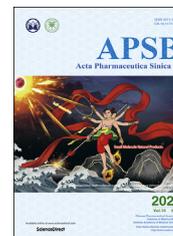




Chinese Pharmaceutical Association
Institute of Materia Medica, Chinese Academy of Medical Sciences

Acta Pharmaceutica Sinica B

www.elsevier.com/locate/apsb
www.sciencedirect.com



ORIGINAL ARTICLE

NF- κ B/HDAC1/SREBP1c pathway mediates the inflammation signal in progression of hepatic steatosis



Yunwei Guo^{a,c}, Xiaoying Zhang^b, Zhiyun Zhao^c, Hongyun Lu^c,
Bilun Ke^c, Xin Ye^c, Bin Wu^a, Jianping Ye^{b,c,*}

^aDepartment of Gastroenterology, Third Affiliated Hospital, Sun Yat-sen University, Guangzhou 510630, China

^bShanghai Diabetes Institute, Shanghai Sixth People's Hospital, Shanghai Jiao Tong University, Shanghai 200233, China

^cAntioxidant and Gene Regulation Laboratory, Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, LA 70808, USA

Received 7 September 2019; received in revised form 28 November 2019; accepted 7 January 2020

KEY WORDS

NF- κ B;
HDAC1;
SREBP1c;
Succinylation;
Hepatic steatosis

Abstract The transcription factor nuclear factor kappa B (NF- κ B) is activated in hepatocytes in the pathogenesis of hepatic steatosis. However, the action mechanism of NF- κ B remains to be established in the hepatic steatosis. In this study, the *P50* subunit of NF- κ B was found to promote the hepatic steatosis through regulation of histone deacetylase 1 (HDAC1) in hepatocytes. The activity was supported by the phenotypes of *P50* knockout (*P50*-KO) mice and *P65* knockout (*P65*-KO) mice. Hepatic steatosis was reduced in the *P50*-KO mice, but not in the *P65*-KO mice. The reduction was a result of inhibition of HDAC1 activity in the *P50*-KO cells. Knockdown of *Hdac1* gene led to suppression of hepatocyte steatosis in HepG2 cells. A decrease in sterol-regulatory element binding protein 1c (SREBP1c) protein was observed in the liver of *P50*-KO mice and in cell with *Hdac1* knockdown. The decrease was associated with an increase in succinylation of SREBP1c protein. The study suggests that *P50* stabilizes HDAC1 to support the SREBP1c activity in hepatic steatosis in the pathophysiological condition. Interruption of this novel pathway in the *P50*-KO, but not the *P65*-KO mice, may account for the difference in hepatic phenotypes in the two lines of transgenic mice.

© 2020 Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

*Corresponding author.

E-mail address: yejianping@sjtu.edu.cn (Jianping Ye).

Peer review under responsibility of Institute of Materia Medica, Chinese Academy of Medical Sciences and Chinese Pharmaceutical Association.

<https://doi.org/10.1016/j.apsb.2020.02.005>

2211-3835 © 2020 Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Nuclear factor kappa B (NF- κ B) was reported to promote lipid accumulation in hepatocytes in the progression of hepatic steatosis^{1,2}. However, the exact mechanism of NF- κ B action remains unknown. In adipocytes, NF- κ B inhibits lipid accumulation through suppression of the transcriptional activity of peroxisome proliferator activated receptor gamma (PPAR γ) in the lipogenic transcriptional network³. Sustained NF- κ B activation leads to a reduction in lipid accumulation of adipocytes, which accounts for a resistance to obesity in the transgenic mice with overexpression of P65 (RelA) or inhibitor of kappa B kinase beta (IKK β) in the adipocytes^{4,5}. The lean phenotype together with improved insulin sensitivity^{6,7} is consistent with those observed in mice of global *P50-KO*^{8,9}, which exhibit an increase in NF- κ B activity. These studies suggest that NF- κ B have opposite activities in the control of lipid accumulation between hepatocytes and adipocytes. The molecular basis is unknown for the opposite activities. NF- κ B is generally formed by two different subunits, P65 and P50. The two subunits have different activities in the control of gene transcription, the P65 subunit triggers transactivation of target genes, and the P50 subunit binds to DNA to inhibit the P65 activity. The difference in the two subunits may provide a mechanism for the distinct activities of NF- κ B in hepatocytes vs. adipocytes.

Histone deacetylase 1 (HDAC1) is a member of the class I histone deacetylase, and is located in the nucleus to catalyse the deacetylation reaction of histone proteins in the regulation of gene transcription. HDAC1 binds to NF- κ B protein in the promoter DNA to perform the deacetylase activity. However, there is no literature about regulation of HDAC1 activity by NF- κ B. HDAC1 activity has been reported in variety of systems. Global inactivation of *Hdac1* gene results in embryonic lethality from severe developmental defects¹⁰, which is associated with a reduction in cell cycle progression. In the liver, HDAC1 is required for generation of new hepatocytes from the progenitor cells¹¹. In the adipose tissue, HDAC1 controls differentiation of white adipocytes through an interaction with CCAAT-enhancer binding protein alpha (C/EBP α)¹², or thermogenesis of brown adipocytes by suppression of uncoupling protein 1 (UCP-1) and PPAR γ co-activator 1 (PGC-1) expression¹³. These studies suggest that HDAC1 regulates activities of multiple transcription factors in adipocytes. Unfortunately, there is no report about a feedback regulation of HDAC1 activity by the transcription factors.

Sterol-regulatory element binding protein 1c (SREBP1c) is one of the key transcription factors in the regulation of *de novo* lipogenesis in hepatocytes through induction of lipogenic gene expression¹⁴. SREBP1c activity is regulated at the transcriptional and post-translational levels. The transcriptional expression is well documented in the lipogenic effects of insulin. The post-translational modification includes protease cleavage of SREBP1c for activation and modification by phosphorylation¹⁵ and acetylation¹⁶. Succinylation is one of the metabolism-associated post-translational modifications, whose activity is enhanced in non-alcoholic fatty liver¹⁷ and decreased by sirtuin 5 (SIRT5) through de-succinylation¹⁸. There is no report of SREBP1c regulation by succinylation.

In current study, we observed that HDAC1 protein decreased in the liver of *P50-KO* mice for a reduction in hepatic steatosis. HDAC1 may promote the hepatic steatosis through de-succinylation of SREBP1c protein in the nucleus. The study reveals a new mechanism of P50/HDAC1/SREBP1c for hepatic steatosis.

2. Materials and methods

2.1. Animals

The male *P50-KO* mice (strain name: B6.Cg-Nfkb1 tm Bal/J, Stock number: 006097) were purchased from the Jackson Laboratory at 4 weeks of age. The mice were crossed with wild-type (WT) C57BL/6J mice to generate heterozygous breeders. The male homozygous *P50-KO* mice (Experiment group) and wild-type littermates (Control group) were obtained by crossing the heterozygous mice. The male mice were fed on chow diet (5001, 13% calories in fat, LabDiet, Richmond, IN, USA) to 12 weeks of age, and then fed on a high-fat diet (HFD, D12331, 58% calories in fat, Research Diets, New Brunswick, NJ, USA) for 16 weeks to establish the fatty liver model. Tissue samples were collected from the mice at the end of HFD feeding. The male liver specific *P65-KO* mice were described in an early study⁶ and fed on HFD diet at 8 weeks of age for 14 weeks to induce the hepatic steatosis. The mice were housed at 4/cage with free access to water and diet. All procedures were performed in accordance with National Institutes of Health guidelines and approved by the Institute Animal Care and Use Committee (IACUC) at the Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, LA, USA.

2.2. Tissue collection

Liver tissues of mice were collected at the age of 9 months without fast. One part of liver was frozen in liquid nitrogen for protein and mRNA analysis and then kept at -80°C , the other part of liver was treated with 4% formaldehyde for histological analysis and then kept at room temperature. The whole-body weight was collected at the same time.

2.3. Cell culture, reagents and treatment

HepG2 cells were purchased from American Type Culture Collection (Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS at 37°C in a 5% CO_2 humidified atmosphere. MEF cells of *P50-KO*, *I κ B α -KO* and *Ikk β -KO* were described in the early studies^{3,19}. The proteasome inhibitor MG-132 (SML1135, Sigma, St. Louis, MO, USA) was used at the final concentration of $50\ \mu\text{mol/L}$ in the cell culture.

P50 shRNA. HepG2 cells were electrical transfected with mouse *P50*-shRNA expression vector (P/N: 9033, L/N: P04122008, Panomics, Redwood, CA, USA) through Amaxa Nucleofector™ II/2b Device, using cell line nucleofector Kit V (VCA-1003, Lonza cologne AG) with the program T-031. shRNA was used at 1–3 $\mu\text{g/well}$ in the 6-well plate. The control cells were transfected with the empty vector (P/N: 8524, L/N: P04120901, Panomics). The transfected cells were cultured for 24 h and then treated with 0.3 mmol/L oleic acid (P5585, Sigma) for additional 24 h. The cells were harvested for Western blotting of SREBP1c, stearoyl-coenzyme A desaturase 1 (SCD1) and PPAR γ and oil red O staining for triglyceride quantification.

Hdac1 shRNA. HepG2 cells were electrically transfected with *Hdac1*-shRNA expression vector through Amaxa Nucleofector™ II/2b Device, using the cell line nucleofector Kit V and program T-031. Control cells were transfected with the empty vector. The transfected cells were treated with 0.5 mmol/L oleic acid for 24 h

Hdac1 expression vector together with the control vector were described elsewhere²⁰.

HDAC1 inhibitor. HepG2 cells were pretreated with 0.4 mmol/L HDAC1 chemical inhibitor (SML0296, Sigma) for 30 min and then treated with 0.5 mmol/L oleic acid for 24 h to determine the impact on hepatic steatosis.

2.4. Oil red O staining

At the end of oleic acid treatment, HepG2 cells were fixed with 10% formalin for 30 min and stained oil red O dye (Fisher Biotech BP112-10, Fair Lawn, NJ, USA) as described elsewhere²¹.

2.5. Triglyceride assay

Liver tissues were homogenized in phosphate buffered saline (PBS; 1 g:20 mL) and the cell lysate was made with the cell lysis buffer on ice. The lipids were extracted from the liver tissue homogenization or cell lysate using a chloroform/methanol (2:1) mixture. Triglyceride was determined using the Serum Triglyceride Determination Kit (TR0100, Sigma) according to the manufacturer's instructions. The triglyceride concentration was also measured with the method of oil red O staining in HepG2 cells.

2.6. Western blot and immunoprecipitation (IP)

The whole cell lysate was prepared in a lysis buffer with sonication as described elsewhere²¹. In IP, the lysate protein (500 μ g at 1 μ g/ μ L) was mixed with 2 μ g antibody in 100 μ L protein G agarose (16–266, Millipore, Billerica, MA, USA) and rotated overnight at 4 °C. The agarose beads were washed with 800 μ L washing buffer three times and collected as IP product after centrifugation at 13,000 \times g for 30 s. The succinylation signal was detected in the IP product after resolution in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Antibodies included: HDAC1 (H6287, Sigma), histone deacetylase 3 (HDAC3, ab7030; Abcam, Cambridge, UK), sirtuin 1 (SIRT1, ab12193; Abcam), SCD1 (ab19862, Abcam), SREBP1 (sc-8984, Santa Cruz, CA, USA), pan anti-succinyllysine polyclonal (106,768, NovoPro, Shanghai, China), PPAR γ (made in our laboratory), *P50* (sc-8414, Santa Cruz, CA, USA), and β -actin (sc-47778, Santa Cruz, CA, USA).

2.7. Succinylation assay

Succinylation proteins were examined with the anti-succinyllysine antibody in the cell or tissue lysates. Succinylation of SREBP1c protein was determined in the IP product of SREBP1c antibody. The de-succinylation assay was conducted with the recombinant HDAC1 protein (BML-SE456, Enzo Life Sciences Inc., New York, NY, USA) in the IP product of SREBP1c. The succinylation signal of SREBP1c was determined with the anti-succinylation antibody in a Western blot.

2.8. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from frozen tissues using Tri-Reagent (T9424, Sigma) as described elsewhere²². The expression of mRNA was determined by one-step quantitative real-time PCR using TaqMan Master Mix (Applied Biosystems, Foster City, CA, USA). TaqMan primers and the probe were acquired from Applied Biosystems as followings: *Hdac1* (Mm02391771_g1), *Hdac3* (Mm00515916_m1), *Sirt1* (Mm00493421_m1), fatty acid synthase

(*Fas*) (Mm00662319_m1), *Ppar γ* (Mm00440945_m1), *Scd1* (Mm00772290_m1), *Srebp1* (Mm00550338_m1), phosphodiesterase 3B (*Pde3b*, Mm00691635_m1), glucose-6-phosphatase (G6Pase, Mm00839363_m1), fatty acid translocase (*Cd36*, Mm00432403_m1), *Pgc1 α* (Mm00447183_m1), phosphoenolpyruvate carboxykinase 1 (*Pck1*, Mm00440636_m1), tumor necrosis factor alpha (*Tnf- α* , Mm00443258_m1), interleukin 6 (*Il-6*, Mm00446190_ml), interleukin 1 beta (*Il-1 β* , Mm00434228_ml). Mouse ribosome 18S rRNA_s1 (AIQJA2B) was used as an internal control.

2.9. Statistical analysis

The *in vivo* data were analyzed with two-way ANOVA. The *in vitro* experiments were conducted at least three times. Two-tailed, unpaired Student's *t* test was used in the statistical analysis of *in vitro* data. The data was presented as mean \pm SE. Significance was considered at $P < 0.05$ in the statistical analysis.

3. Results

3.1. Hepatic steatosis was decreased in *P50*-KO mice

To explore the role of *P50* in the control of hepatic steatosis, *P50*-KO mice were fed on HFD to establish the hepatic steatosis model. Compared to WT mice, the *P50*-KO mice exhibited a reduced degree in hepatic steatosis at the end of 4 months on HFD, which was observed in the liver appearance, liver weight and liver triglyceride content (Fig. 1A–D). The difference was observed in the mice on HFD, not on chow diet. The *P50*-KO mice exhibited a lower body weight on either chow diet or HFD with more significant difference on HFD (Fig. 1E). However, the liver to body ratio was significantly reduced in the *P50*-KO mice on HFD (Fig. 1F), suggesting that *P50* has a liver-specific effect in the regulation of hepatic steatosis. The data suggest that *P50*-KO mice are resistant to the hepatic steatosis on HFD.

3.2. Reduction of lipogenic gene expression in *P50*-KO mice

In order to understand the mechanism of reduced steatosis in *P50*-KO mice, lipogenesis capacity was examined in the liver by expression of lipogenic proteins including SREBP1c, PPAR γ , and SCD1. A significant reduction was observed in SREBP1c and SCD1, but not PPAR γ in the *P50*-KO mice on chow diet (Fig. 2A). The reduction status was maintained in the *P50*-KO mice on HFD (Fig. 2B). The SCD1 protein reduction suggests a decrease in *de novo* lipogenesis. *Scd1* gene expression is subject to regulation by the transcription factor SREBP1c in the liver. The decreased abundance of SREBP1c protein provides a mechanism for the SCD1 reduction in *P50*-KO mice. The mRNA level of *Scd1* was reduced in support of the mechanism (Fig. 2C). In addition, other lipogenic genes such as *Fas* and *Cd36* also decreased in the liver of *P50*-KO mice (Fig. 2C). The mRNA level of *Srebp1c* was not reduced in the *P50*-KO mice (Fig. 2C). This group of data suggests that a reduction in lipogenesis occurs in the liver of *P50*-KO mice for the reduced steatosis.

3.3. Knockdown of *P50* gene led to reduction of SREBP1c

Above data suggest that the reduction in SREBP1c protein accounts for the attenuation of hepatic steatosis in the *P50*-KO

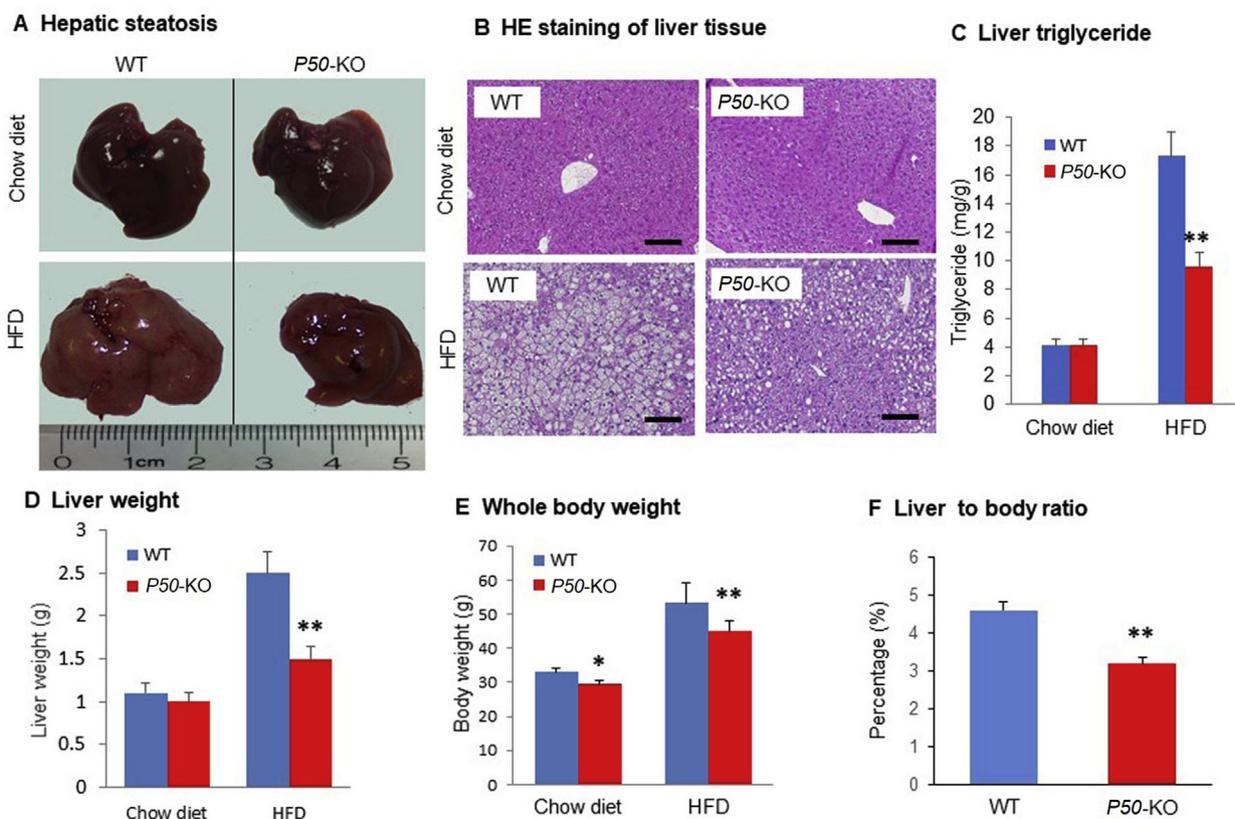


Figure 1 Hepatic steatosis was decreased in *P50*-KO mice. Liver was examined in *P50*-KO and WT mice fed on HFD for 16 weeks. (A) Liver image. (B) Microscope image of liver tissue with H&E staining. Scale bar: 200 μ m. (C) Triglyceride in liver tissue. (D) Liver weight. (E) Body weight of mice. (F) The liver weight to body weight ratio. In the bar figure, each data represents mean \pm SE ($n = 8$). * $P < 0.05$, ** $P < 0.01$ over WT mice.

mice. This led us to investigate the impact of P50 in SREBP1c protein in the cellular system with the knockdown strategy in HepG2 cells. The *P50* knockdown was performed with an expression vector of *P50* shRNA in transient transfection. shRNA was highly expressed in the cells following transfection (Fig. 3A), and P50 protein abundance was significantly reduced in the transfected cells (Fig. 3C). Steatosis model was established in the cells with the oleic acid treatment as indicated by the increased oil red O staining (Fig. 3C). In the model, the steatosis was reduced by *P50* shRNA (Fig. 3B). Abundance of SREBP1c protein was significantly reduced in the knockdown cells, and the reduction was more significant in the steatosis condition (Fig. 3C). These data suggest that inhibition of P50 activity by gene knockdown reduces steatosis in HepG2 cells with a reduction in SREBP1c protein, which recapitulates the changes in the liver of *P50*-KO mice. The data supports a specific-connection of P50 and SREBP1c in the regulation of steatosis.

3.4. *P50*-KO led to down-regulation of HDAC1 protein in hepatocytes

Above data suggest that SREBP1c protein reduction is not a result of a mRNA expression, suggesting a mechanism of protein level regulation. Given that stability of SREBP1c protein is regulated by acetylation¹⁶, we examined histone deacetylases including HDAC1, HDAC3 and SIRT1 in the liver for the SREBP1c

reduction. HDAC1 protein was dramatically reduced in the KO mice relative to the WT mice, and the reduction was observed under both lean and obese conditions (Fig. 4A and B). HDAC3 and SIRT1 proteins were not changed in the same condition. Interestingly, *Hdac1* mRNA was not decreased, but slightly increased in the *P50*-KO mice (Fig. 4C), suggesting that the HDAC1 reduction is unlikely a result of the transcriptional suppression. HDAC1 protein was elevated in the steatosis liver of WT mice together with SREBP1c (Fig. 4D), suggesting that HDAC1 may be required for the SREBP1c elevation in the hepatic steatosis. In contrast to HDAC1, SIRT1 protein was down-regulated and HDAC3 was not changed in the steatosis of WT mice (Fig. 4D). The association of HDAC1 and SREBP1c suggests that HDAC1 may regulate SREBP1c in the hepatocytes.

3.5. Knockdown of HDAC1 decreased SREBP1c in hepatocytes

To test above possibility, HDAC1 was knocked down in HepG2 cells by shRNA to mimic the liver condition of *P50*-KO mice. The HDAC1 protein was significantly reduced by the highly expressed shRNA in the transfected cells (Fig. 5A and D). The hepatic steatosis was decreased in the knockdown cells as indicated by the oil red O staining and total triglyceride quantification (Fig. 5B and C). A reduction in SREBP1c and SCD1 proteins was detected in the *Hdac1* knockdown cells (Fig. 5D). PPAR γ and HDAC3 were not changed in the cells, which enforces the relationship of HDAC1 and SREBP1c. A similar reduction in SREBP1c protein

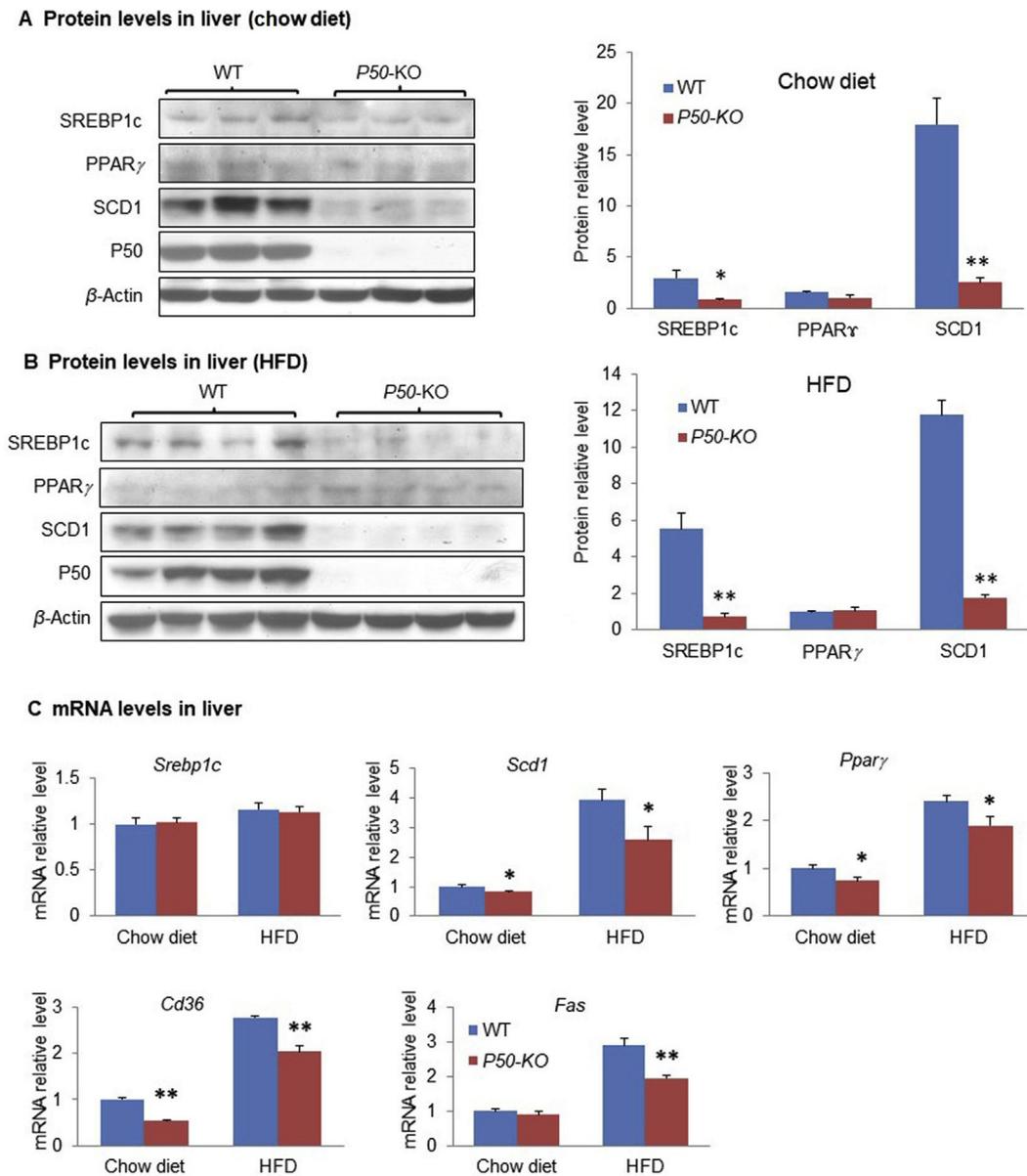


Figure 2 Expression of lipogenic genes was down-regulated in the liver of *P50-KO* mice. (A) Protein levels on chow diet. Western blot was performed for proteins of SREBP1, SCD1, PPAR γ , and *P50* in the liver tissue. (B) Protein level on HFD. Liver tissue was obtained at 16 weeks on HFD and used in the assay. (C) mRNA level of lipogenic genes. *Srebp1*, *Scd1*, *Ppar γ* , *Cd36* and *Fas* were examined by qRT-PCR in the liver tissue. Each experiment was repeated at least three times with consistent results. Representative blots were shown. In the bar figure, each data represents mean \pm SE ($n = 8$). * $P < 0.05$, ** $P < 0.01$.

was observed with HDAC1 suppression by a chemical inhibitor (Fig. 5D). These data suggest that HDAC1 activity is required for the maintenance of SREBP1c protein abundance in hepatocytes.

3.6. Succinylation of SREBP1c was increased in hepatic steatosis of *P50-KO* mice

Succinylation is a protein post-translational modification originally reported in 2011^{18,23}. It modifies proteins in both nucleus and cytoplasm^{24–26}. Succinylation modification is extensive in hepatocytes, and an elevation is reported in some proteins under the hepatic steatosis^{27,28}. However, the succinylation status was

not known in the liver of *P50-KO* mice. To address this issue, we examined protein succinylation in the liver tissue with the anti-succinyllysine antibody in Western blot. The signal was observed in 3–4 major bands at the molecular weights between 130 and 55 kDa, which were significantly reduced by hepatic steatosis in the obese WT mice (Fig. 6A). However, the signal was significantly increased in the obese *P50-KO* mice (Fig. 6B). The enhanced signal was observed in all bands in the *P50-KO* mice. In the lean mice, the increase was observed only in the 130 kDa band in the *P50-KO* mice (Fig. 6C). The data suggest that the succinylation signals were increased in the liver of *P50-KO* mice.

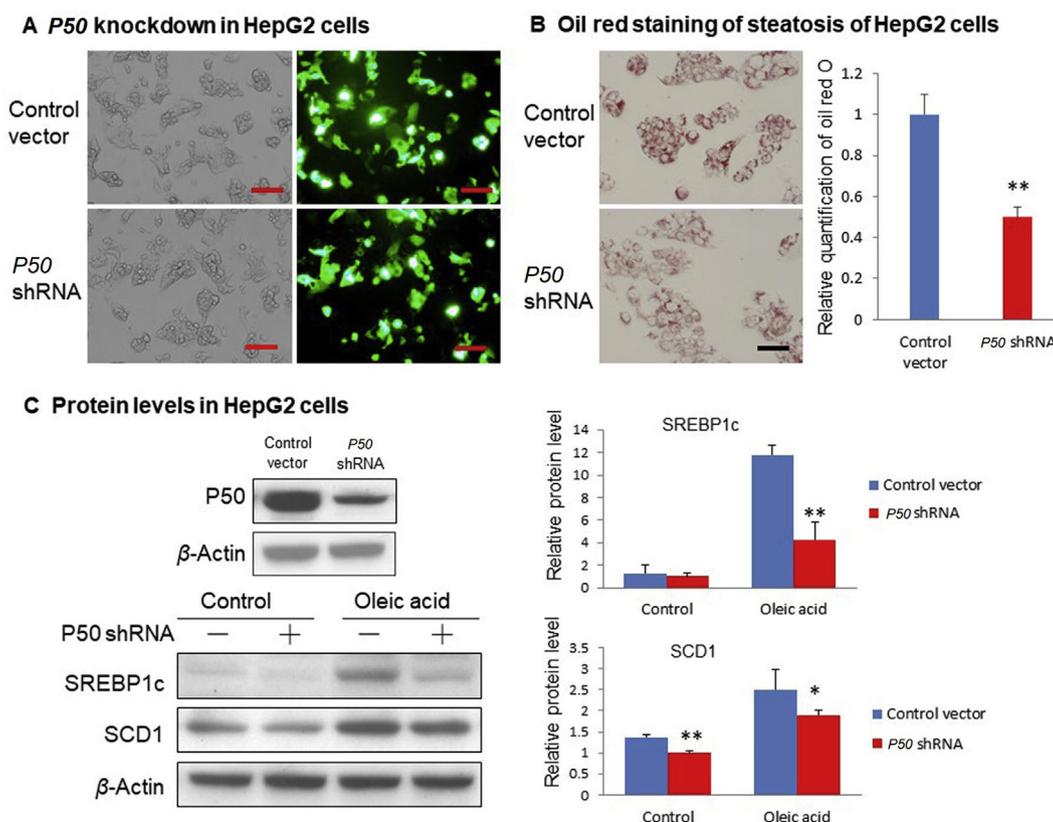


Figure 3 Impact of *P50* knockdown on lipogenic proteins in HepG2 cells. (A) HepG2 cells were electrically transfected with *P50* shRNA expression plasmid using cell line nucleofector Kit L, program T031. Pictures were taken using a microscopy with 10 × object lenses under fluorescent light. Scale bar: 200 μm. (B) Oil red staining of triglyceride in transfected HepG2 cells after 0.3 mmol/L oleic acid treatment for 24 h. Relative triglyceride content was quantified in the HepG2 cells by measuring the oil red O amount in the samples. Scale bar: 100 μm. (C) Protein levels of *P50*, *SREBP1c*, *SCD1* and *PPARγ* were detected by Western blot in HepG2 cells. In the control group, HepG2 cells were transfected with *P50* shRNA plasmid or control vector, and then cultured with DMEM only. In the oleic acid group, transfected cells were treated with 0.3 mmol/L oleic acid in DMEM for 24 h. In the bar figure, each data represents mean ± SE ($n = 3$). * $P < 0.05$, ** $P < 0.01$.

The *SREBP1c* protein has a molecular weight (125 kDa) close to the succinylation band of 130 kDa. Succinylation of *SREBP1c* protein was examined in the IP product of *SREBP1c* protein with the succinylation antibody. The succinylation signal was observed in *SREBP1c*, and an increase was observed in *P50*-KO mice (Fig. 6D). The increase was associated with *HDAC1* reduction, suggesting a substrate/enzyme relationship for *SREBP1c* and *HDAC1*. To test the relationship, the *HDAC1* enzyme assay was conducted for de-succinylation of *SREBP1c* using the recombinant protein. The de-succinylation reaction was catalysed by *HDAC1* in a dose-dependent manner (Fig. 6E). The data suggest that *SREBP1c* protein is subjected to modification by succinylation, which is modulated by *HDAC1* through de-succinylation. In the liver of *P50*-KO mice, the succinylation was increased due to the lack of *HDAC1* activity.

3.7. Regulation of *HDAC1* oscillation by *NF-κB*

Above data suggest that regulation of *HDAC1* by *NF-κB* is the key in the liver phenotype of *P50*-KO mice. However, the physiological relevance of the finding remains unknown. To address this issue, we tested *HDAC1* response to *TNF-α* in the mouse embryonic fibroblast (MEF) cells. *Tnf-α* expression was elevated together with *Il-1β* and *Il-6* in the liver of *P50*-KO mice (Fig. 7A),

suggesting an enhanced inflammatory status. *HDAC1* protein exhibited a pattern of oscillation in the wild-type MEF cells in response to *NF-κB* activation by *TNF-α* (Fig. 7B). *HDAC1* was reduced by *TNF-α* at three time points (30, 120 and 480 min) with a recovery to the normal level between the reductions. MEF cells with different gene knockout in the *NF-κB* family were used to test the role of *NF-κB* in the *TNF-α* effect. In the *P50*-KO MEF cells, the *HDAC1* oscillation disappeared, and a sustained *HDAC1* reduction was observed under the *TNF-α* treatment (Fig. 7C). The *HDAC1* reduction was blocked by the proteasome inhibitor MG-132 (Fig. 7D). In *IκBα*-KO cells in which *NF-κB* activation by *TNF-α* remains normal, the oscillation was not affected (Fig. 7E). In *Ikkβ*-KO cells, in which *TNF-α* could not activate *NF-κB*, *TNF-α* did not affect *HDAC1* (Fig. 7F). The data suggest that *NF-κB* mediates the *TNF-α* signal in regulation of *HDAC1*, and *P50* is required for the *TNF-α* effect. The *HDAC1* protein degradation was dependent on proteasome in the absence of *P50*.

3.8. Inactivation of *NF-κB* *P65* did not alter the hepatic steatosis

Above data suggests that the *P50* subunit of *NF-κB* may regulate hepatic steatosis through an impact in the *HDAC1/SREBP1c* pathway. However, the role of *P65* subunit remains unknown in

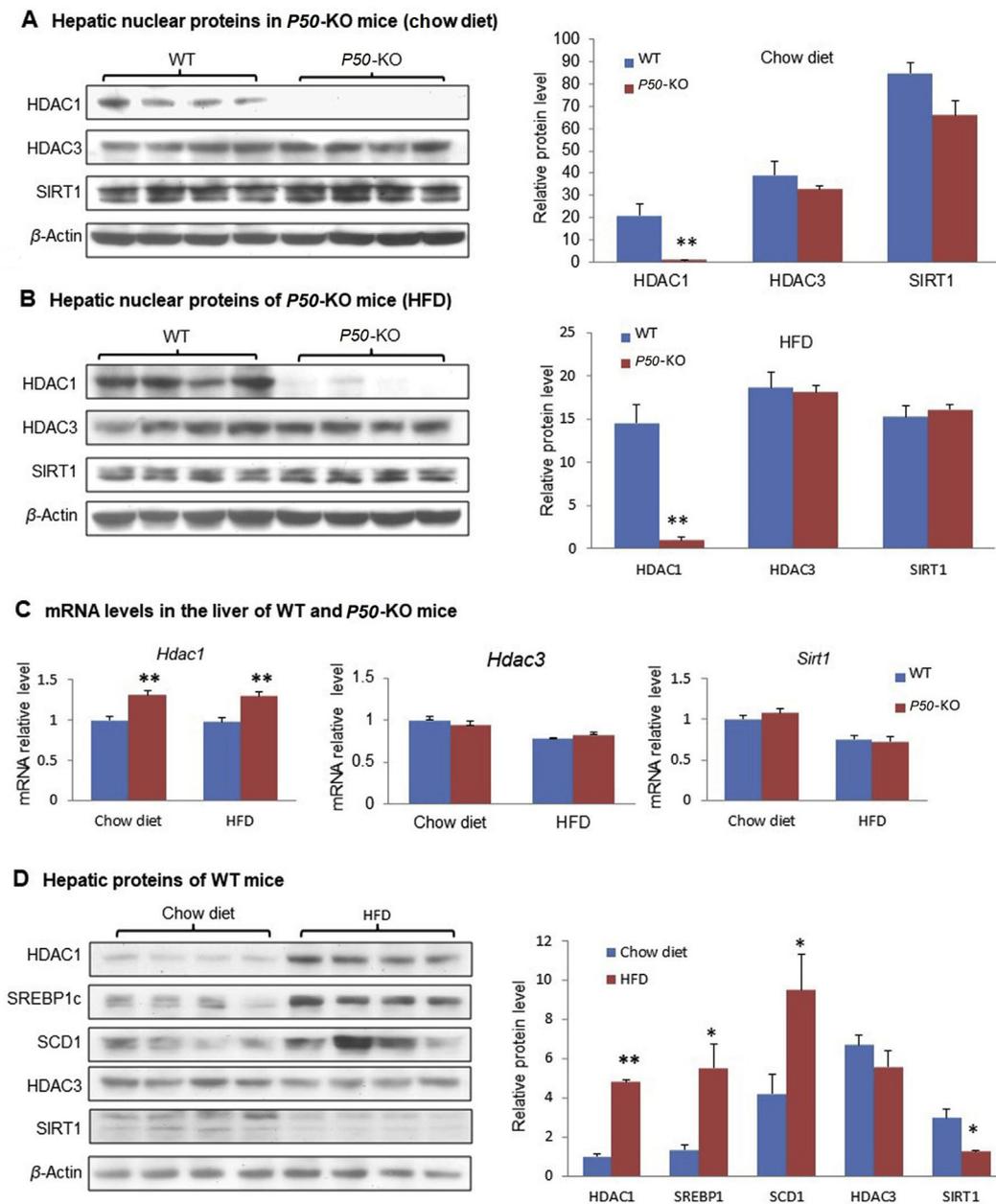


Figure 4 Reduction of HDAC1 protein in the liver of *P50*-KO mice. HDAC1 protein was decreased in the liver of *P50*-KO mice compared with that of WT mice. HDAC1 down-regulation was associated with the decrease in hepatic steatosis. (A) Nuclear protein in the liver tissue of *P50*-KO mice on chow diet. (B) Nuclear protein in the liver of *P50*-KO mice on HFD. (C) mRNA expression. mRNA level of HDAC1, HDAC3, SIRT1 was determined in the liver tissue of *P50*-KO mice on chow diet and HFD, respectively. (D) Proteins in liver tissue of WT mice on HFD. Western blot was performed for HDAC1, HDAC3, SIRT1, SCD1 and SREBP1 proteins in liver tissue of WT mice on HFD for 4 months. In the bar figure, each data represents mean \pm SE ($n = 8$). * $P < 0.05$, ** $P < 0.01$.

the steatosis. To address this issue, P65 protein was examined in *P50*-KO mice and an elevation was observed in the liver (Fig. 8A). The P65 activity was tested in the liver-specific *P65*-KO mice. The *P65*-KO mice did not exhibit any significant difference in the hepatic steatosis over the WT mice (Fig. 8B and C). A modest increase in the number of large lipid droplets was observed in the hepatocytes of *P65*-KO mice, but the difference did not lead to an increase in the triglyceride content (Fig. 8C). HDAC1 and

SREBP1c did not show a difference in the liver between the *P65*-KO and WT mice (Fig. 8D). No difference was detected in mRNA of lipogenic genes and proinflammatory genes in the livers of *P65*-KO over WT mice except *Ppar γ* (Fig. 8E). The mRNA of *Ppar γ* was increased in the liver of *P65*-KO mice, which might account for the increased number of large lipid droplets. This group of data suggest that the P65 subunit did not play a direct role in the control of hepatic steatosis.

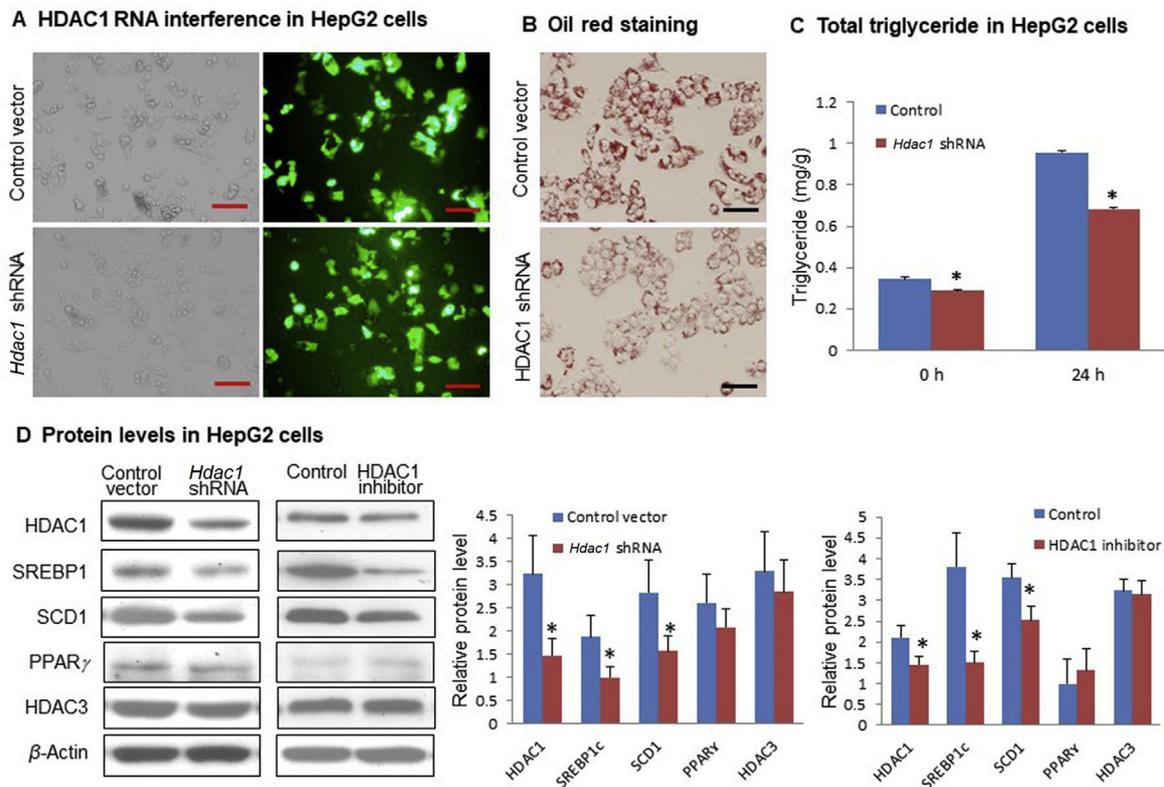


Figure 5 Inhibition of steatosis by *Hdac1* knockdown in HepG2 cells. (A) *Hdac1* shRNA transfection. HepG2 cells were electroporated with *Hdac1* shRNA plasmid or control vector using cell line nucleofector Kit L, program T031. Pictures were taken using a microscopy with 10 × objective lenses for transfection efficiency. Scale bar: 200 μm. (B) Impact of *Hdac1* knockdown in steatosis. Oil red staining of triglyceride in transfected HepG2 cells after 0.5 mmol/L oleic acid treatment for 24 h. Scale bar: 100 μm. (C) Triglyceride quantification in the knockdown cells. (D) Suppression of lipogenic proteins by *Hdac1* knockdown or chemical inhibitor. Western blot of HDAC1, HDAC3, SREBP1, SCD1, PPAR γ protein in HepG2 cells after 0.5 mmol/L oleic acid treatment for 24 h. In the first column, *Hdac1* was knocked down by shRNA. In the second column, HDAC1 was inhibited with HDAC1-specific inhibitor (0.4 mmol/L) for 30 min before oleic acid treatment in HepG2 cells. In the bar figure, each data represents mean \pm SE ($n = 3$). * $P < 0.05$.

4. Discussion

Current study suggests that NF- κ B may regulate hepatic steatosis through an impact in the HDAC1 activity by the P50 subunit. NF- κ B is activated in response to multiple signals including TNF- α , endotoxin and reactive oxygen species, etc. It is known that the P50 subunit of NF- κ B associates with HDAC1 on the promoter DNA of NF- κ B target genes to regulate gene transcription. It is unknown if NF- κ B regulates the abundance of HDAC1 protein in cells. Current study provides evidence that NF- κ B indeed regulates the HDAC1 protein. The observation was made in the hepatocytes of *P50*-KO mice, in which HDAC1 protein was significantly decreased. The P50 impact on HDAC1 was confirmed in cells with *P50* gene knockdown. The P50 impact was only observed in protein, but not mRNA of *Hdac1*, suggesting a role of post-translational modification in the control of HDAC1 protein by P50. The reduction is unlikely a result of suppression of protein synthesis as the liver development is normal in *P50*-KO mice. HDAC1 is required for normal differentiation of hepatocytes¹¹. If HDAC1 protein synthesis was blocked, *P50*-KO liver should suffer a defect in development. Our data suggests that the HDAC1 reduction was a result of protein destabilization as the

change was blocked by MG-132. The HDAC1 degradation is mediated by ubiquitination²⁹. Other HDACs (HDAC3 and SIRT1) were not significantly altered in the liver of *P50*-KO mice, suggesting that P50 specifically regulates HDAC1. We speculate that P50 may stabilize HDAC1 through a protein–protein association to prevent HDAC1 degradation. However, the exact molecular event remains to be investigated for the HDAC1 degradation. The P65 subunit was reported to associate with HDAC1³⁰. However, the possibility is not supported by current study in *P65*-KO mice.

This study demonstrates that P65 acts in hepatocytes in the control of hepatic steatosis. Global P50 inactivation in mice was reported to generate several metabolic changes including resistance to diet-induced obesity and improvement in insulin resistance with an elevation in energy expenditure^{4,8,9,31}. *P50*-KO mice exhibited an increase in energy expenditure and resistance to obesity as reported by this and other groups^{4,8,9}. The reduction in hepatic steatosis may be a result of global increase in energy expenditure. However, the reduction in the liver to body weight ratio and the data of cellular models consistently support the direct role of P50 in hepatocytes. The hepatic effect may contribute to the global phenotype of *P50*-KO mice. The HDAC1 reduction is a potential mechanism for the reduced steatosis in *P50*-KO mice.

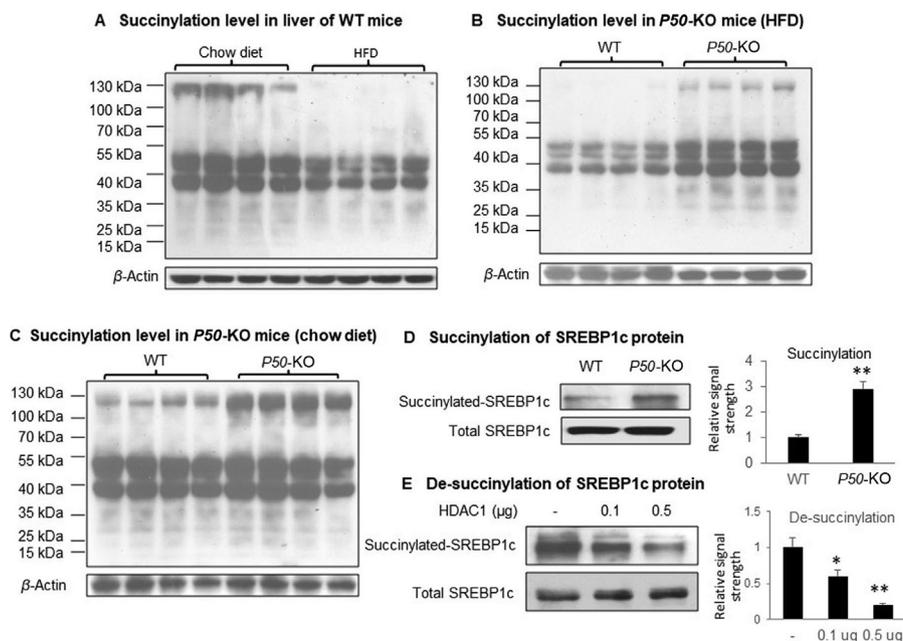


Figure 6 Succinylation of SREBP1c protein. Liver tissue was obtained from mice on HFD for 4 months. (A) Succinylation signal in the liver tissue of WT mice on chow diet or HFD, respectively. (B) Succinylation signal in the liver tissue of WT and *P50*-KO mice on HFD. (C) Succinylation signal in the liver tissue of WT and *P50*-KO mice on chow diet. (D) Succinylation of SREBP1c protein in the liver tissue of *P50*-KO mice on HFD. SREBP1c was isolated from the liver tissue by immunoprecipitation and resolved in SDS-PAGE gel. Succinylation was detected in the IP product with the succinylation antibody. (E) De-succinylation of SREBP1c protein by HDAC1. The de-succinylation assay was conducted with the recombinant HDAC1 in the IP product of SREBP1c of *P50*-KO mice. In the bar figure, each data represents mean \pm SE ($n=3$). * $P < 0.05$, ** $P < 0.01$.

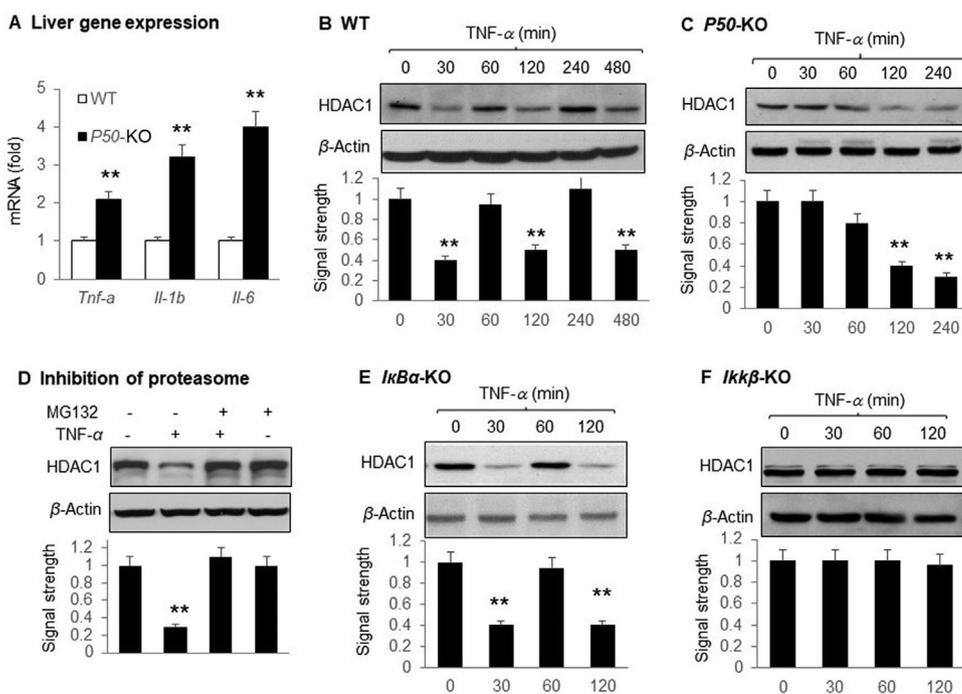


Figure 7 Maintenance of HDAC1 protein level was dependent on *P50*. MEF cells with *P50*-KO, *I κ B α* -KO and *I κ B β* -KO were examined for HDAC1 after TNF- α (20 ng/mL) treatment at different times as indicated. The signal of HDAC1 protein was quantified and normalized with the signal of actin in the blot. (A) Liver gene expression. The expression was determined in the liver of *P50*-KO mice by qRT-PCR. (B) HDAC1 in the wild type MEF cells treated with TNF- α . (C) HDAC1 in the *P50*-KO MEF cells treated with TNF- α . (D) Blockage of HDAC1 reduction by proteasome inhibitor. HDAC1 protein was examined in *P50*-KO MEF cells that were pretreated with the proteasome inhibitor MG132 for 30 min followed by TNF-treatment for 120 min. (E) HDAC1 in the *I κ B α* -KO MEF cells treated with TNF- α . (F) HDAC1 in the *I κ B β* -KO MEF cells treated with TNF- α . Each experiment was repeated at least three times with consistent results. Representative blots were shown. In the bar figure, each data represents mean \pm SE ($n=3$). ** $P < 0.01$.

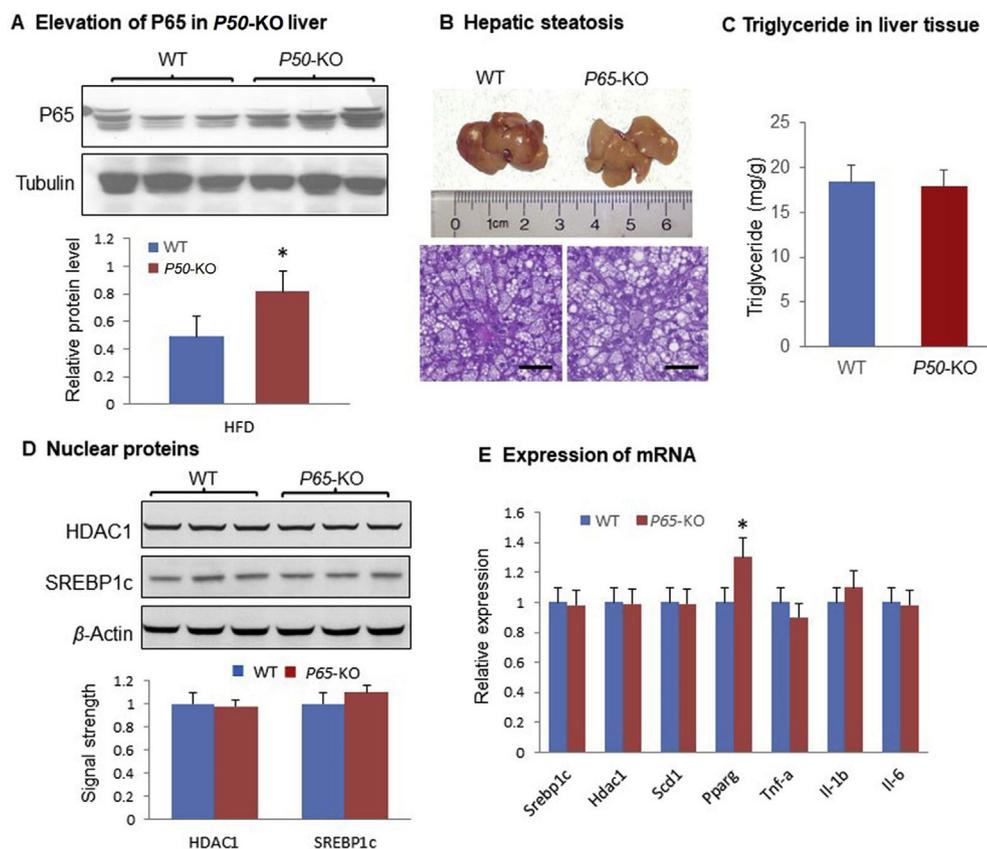


Figure 8 Hepatic steatosis of *P65*-KO mice. (A) P65 protein in *P50*-KO liver. The P65 protein was examined in the liver of *P50*-KO mice on HFD. (B) Liver weight of *P65*-KO mice on HFD. The liver was collected from the mice at 14 weeks on HFD and the tissue image was taken under a microscope at 20 \times from the tissue slide with H&E staining. Scale bar: 200 μ m. (C) Triglyceride in liver tissue of mice on HFD. (D) Protein level in the liver tissues. (E) mRNA of lipogenic genes in the liver tissue. In the bar figure, each data represents mean \pm SE ($n = 7$). * $P < 0.05$.

Consistently, an increase in HDAC1 activity by gene over-expression enhances hepatic steatosis in mice although the mechanism was not clear³². In contrast to HDAC1, HDAC3 inhibits lipogenesis in the physiological conditions. The liver-specific *Hdac3* knockout mice suffer severe hepatic steatosis³³. HDAC3 may not contribute to the phenotype in this study for lack of HDAC3 alteration in the liver of *P50*-KO mice. In brown adipocytes, HDAC1 promotes lipid accumulation through inhibition of PGC-1 α (peroxisome proliferator-activated receptor gamma co-activator 1 α) activity¹³. Our data suggest that the P50 subunit of NF- κ B promotes hepatic steatosis in the physiological condition by stabilization of HDAC1 protein in hepatocytes.

Our data suggests that HDAC1 promotes hepatic steatosis through de-succinylation of SREBP1c protein in hepatocytes. There are two SREBP genes in mammals. SREBP1 promotes fatty acid and triglyceride synthesis, and SREBP2 controls cholesterol synthesis. SREBP1c activity is induced by insulin through the PI3K/AKT pathway and by protein acetylation³⁴. The activity of SREBP1c protein is inhibited by phosphorylation and ubiquitination-mediated protein degradation^{15,35}. Succinylation was found as a new mechanism in the regulation of SREBP1c activity in current study. SREBP1c succinylation was found in *P50*-KO mice, and over modification of SREBP1c protein by succinylation was observed with the SREBP1c reduction. Succinylation occurs at the lysine residue in variety of proteins²⁸. The succinylation status is regulated by SIRT5 through de-

succinylation in mitochondria^{18,24}. SIRT5 is located in the mitochondrion, and the identity of nuclear de-succinylation enzyme remains unknown. In current study, the succinylation signals were observed in the liver at molecules of 35–130 kDa, which was reduced by fatty liver in the WT mice. In *P50*-KO mice, the succinylation was increased under the non-obese and obese conditions. HDAC1 was found to de-succinylate SREBP1c protein in the enzyme assay, and lack of HDAC1 was responsible for the increased succinylation of SREBP1c in the *P50*-KO mice. The relationship among succinylation, acetylation and phosphorylation remains to be investigated in the SREBP1c protein in the future.

The P65 protein was elevated in the absence of P50 to induce expression of cytokines including TNF- α , IL-1 and IL-6 in the *P50*-KO liver. In the NF- κ B dimer, P50 enhance the binding ability of P65 in the target gene DNA. However, the association reduces the transcriptional activity of P65 for lack of a transactivation domain in P50. In the absence of P50, the P50 homodimer binds to DNA with a low affinity, but higher transactivation ability as a result of doubled number of transactivation domain. This contributes to the elevated cytokine expression in the liver of *P50*-KO mice in current study. Kupffer cells are more active in the expression of the cytokines. When this happens in Kupffer cells and hepatocytes, more pro-inflammatory cytokines are produced. Current study suggests that the P65 subunit of NF- κ B is not a major player in the pathogenesis of hepatic steatosis. In activation of P65 in the liver-specific *P65*-KO mice did not change the liver

size, triglyceride content and body weight. The hepatic steatosis was not significantly altered in the *P65*-KO mice on HFD except the modest increase in the lipid droplet size, suggesting that *P65* is not required for the hepatic steatosis.

5. Conclusions

This study demonstrates that the *P50* subunit, not the *P65* subunit, plays a direct role in hepatocytes in the regulation of steatosis. *P50* protected HDAC1 activity through stabilization of the HDAC1 protein. The HDAC1 protein was reduced in the liver of *P50*-KO mice due to lack of the protection. HDAC1 preserved SREBP1c activity through de-succinylation, which was interrupted in the liver of *P50*-KO mice for super succinylation of SREBP1c and inhibition of hepatic steatosis. In the physiological condition, HDAC1 may stabilize SREBP1c protein through the de-succinylation activity to promote hepatic steatosis. These data suggest a novel signaling pathway of *P50*/HDAC1/SREBP1c in hepatocytes in the control of hepatic steatosis. The pathway may apply to the pathogenesis of hepatic steatosis under endotoxin challenge or inflammatory status. However, this pathway remains to be explored further to identify the exact mechanisms for degradation of HDAC1 and SREBP1c proteins. A role of the gut microbiota, which influences hepatic steatosis³⁶, remains to be investigated in the phenotype of *P50*-KO mice.

Acknowledgments

This study was funded by the National Key Research and Development Program of China (2018YFA0800603 to JY) the starting fund at Shanghai Jiao Tong University Affiliated Sixth People's Hospital to Jianping Ye. The animal study was conducted at the Pennington Biomedical Research Center, Louisiana State University, Baton Rouge, LA 70808, USA.

Author contributions

Yunwei Guo, Xiaoying Zhang, Zhiyun Zhao, Hongyun Lu, Bilun Ke and Xin Ye conducted the experiments and data analysis, involved in preparation of the figures and manuscript. Bin Wu and Jianping Ye designed the study and prepared the manuscript. All authors have read the manuscript and agreed to the conclusion.

Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supporting information

Supporting data to this article can be found online at <https://doi.org/10.1016/j.apsb.2020.02.005>.

References

- Wang XA, Zhang R, She ZG, Zhang XF, Jiang DS, Wang T, et al. Interferon regulatory factor 3 constrains IKK β /NF- κ B signaling to alleviate hepatic steatosis and insulin resistance. *Hepatology* 2014;**59**: 870–85.
- Liu Y, Sheng L, Xiong Y, Shen H, Liu Y, Rui L. Liver NF- κ B-inducing kinase promotes liver steatosis and glucose counterregulation in male mice with obesity. *Endocrinology* 2017;**158**:1207–16.
- Gao Z, He Q, Peng B, Chiao PJ, Ye J. Regulation of nuclear translocation of HDAC3 by I κ B α is required for tumor necrosis factor inhibition of peroxisome proliferator-activated receptor γ function. *J Biol Chem* 2006;**281**:4540–7.
- Tang T, Zhang J, Yin J, Staszkievicz J, Gawronska-Kozak B, Mynatt R, et al. Uncoupling of inflammation and insulin resistance by NF- κ B in transgenic mice through induction of energy expenditure. *J Biol Chem* 2010;**285**:4637–44.
- Jiao P, Feng B, Ma J, Nie Y, Paul E, Li Y, et al. Constitutive activation of IKK β in adipose tissue prevents diet-induced obesity in mice. *Endocrinology* 2012;**153**:154–65.
- Ke B, Zhao Z, Ye X, Gao Z, Manganiello V, Wu B, et al. Inactivation of NF- κ B p65 (RelA) in liver improved insulin sensitivity and inhibited cAMP/PKA pathway. *Diabetes* 2015;**64**:E496–505.
- Liu J, Ibi D, Taniguchi K, Lee J, Herrema H, Akosman B, et al. Inflammation improves glucose homeostasis through IKK β -XBPIs interaction. *Cell* 2016;**167**:1052–1066 e18.
- Minegishi Y, Haramizu S, Misawa K, Shimotoyodome A, Hase T, Murase T. Deletion of nuclear factor- κ B p50 upregulates fatty acid utilization and contributes to an anti-obesity and high-endurance phenotype in mice. *Am J Physiol Endocrinol Metab* 2015;**309**: E523–33.
- Bhatt BA, Dedousis N, Sipula IJ, O'Doherty RM. Elevated metabolic rate and skeletal muscle oxidative metabolism contribute to the reduced susceptibility of NF- κ B p50 null mice to obesity. *Phys Rep* 2018;**6**:e13836.
- Lagger G, O'Carroll D, Rembold M, Khier H, Tischler J, Weitzer G, et al. Essential function of histone deacetylase 1 in proliferation control and CDK inhibitor repression. *EMBO J* 2002;**21**:2672–81.
- Ko S, Russell JO, Tian J, Gao C, Kobayashi M, Feng R, et al. HDAC1 regulates differentiation of bipotent liver progenitor cells during regeneration via SOX9B and CDK8. *Gastroenterology* 2019;**156**: 187–202. e14.
- Wiper-Bergeron N, Wu D, Pope L, Schild-Poulter C, Hache RJ. Stimulation of preadipocyte differentiation by steroid through targeting of an HDAC1 complex. *EMBO J* 2003;**22**:2135–45.
- Li F, Wu R, Cui X, Zha L, Yu L, Shi H, et al. Histone deacetylase 1 (HDAC1) negatively regulates thermogenic program in brown adipocytes via coordinated regulation of histone h3 lysine 27 (h3k27) deacetylation and methylation. *J Biol Chem* 2016;**291**:4523–36.
- Gan L, Xiang W, Xie B, Yu L. Molecular mechanisms of fatty liver in obesity. *Front Med* 2015;**9**:275–87.
- Sundqvist A, Bengoechea-Alonso MT, Ye X, Lukiyanchuk V, Jin J, Harper JW, et al. Control of lipid metabolism by phosphorylation-dependent degradation of the srebp family of transcription factors by scf(fbw7). *Cell Metabol* 2005;**1**:379–91.
- Giandomenico V, Simonsson M, Gronroos E, Ericsson J. Coactivator-dependent acetylation stabilizes members of the srebp family of transcription factors. *Mol Cell Biol* 2003;**23**:2587–99.
- Du Y, Hu H, Qu S, Wang J, Hua C, Zhang J, et al. Sirt5 deacetylates metabolism-related proteins and attenuates hepatic steatosis in *ob/ob* mice. *EBioMedicine* 2018;**36**:347–57.
- Du J, Zhou Y, Su X, Yu JJ, Khan S, Jiang H, et al. Sirt5 is a NAD-dependent protein lysine demalonylase and desuccinylase. *Science* 2011;**334**:806–9.
- Gao Z, Hwang D, Bataille F, Lefevre M, York D, Quon MJ, et al. Serine phosphorylation of insulin receptor substrate 1 by inhibitor kappaB kinase complex. *J Biol Chem* 2002;**277**:48115–21.
- Gao Z, Chiao P, Zhang X, Zhang X, Lazar MA, Seto E, et al. Coactivators and corepressors of NF- κ B in I κ B α gene promoter. *J Biol Chem* 2005;**280**:21091–8.
- Xu F, Gao Z, Zhang J, Rivera CA, Yin J, Weng J, et al. Lack of SIRT1 (mammalian sirtuin 1) activity leads to liver steatosis in the SIRT1^{+/-} mice: a role of lipid mobilization and inflammation. *Endocrinology* 2010;**151**:2504–14.
- Le J, Zhang X, Jia W, Zhang Y, Luo J, Sun Y, et al. Regulation of microbiota-GLP1 axis by sennoside a in diet-induced obese mice. *Acta Pharm Sin B* 2019;**9**:758–68.

23. Zhang Z, Tan M, Xie Z, Dai L, Chen Y, Zhao Y. Identification of lysine succinylation as a new post-translational modification. *Nat Chem Biol* 2011;**7**:58–63.
24. Park J, Chen Y, Tishkoff DX, Peng C, Tan M, Dai L, et al. Sirt5-mediated lysine desuccinylation impacts diverse metabolic pathways. *Mol Cell* 2013;**50**:919–30.
25. Colak G, Xie Z, Zhu AY, Dai L, Lu Z, Zhang Y, et al. Identification of lysine succinylation substrates and the succinylation regulatory enzyme CobB in *Escherichia coli*. *Mol Cell Proteomics* 2013;**12**:3509–20.
26. Xie Z, Dai J, Dai L, Tan M, Cheng Z, Wu Y, et al. Lysine succinylation and lysine malonylation in histones. *Mol Cell Proteomics* 2012;**11**:100–7.
27. Rardin MJ, He W, Nishida Y, Newman JC, Carrico C, Danielson SR, et al. Sirt5 regulates the mitochondrial lysine succinylome and metabolic networks. *Cell Metabol* 2013;**18**:920–33.
28. Cheng Y, Hou T, Ping J, Chen G, Chen J. Quantitative succinylome analysis in the liver of non-alcoholic fatty liver disease rat model. *Proteome Sci* 2016;**14**:3.
29. Ma Y, Yuan S, Tian X, Lin S, Wei S, Hu T, et al. ABIN1 inhibits HDAC1 ubiquitination and protects it from both proteasome- and lysosome-dependent degradation. *J Cell Biochem* 2018;**119**:3030–43.
30. Lee S-K, Kim J-H, Lee YC, Cheong J, Lee JW. Silencing mediator of retinoic acid and thyroid hormone receptors, as a novel transcriptional corepressor molecule of activating protein-1, nuclear factor-kappa B, and serum response factor. *J Biol Chem* 2000;**275**:12470–4.
31. Gao Z, Yin J, Zhang J, He Q, McGuinness OP, Ye J. Inactivation of NF- κ B p50 leads to insulin sensitization in liver through post-translational inhibition of p70s6k. *J Biol Chem* 2009;**284**:18368–76.
32. Wang AG, Seo SB, Moon HB, Shin HJ, Kim DH, Kim JM, et al. Hepatic steatosis in transgenic mice overexpressing human histone deacetylase 1. *Biochem Biophys Res Commun* 2005;**330**:461–6.
33. Papazyan R, Sun Z, Kim YH, Titchenell PM, Hill DA, Lu W, et al. Physiological suppression of lipotoxic liver damage by complementary actions of HDAC3 and SCAP/SREBP. *Cell Metabol* 2016;**24**:863–74.
34. You M, Liang X, Ajmo JM, Ness GC. Involvement of mammalian sirtuin 1 in the action of ethanol in the liver. *Am J Physiol Gastrointest Liver Physiol* 2008;**294**:G892–8.
35. Li Y, Xu S, Mihaylova MM, Zheng B, Hou X, Jiang B, et al. AMPK phosphorylates and inhibits SREBP activity to attenuate hepatic steatosis and atherosclerosis in diet-induced insulin-resistant mice. *Cell Metabol* 2011;**13**:376–88.
36. Sun L, Pang Y, Wang X, Wu Q, Liu H, Liu B, et al. Ablation of gut microbiota alleviates obesity-induced hepatic steatosis and glucose intolerance by modulating bile acid metabolism in hamsters. *Acta Pharm Sin B* 2019;**9**:702–10.