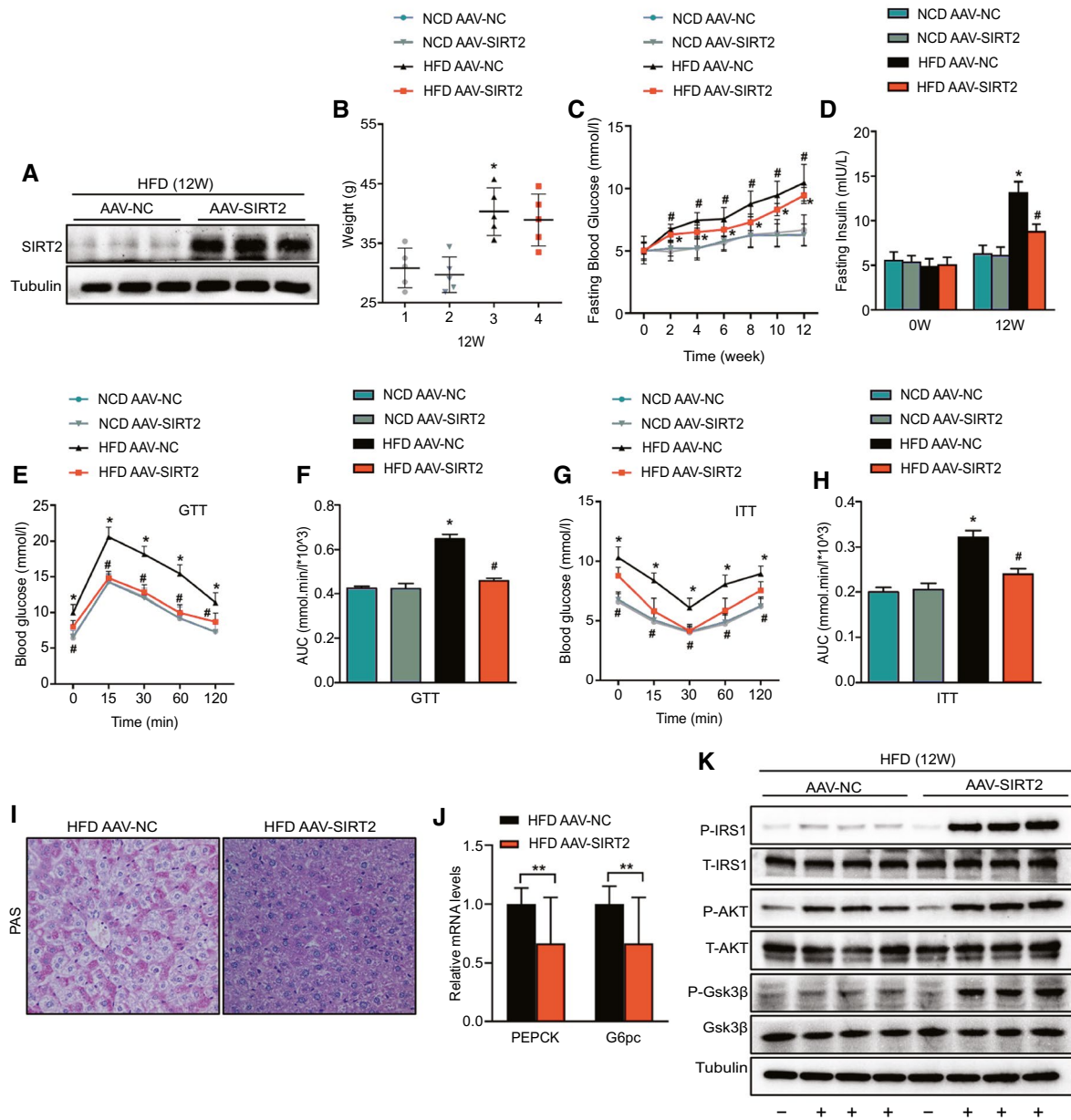
FIG. 3. Hepatic SIRT2 overexpression (AAV-SIRT2) alleviates HFD-induced insulin resistance. (A) Immunoblotting of SIRT2 protein expression in livers of mice with AAV-SIRT2 transfection. Data are representative of three samples for each protein. (B) Body weights of AAV-SIRT2 mice or their controls (AAV-NC) after 12 weeks of NCD or HFD feeding (n = 5/group). Fasting blood glucose levels (C) and fasting insulin levels (D) in NCD-fed or HFD-fed AAV-NC or AAV-SIRT2 mice at indicated time points (n = 5/group). (E,G) Blood glucose levels after NCD or HFD feeding for 12 weeks in AAV-NC or AAV-SIRT2 mice during GTT and ITT assays. (F,H) AUC of GTTs (F) and ITTs (H) were calculated. (I) Representative images of PAS in liver sections from HFD-fed AAV-shNC or AAV-shSIRT2 mice (n = 5/group). (J) mRNA expression of PEPCK and G6pc was measured in AAV-NC or AAV-SIRT2 mouse liver with HFD feeding for 12 weeks by real-time quantitative PCR (n = 5/group). (K) Mice were injected i.p. with insulin after overnight fasting. Activation of insulin signaling (IRS1/AKT/Gsk3β activities) in livers from HFD-fed AAV-NC or AAV-SIRT2 mice was assayed by western blotting (n = 5/group). All results are representative of three independent experiments. Values are presented as mean ± SD. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparisons test. For (A-H), *$P < 0.05$, AAV-NC HFD group versus AAV-NC NCD group; #$P < 0.05$, AAV-SIRT2 HFD group versus AAV-NC HFD group. For other data, *$P < 0.05$, **$P < 0.01$.

HEK293T cells suggested similar results (Fig. 5C). SIRT2 and HNF4α protein colocalization was further confirmed by immunofluorescence analysis in hepatoma cells and quantified (Fig. 5D; Supporting Fig. S6). An immunofluorescence assay was also conducted to identify SIRT2 and HNF4α interactions in NCD liver tissues, suggesting that SIRT2 displayed a cytoplasmic and predominantly nuclear distribution. Colocalization of SIRT2 and HNF4α was observed (Supporting Fig. S7). Western blotting analysis was performed to detect HNF4α expression in livers from SIRT2-deficient or overexpressed mice. The results
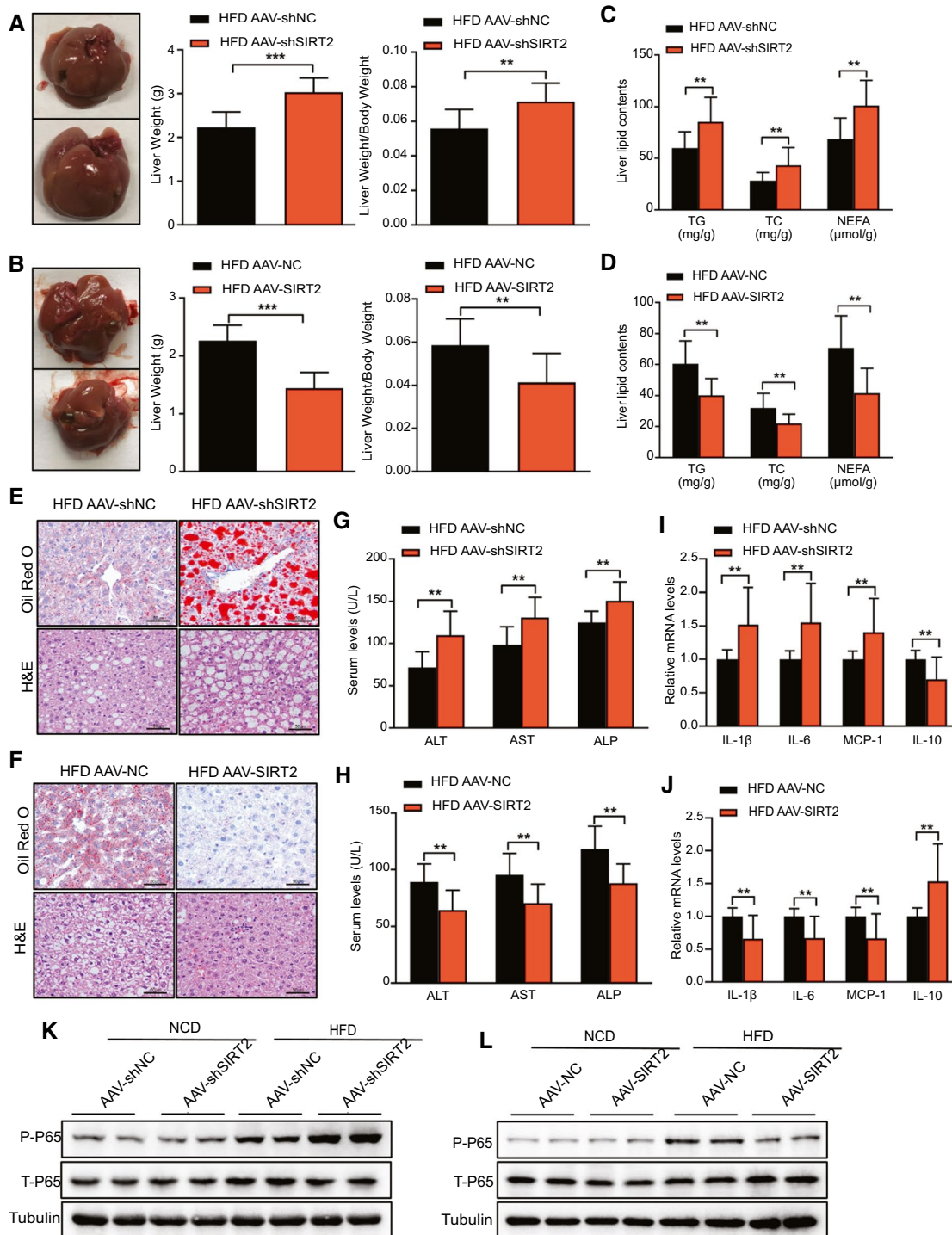
FIG. 4. SIRT2 protects against HFD-induced hepatic steatosis and inflammation. (A) Liver weight and liver-to-body weight ratios of AAV-shSIRT2 mice and their corresponding littermate controls after HFD feeding for 12 weeks (n = 5/group). (B) Liver weight and liver-to-body weight ratios of AAV-SIRT2 mice and their corresponding littermate controls after HFD feeding for 12 weeks (n = 5/group). (C) Hepatic TG, TC, and NEFA contents in AAV-shNC or AAV-shSIRT2 mice after HFD feeding for 12 weeks (n = 5/group). (D) Hepatic TG, TC, and NEFA contents in AAV-NC or AAV-SIRT2 mice after HFD feeding for 12 weeks (n = 5/group). (E,F) Representative images of ORO (upper) and H&E (bottom) staining in liver sections from AAV-shNC or AAV-shSIRT2 mice (E) and AAV-NC or AAV-SIRT2 mice (F) after HFD feeding for 12 weeks (n = 5/group). (G,H) Serum ALT, AST, and ALP levels in AAV-shNC or AAV-shSIRT2 mice (G) and AAV-NC or AAV-SIRT2 mice (H) after HFD feeding for 12 weeks (n = 5/group). (I,J) mRNA expression of inflammation-related cytokines (IL-1β, IL-6, MCP-1, IL-10) was measured in AAV-shNC or AAV-shSIRT2 mice (I) and AAV-NC or AAV-SIRT2 mice (J) liver with HFD feeding for 12 weeks by real-time quantitative PCR (n = 5/group). (K,L) Hepatic P65 expression in AAV-shNC or AAV-shSIRT2 mice (K) and AAV-NC or AAV-SIRT2 mice (L) after HFD feeding for 12 weeks (n = 5/group). All results are representative of three independent experiments. Values are presented as mean ± SD. Data were analyzed by a two-tailed Student t test. $*P < 0.05$, $**P < 0.01$.

suggested that SIRT2 deficiency attenuated HNF4α protein expression levels (Fig. 5E), with SIRT2 overexpression exhibiting opposite effects (Fig. 5G). Interestingly, real-time quantitative PCR results suggested that SIRT2 deficiency significantly decreased HNF4α target gene expression (Fig. 5F), with SIRT2 overexpression increasing HNF4α target gene expression (Fig. 5H) but not altering HNF4α mRNA levels (Fig. 5F,H). In accordance with the *in vivo* results, siRNA-mediated SIRT2 knockdown *in vitro* significantly decreased HNF4α protein levels and HNF4α target gene mRNA levels, while cells with ectopic SIRT2 overexpression showed the opposite effect; no significant effect was observed on HNF4α mRNA levels (Supporting Fig. S8A-D). These data suggest that HNF4α regulation is posttranscriptionally controlled.

Thus, it was proposed that SIRT2 interacts with HNF4α to alter its protein stability. To test this hypothesis, HNF4α protein levels in HepG2 cells were tested with or without SIRT2 knockdown after treatment with proteasome inhibitor MG132. SIRT2 knockdown promoted HNF4α protein destabilization (Fig. 5I; Supporting Fig. S8E). To study the effect of SIRT2 on HNF4α protein stability in more detail, the half-life of HNF4α after treatment with CHX was measured. SIRT2 knockdown reduced the half-life of HNF4α protein (Fig. 5J; Supporting Fig. S8F). Overall, these findings suggested that SIRT2 and HNF4α interaction was responsible for its degradation in the cell.

SIRT2 DEACETYLATED HNF4α AND INCREASED ITS STABILITY

As one of the most important posttranslational modifications, deacetylation plays a critical role in protein stability. Hepatic HNF4α acetylation status was evaluated in liver tissues of HFD-fed and ob/ob mice, as well as in PA-treated hepatoma cells. HFD significantly decreased SIRT2 and HNF4α levels in livers compared with control mice, while increasing HNF4α acetylation levels. Similar results were observed in ob/ob mice and PA-treated HepG2 cells (Supporting Fig. S9A-D). To determine whether SIRT2 regulates the function of HNF4α through its deacetylase activity, an *in vitro* deacetylation assay was performed. HepG2 cells were incubated with either WT or catalytic mutants of SIRT2 (SIRT2-H187Y or SIRT2-N168A). Western blotting analysis indicated that WT, but not catalytic mutant-SIRT2, markedly increased HNF4α protein levels (Fig. 6A), indicating that SIRT2 deacetylase activity was required for HNF4α protein-level regulation. Additionally, an acetylation assay was performed to evaluate the effects of SIRT2 mutants on HNF4α acetylation levels. SIRT2 overexpression significantly reduced HNF4α acetylation levels; however, SIRT2 mutants had no effect on HNF4α acetylation (Fig. 6B). Subsequently, whether HNF4α was deacetylated by SIRT2 *in vivo* and *in vitro* was investigated. SIRT2 were overexpressed in HepG2 cells, followed by IP using HNF4α or IgG antibody and blotting with an antiacetylated lysine antibody. SIRT2 overexpression significantly reduced HNF4α acetylation status (Fig. 6C,E); in contrast, SIRT2 knockdown increased HNF4α acetylation (Fig. 6D,F). Additionally, HNF4α protein acetylation was analyzed by immunoprecipitating acetyl lysine, followed by western blotting for HNF4α *in vitro* and *in vivo*. Similar results were obtained (Supporting Fig. S10A-E). Taken together, these results demonstrated that HNF4α is a substrate of SIRT2-mediated deacetylation.
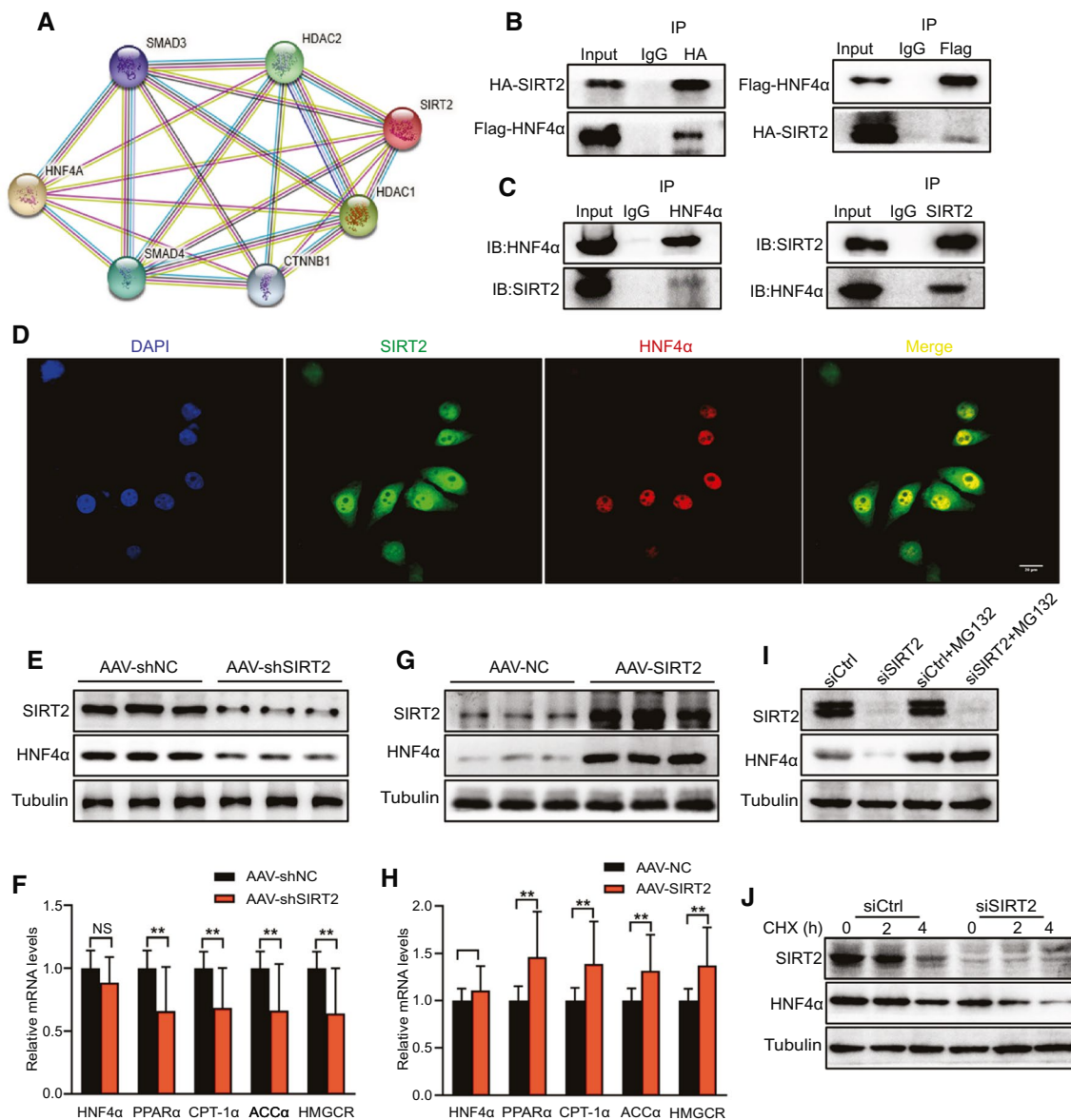
FIG. 5. SIRT2 interacts with HNF4α and suppresses its degradation. (A) Prediction of protein interactions using the STRING database showing two partner proteins, SIRT2 and HNF4α. (B) Co-IP experiments of HEK293T cells cotransfected with hemagglutinin (HA)-SIRT2 and flag-HNF4α, using an anti-HA antibody for IP and anti-Flag antibody for immunoblotting (left) or anti-flag antibodies for IP and anti-HA antibody for immunoblotting (right). (C) HepG2 cell lysates were subjected to co-IP with anti-SIRT2 or anti-HNF4α antibody, respectively; and normal rabbit IgG was used as the control, followed by western blotting using indicated antibodies. (D) Representative immunofluorescence staining of HepG2 cells costained with SIRT2 antibody (green) and HNF4α antibody (red). Nuclei were stained with DAPI (blue). Yellow indicates SIRT2 colocalization with HNF4α in HepG2 cells. Scale bar, 20 μm. (E) SIRT2 and HNF4α protein expression levels in livers from AAV-shNC or AAV-shSIRT2 mice after HFD feeding for 12 weeks (n = 5/group). (F) Representative mRNA levels of HNF4α and its target genes in livers of AAV-shNC or AAV-shSIRT2 mice after HFD feeding for 12 weeks (n = 5/group). (G) SIRT2 and HNF4α protein levels in livers from AAV-NC or AAV-SIRT2 mice after HFD feeding for 12 weeks (n = 5/group). (H) Representative mRNA levels of HNF4α and its target genes in livers of AAV-NC or AAV-SIRT2 mice after HFD feeding for 12 weeks (n = 5/group). (I) HNF4α protein expression levels in siCtrl-transfected or siSIRT2-transfected HepG2 cells treated with protease inhibitor MG132 (20 μM) for 10 hours. (J) HNF4α protein stability time course in siCtrl-transfected or siSIRT2-transfected HepG2 cells after treatment with 100 μg/mL CHX for indicated times. All results are representative of three independent experiments. Values are presented as mean ± SD. *$P < 0.05$, **$P < 0.01$. Abbreviations: ACCα, acetyl-CoA carboxylase α; Cpt-1α, carnitine palmitoyltransferase 1α; CTNNB1, catenin β1; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; IB, immunoblotting; NS, not significant; PPARα, peroxisome proliferator–activated receptor α.

MAPPING DEACYLATED LYSINE RESIDUES REGULATED BY SIRT2

To identify deacetylation sites of HNF4α that mediate its interaction with SIRT2, acetylation prediction tools and existing scholarly literature were consulted to predict acetylated sites. Five preferentially deacetylated mimetic mutants were generated by mutating lysine (K) to arginine (R) at the following five sites: Lys106, Lys108, Lys126, Lys127, and Lys458. HEK293T cells were cotransfected with si-Ctrl or si-SIRT2, together with vectors containing either WT-HNF4α (control)
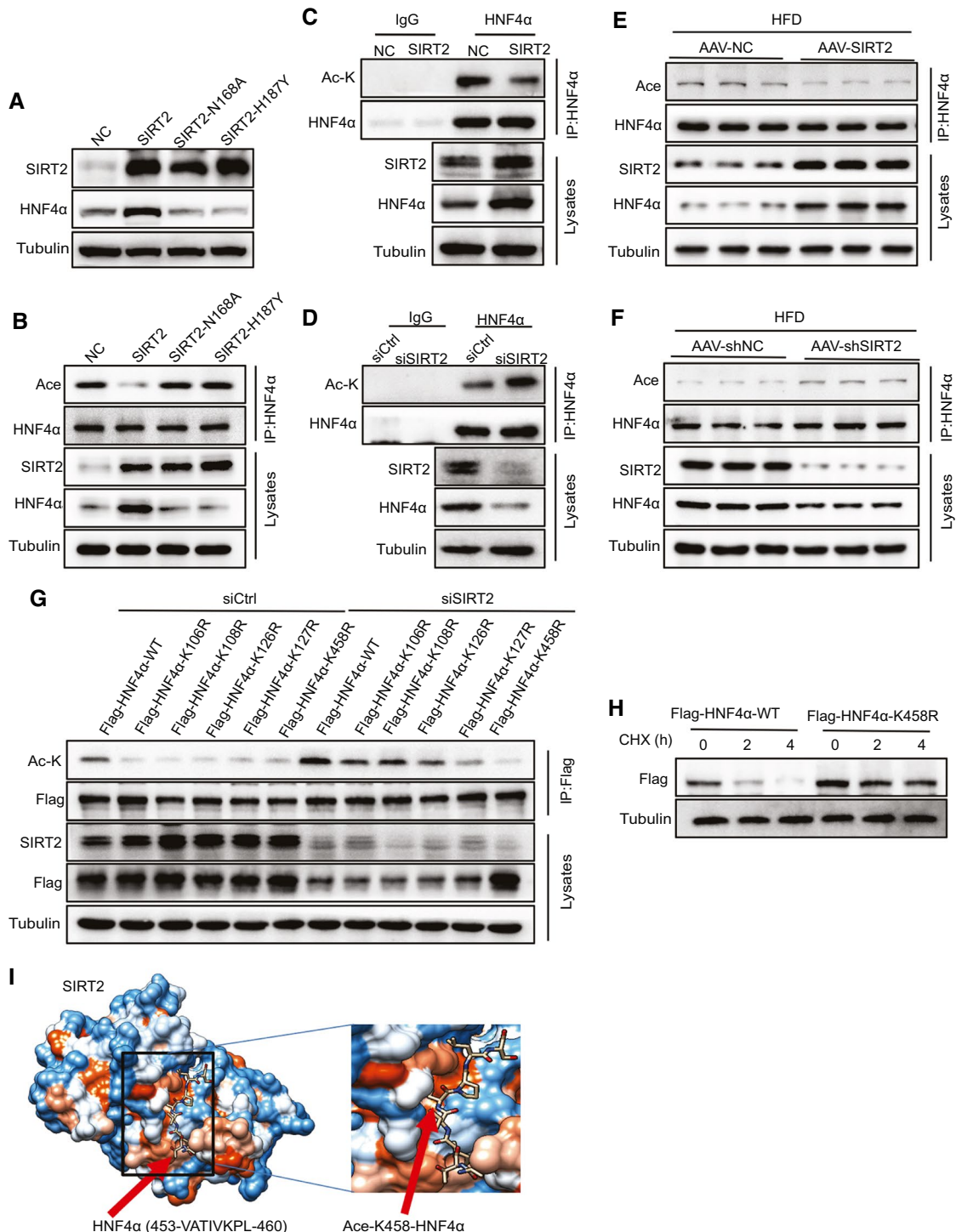
FIG. 6. SIRT2 deacetylates HNF4α and suppresses its degradation. (A) SIRT2 and HNF4α protein expression levels in HepG2 cells expressing empty vector (NC), SIRT2, or mutant plasmids (SIRT2-N168A, SIRT2-H187Y). (B) Co-IP experiments of HepG2 cells expressing NC or SIRT2 or mutant plasmids (SIRT2-N168A, SIRT2-H187Y), using an anti-HNF4α antibody for IP and anti-acetyl-lysine antibodies for immunoblotting. (C) Co-IP experiments of HepG2 cells overexpressing NC or SIRT2 plasmids, using an anti-HNF4α antibody for IP and anti-acetyl-lysine antibodies for immunoblotting. (D) Co-IP experiments of HepG2 cells transfected with siCtrl or siSIRT2, using an anti-HNF4α antibody for IP and anti-acetyl-lysine antibodies for immunoblotting. (E) Co-IP assay of hepatocytes from AAV-NC or AAV-SIRT2 mice, using an anti-HNF4α antibody for IP and anti-acetyl-lysine antibodies for immunoblotting. (F) Co-IP assay of hepatocytes from AAV-shNC or AAV-shSIRT2 mice, using an anti-HNF4α antibody for IP and anti-acetyl-lysine antibodies for immunoblotting. (G) siCtrl or siSIRT2 were transfected into HEK293T cells with either HNF4α-WT or different mutant plasmids including K106R, K108R, K126R, K127R, and K458R. Lysates were immunoprecipitated with anti-Flag antibody and blotted with anti-acetyl-lysine antibody. (H) HNF4α protein stability time course of overexpressed HNF4α-WT and K459R mutants after treatment with 100 μg/mL CHX in HEK293T cells. (I) SIRT2-HNF4α K458 docking with the HDOCK server. High magnification of boxed areas is presented on the right. Arrow (left), HNF4α protein 453-460 peptide; arrow (right), HNF4α protein K458 site. All results are representative of three independent experiments. Abbreviation: Ac-K, acetyl-lysine.

or different mutants, including HNF4α-K106R, HNF4α-K108R, HNF4α-K126R, HNF4α-K127R, and HNF4α-K458R, followed by IP using SIRT2 antibody and blotting with antiacetylated lysine antibody. HNF4α-K106R, HNF4α-K108R, HNF4α-K126R, and HNF4α-K127R acetylation levels were significantly increased in SIRT2 knockdown cells, whereas no difference was observed in HNF4α-K458R acetylation levels, indicating that HNF4α could be acetylated at Lys458 (Fig. 6G). To determine whether acetylation mutation modulates HNF4α protein stability, HEK293T cells transfected with HNF4α-WT or the mutant construct HNF4α-K458R were treated with CHX. HNF4α-K458R displayed a shorter half-life compared with HNF4α-WT, suggesting that protein acetylation at the Lys458 residue is responsible for HNF4α protein stability (Fig. 6H). The HDOCK server (http://hdock.phys.hust.edu.cn/) is a highly integrated suite of protein–protein docking information.[17] The server was exploited to predict the SIRT2 and HNF4α-K458 interaction interface. The server provided docking information about SIRT2-HNF4α protein 453-460 peptide, indicating SIRT2 and HNF4α-K458 interaction (Fig. 6I). Collectively, these data indicated that SIRT2 deacetylated Lys458 on HNF4α and increased its protein stability.

SIRT2 INHIBITED INSULIN RESISTANCE, HEPATIC STEATOSIS, AND INFLAMMATORY RESPONSES THROUGH AN HNF4α-DEPENDENT MECHANISM

To confirm the beneficial effect of hepatic SIRT2 overexpression mediated through HNF4α, we investigated whether HNF4α deficiency reversed hepatic steatosis alleviation and insulin resistance following SIRT2 overexpression in an *in vivo* model. Accordingly, AAV-shNC+AAV-HNF4α and AAV-shSIRT2+AAV-HNF4α mice were established (Fig. 7A) and subjected to HFD challenge. In the setting of HNF4α silence, SIRT2 overexpression failed to improve indexes reflecting insulin resistance, including fasting glucose and insulin levels, glucose and insulin tolerance, liver mass index, and liver lipid accumulation (Fig. 7C-K; Supporting Fig. S11A-D).

Subsequently, it was determined whether HNF4α-458K deacetylation by SIRT2 is essential for the protective role of SIRT2 in NAFLD. For these studies, a deacetylation mimetic mutant was generated by mutating lysine (K) to arginine (R), as well as a mutation of HNF4α-458K to glutamine (Q) to mimic the acetylated form. AAV expressing HNF4α, HNF4α-K458R, or HNF4α-K458Q was delivered into AAV-shSIRT2 mice. HNF4α protein levels in AAV-shSIRT2 mice were significantly increased with AAV-HNF4α and AAV-HNF4-K458R injection, while there was no increase with AAV-HNF4-K458R injection (Fig. 7B). Further assessment indicated that AAV-HNF4α and AAV-HNF4-K458R injection significantly ameliorated fasting glucose and insulin levels, glucose and insulin tolerance, liver mass index, and liver lipid accumulation in AAV-shSIRT2 mice (Fig. 7C-K; Supporting Fig. 11E-H), which was more evident with AAV-HNF4-K458R injection. Collectively, these data indicated that SIRT2 inhibits lipid accumulation and metabolic profile impairments partially by deacetylation of the HNF4α-mediated mechanism.
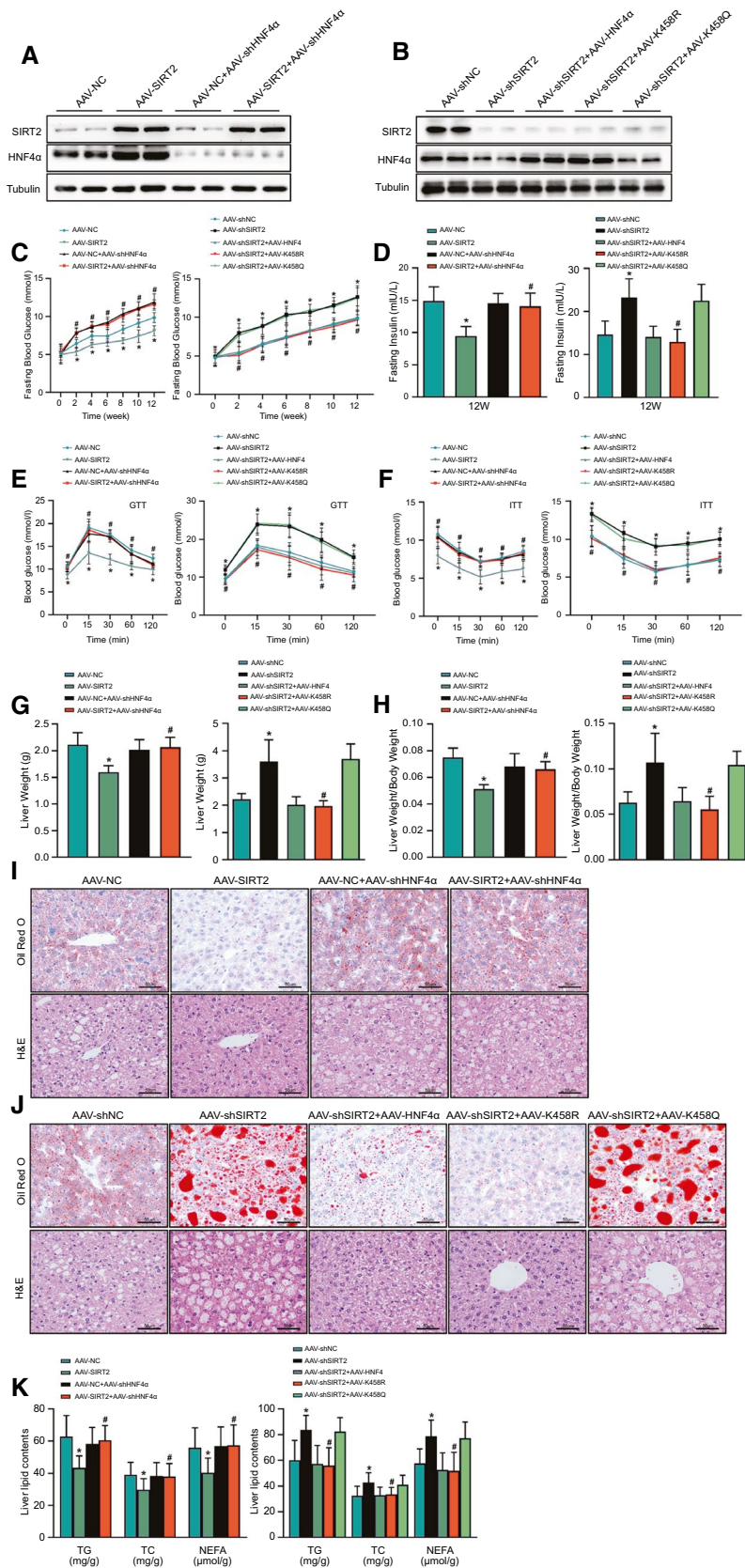
FIG. 7. HNF4α mediates the effects of SIRT2 in NAFLD. (A) SIRT2 and HNF4α protein expression levels in livers from AAV-NC, AAV-SIRT2, AAV-NC+AAV-shHNF4α, and AAV-SIRT2+AAV-shHNF4α mice (n = 5/group). (B) SIRT2 and HNF4α protein expression levels in livers from AAV-shNC, AAV-shSIRT2, AAV-shSIRT2+AAV-K458R, and AAV- shSIRT2+AAV-K458Q mice (n = 5/group). (C,D) FBG levels (C) and FINS levels (D) in the current model at indicated time points in response to HFD feeding (n = 5/group). (E,F) GTTs (E) and ITTs (F) were performed in indicated groups after HFD feeding for 12 weeks (n = 5/group). (G,H) Liver weight and liver-to-body weight ratios of indicated groups at 12 weeks of HFD feeding (n = 5/group). (I,J) Representative images of ORO-stained (upper) and H&E-stained (lower) liver sections from indicated groups after HFD feeding for 12 weeks (n = 5/group; bar, 50 μm). (K) Hepatic TG, TC, and NEFA contents in indicated groups after HFD feeding for 12 weeks (n = 5/group). All results are representative of three independent experiments. Values are presented as mean ± SD. *$P < 0.05$, AAV-SIRT2 group versus AAV-NC group, AAV-shSIRT2 group versus AAV-shNC group; #$P < 0.05$, AAV-SIRT2+AAV-shHNF4α group versus AAV-NC+AAV-shHNF4α group, AAV-shSIRT2+AAV-K458R group versus AAV-shSIRT2 group.

HEPATIC SIRT2 EXPRESSION RESTORATION ATTENUATED HEPATIC INSULIN RESISTANCE AND STEATOSIS IN ob/ob MICE

A genetic obesity model (ob/ob mice) was used to further determine whether SIRT2 exerted a protective function in NAFLD. SIRT2 was overexpressed in ob/ob mice using an AAV vector through the tail vein, confirmed in AAV-SIRT2 ob/ob mice by comparing with AAV-green fluorescent protein (GFP) ob/ob mice, along with enhanced HNF4α expression (Fig. 8A). SIRT2 overexpression in AAV-SIRT2 ob/ob mice livers reduced liver weights and liver weight to body weight ratios, decreased FBG and FINS levels and HOMA-IR indexes, and improved GTTs and ITTs compared with AAV-GFP mice, with no significant differences in body weight observed between AAV-GFP and AAV-SIRT2 ob/ob mice (Fig. 8B-I). Moreover, AAV-SIRT2 ob/ob mice exhibited enhanced insulin signaling and gluconeogenesis and ameliorated lipid accumulation in livers compared with AAV-GFP ob/ob mice (Fig. 8J-L). Additionally, lower liver contents of TG, TC, and NEFA were observed in AAV-SIRT2 ob/ob mice than in AAV-GFP ob/ob mice (Fig. 8M). These data indicated that SIRT2 can reverse hepatic insulin resistance and steatosis in ob/ob mice.

Discussion

The present study identified a protective role of SIRT2 in obesity-related insulin resistance, hepatic steatosis, and inflammation. First, markedly reduced SIRT2 expression was observed in obese mouse fatty livers and patients with NAFLD. Second, liver-specific ablation of SIRT2 resulted in significant lipid accumulation, excessive inflammation, and insulin resistance in mice fed the HFD, while overexpression of hepatic SIRT2 attenuated these metabolic dysfunctions in ob/ob mice and HFD-fed mice. Third, the results revealed that HNF4α could be a deacetylated target to mediate SIRT2 effects. Thus, targeting SIRT2 could be a potential therapeutic strategy for fatty liver diseases and related metabolic disorder treatment.

Sirtuin is a homolog of the yeast silent information regulator (SIR2) gene, which increases the yeast life span when up-regulated. Sir2 was also identified as an NAD+-dependent deacetylase, and seven sirtuins in mammals (SIRT1-7) were identified. SIRT2 is a less studied sirtuin, residing in the cytosol and nucleus, playing major roles in aging and metabolic regulation. SIRT2 regulates cellular responses to oxidative stress by interacting with and deacetylating a forkhead box O transcription factor, FOXO3a.[18] In an *in vitro* study, SIRT2 overexpression in hepatocytes improved insulin sensitivity, decreased cellular reactive oxygen species levels, and ameliorated mitochondrial dysfunction.[15] A recent report observed the anti-inflammatory effect of SIRT2 by directly binding and deacetylating the nuclear factor κB (NF-κB) subunit p65 at Lys-310, which resulted in reduced NF-κB-regulated inflammatory gene expression.[19] Hepatic SIRT2 overexpression decreased ATP-citrate lyase acetylation and its protein stability and inhibited lipid accumulation in high-fat/high-sucrose diet–fed mice.[12] Collectively, these data suggested a protective role of SIRT2 in metabolic dysfunction development. Accordingly, it was reasonable to test whether SIRT2 conferred protection against NAFLD, wherein oxidative stress, inflammatory response, insulin resistance, and aberrant lipid metabolism are correlated and create a vicious cycle. This study provided evidence that SIRT2 expression significantly decreased
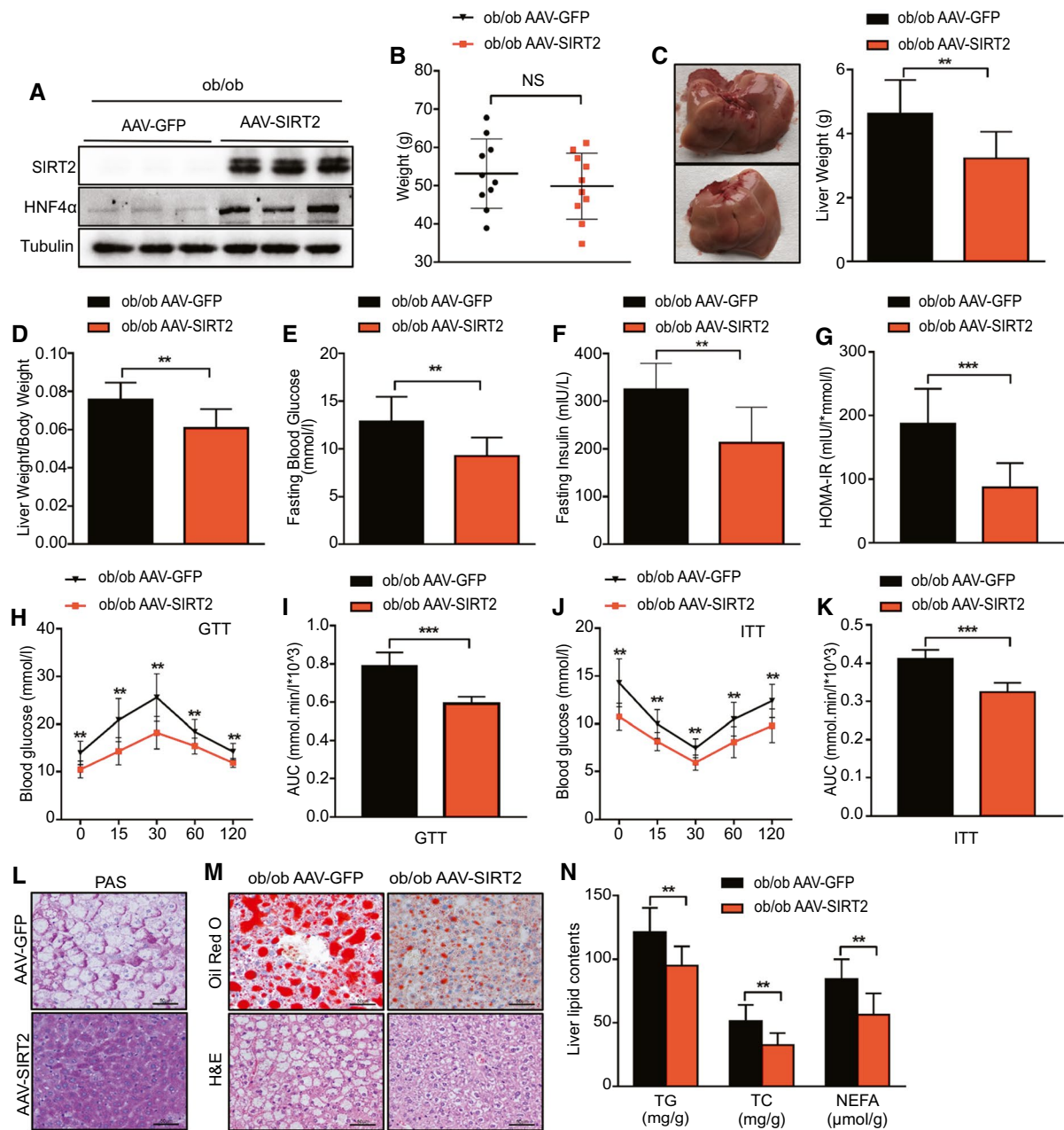
FIG. 8. Hepatic SIRT2 expression restoration attenuates hepatic insulin resistance and steatosis in ob/ob mice. (A) SIRT2 and HNF4 expression in livers from ob/ob mice injected with AAV-GFP and AAV-SIRT2 (n = 10/group). (B-E) Body weights (B), liver weights (C), liver-to-body weight ratios (D), FBG levels (E), FINS levels (F), and HOMA-IR indexes (G) of ob/ob mice were measured 4 weeks after AAV-GFP and AAV-SIRT2 injection. (H) GTTs (H) and ITTs (I) were also performed, and corresponding AUCs were calculated (n = 10/group). (J) Activation of insulin signaling (IRS1/AKT/Gsk3β activities) in livers from AAV-GFP and AAV-SIRT2 mice was assayed by western blotting (n = 10/group). (K) Representative images of PAS in liver sections from ob/ob mice with AAV-GFP or AAV-SIRT2 injection (n = 10/group, bar, 50 μm). (L) Representative images of ORO-stained (upper) and H&E-stained (lower) liver sections from ob/ob mice injected with AAV-GFP and AAV-SIRT2 (n = 10 /group; bar, 50 μm). (M) Hepatic TG, TC, and NEFA contents in ob/ob mice injected with AAV-GFP or AAV-SIRT2. All results are representative of three independent experiments. **$P < 0.01$, **$P < 0.001$, AAV-GFP versus AAV-SIRT2. Abbreviation: NS, not significant.

in multiple models of NAFLD, indicating the potential role of SIRT2 in this disease. Additionally, using AAV-mediated delivery of SIRT2 into the livers of ob/ob mice and HFD mice, it was found that hepatic SIRT2 overexpression markedly alleviated obesity-related hepatic steatosis, insulin resistance, and inflammation. These results suggested that SIRT2 may be a promising clinical therapeutic target for the prevention of hepatic metabolic disorders in the future.

NAFLD development and progression are more likely multifactorial and include a complex molecular network. Multiple insults acting synergistically on predisposed subjects to NAFLD are implicated in the pathogenesis of the disease. Such hits include insulin resistance, lipid species accumulation, inflammatory responses, or reactive species in the liver. Studies have suggested that liver lipid accumulation leads to hepatic insulin resistance. For example, diacylglycerol accumulation, an intermediate in the triglyceride synthesis pathways, inhibits insulin signaling by impairing insulin receptor activation. Insulin resistance promotes hepatic *de novo* lipogenesis and impairs inhibition of adipose lipolysis, thereby increasing free fatty acid (FFA) flux to the liver, contributing to lipid accumulation in hepatocytes.[20] Moreover, increased FFA levels and consequent lipotoxicity lead to hepatic inflammatory activation by enhancing inflammatory cytokine release. Persistent inflammatory cytokine activation in turn not only leads to lipid peroxidation and cell apoptosis but also disrupts insulin signaling and induces insulin resistance, thus indicating a critical role in the vicious cycle in NAFLD treatment.

HNF4α, a member of the nuclear receptor superfamily, is expressed primarily in the liver, with lower levels in the kidney, intestine, and pancreatic β cells.[21,22] HNF4α is considered a master regulator of liver-specific gene expression, including of enzymes involved in lipid, amino acid, and glucose metabolism as well as xenobiotic and drug metabolism.[23,24] Loss of hepatic HNF4α accumulated lipid in the liver and greatly increased serum TC levels but had no effect on serum TG levels.[25] Liver-specific Hnf4α$^{-/-}$ mice exhibited severe hepatosteatosis due to markedly reduced carboxylesterase 2 expression, through lipolysis modulation, endoplasmic reticulum stress, and lipogenesis.[26] Hepatic HNF4α deficiency results in fatty liver by inhibiting VLDL secretion.[27] Thus, hepatic HNF4α is essential for maintaining lipid homeostasis, playing a protective role in NAFLD development.

Notably, in the present study, SIRT2 overexpression in hepatocytes increased HNF4α protein expression and had a favorable effect on hepatic steatosis, insulin resistance, and inflammatory responses in obese animals. Collectively, these results demonstrate that HNF4α is a downstream target of SIRT2. SIRT2 affected NAFLD, at least in part, due to increased HNF4α expression.

HNF4α was originally identified as a homodimer that binds DNA response elements, recruiting transcriptional coactivators and regulating target gene expression.[28] Reversible acetylation is a critical posttranslational modification affecting the DNA binding activity of transcription factors, subcellular distribution, activation/inactivation, or protein–protein interactions.[29,30] However, the role of alternate lysine acetylation in modulating HNF4a function remains elusive. Soutoglou et al. demonstrated that HNF4α acetylation was responsible for HNF4α nuclear retention, increasing its binding activity.[29] Yokoyama et al. identified an acetylation site in the HNF4a protein and found that K458 acetylation was reported to potentiate its DNA binding activity.[31] Of note, we showed that SIRT2 binds to and deacetylates HNF4, thus increasing HNF4α protein levels (through its degradation). More significantly, HNF4α deacetylation at the Lys458 residue by SIRT2 was required for its protein stability. Collectively, this study identified lysine deacetylation as a posttranslational modification regulating HNF4α protein stability, which contributes to the protective role of SIRT2 in fatty liver diseases.

Additional to SIRT2-HNF4α signaling, other mechanisms may contribute to SIRT2-mediated protection against liver steatosis. For instance, p38 mitogen-activated protein kinase (MAPK) increased HNF4α stabilization through its phosphorylation, subsequently exerting effects on bile acid synthesis.[32] Some upstream regulators of HNF4α, including G protein α12 and AMP-activated protein kinase (AMPK), are also known major regulators of metabolic pathways.[33,34] It is interesting to note that most of these factors (AMPK and MAPK) are also downstream of SIRT2.[35,36] These findings suggest a potential crosstalk between these signaling pathways and SIRT2-HNF4α. Multiple signaling pathways could be implicated in SIRT2-HNF4α-mediated regulation of fatty liver diseases and related metabolic disorders. Future studies entail a follow-up on such crosstalk in a detailed analysis of the molecular

mechanisms that govern the crosstalk between these signaling pathways.

In summary, this study demonstrated that SIRT2 is a key metabolic regulator in the liver. Hepatic SIRT2 overexpression improved hepatosteatosis, insulin resistance, and inflammation in obesity, mainly by increasing HNF4α protein stability. Thus, specifically targeting the SIRT2-HNF4α regulatory axis may be useful for the prevention and treatment of fatty liver diseases and related metabolic disorders.

Author Contributions: H.R., F.H., D.W., X.K., and G.Y. participated in research design. H.R., F.H., D.W., X.K., S.L., B.Z., L.Z., X.F., and G.Y. were responsible for conducting experiments and analyzing the data. H.R., F.H., D.W., X.K., S.L., B.Z., L.Z., and X.F. were responsible for data analysis and interpretation. H.R. and F.H. were responsible for drafting the paper. B.Z. and G.Y. were responsible for supervising the study. All authors read and approved the final manuscript.

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Supporting Information

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