Hepatic SREBP-2 and cholesterol biosynthesis are regulated by FoxO3 and Sirt6

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Running title: Sirt6 and FoxO3 in cholesterol homeostasis

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ABSTRACT

Cholesterol homeostasis is crucial for cellular function and organismal health. The key regulator for the cholesterol biosynthesis is sterol regulatory element-binding protein 2 (SREBP-2). The biochemical process and physiological function of SREBP-2 have been well characterized; however, it is not clear how this gene is epigenetically regulated. Here we have identified Sirt6 as a critical factor for Srebp2 gene regulation. Hepatic deficiency of Sirt6 in mice leads to elevated cholesterol levels. On the mechanistic level, Sirt6 is recruited by FoxO3 to the Srebp2 gene promoter where Sirt6 deacetylates histone H3 at lysines 9 and 56, thereby promoting a repressive chromatin state. Remarkably, Sirt6 or FoxO3 overexpression improves hypercholesterolemia in diet-induced or genetic obese mice. In summary, our data suggest an important role of hepatic Sirt6 and FoxO3 in the regulation of cholesterol homeostasis.

Key words: Cholesterol biosynthesis, gene regulation, transcription, histone acetylation, epigenetics, sirtuin, forkhead transcription factor
INTRODUCTION

Cholesterol homeostasis is essential for normal cellular and tissue functions, and hypercholesterolemia can lead to hepatic and cardiovascular abnormalities (1, 2). The balance between cholesterol intake/biosynthesis and metabolism to bile acids is crucial for cholesterol homeostasis (1). In the regulation of cholesterol biosynthesis, sterol regulatory element-binding protein 2 (SREBP-2) is the master regulator (3-6). SREBP-2 is synthesized as a precursor, which is processed to mature nuclear form (nSREBP-2) in Golgi apparatus before it becomes a functional transcription factor (4). In the nucleus, nSREBP-2 controls expression of numerous genes involved in cholesterol homeostasis, including 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR)—the rate-limiting enzyme in cholesterol biosynthesis.

Liver plays a critical role in cholesterol homeostasis, including de novo biosynthesis, efflux, and conversion to bile acids (1, 4, 7-9). Several transcription factors in the liver have been linked to cholesterol regulation, including SREBP-2, SREBP-1a, liver X receptor (LXR), farnesoid X receptor (FXR), and forkhead box O transcription factors (FoxOs) (3, 4, 9-15). With regard to FoxOs, the mechanisms for cholesterol regulation are not well understood (9, 13-17). Some of these factors can also be modulated by post-translational modifications, for instance, deacetylation by an NAD⁺-dependent deacetylase sirtuin 1 (Sirt1) (18-20). It has been shown that deacetylation of SREBP-1/2 by Sirt1 destabilizes these proteins (20, 21). Another sirtuin family member, Sirt6, has been increasingly appreciated for its broad role in biology, including genome maintenance and DNA repair (22-29), cell survival and apoptosis (30-32), inflammation (33-38), cardiac function (39-41), oxidative stress (42), longevity (43), and metabolism and energy homeostasis (44-51). Sirt6 has multiple enzymatic activities including ADP-ribosylation and deacetylation of histone H3 at lysines 9 (H3K9) and 56 (H3K56), respectively (26, 27, 29, 52,
Sirt6 has been shown to regulate glycolysis and fatty acid synthesis and metabolism through deacetylation of H3K9 and H3K56, and Sirt6 liver-specific knockout mice develop fatty liver disease (45). Sirt6 also regulates hepatic gluconeogenesis through modulation of the GCN5 acetyltransferase activity (50). Additionally, Sirt6 has been implicated in cholesterol homeostasis as well since systemic overexpression of Sirt6 in mice lowers LDL-cholesterol under normal and high-fat diet conditions (44). However, it is not yet clear whether Sirt6 has a direct role in cholesterol regulation. Here, we provide in vitro and in vivo evidence to suggest a critical role of Sirt6 in the regulation of hepatic Srebp2 gene expression and cholesterol homeostasis.
MATERIALS AND METHODS

Animal studies

FoxO1, FoxO3, FoxO1/3/4, Sirt1, and Sirt6 liver-specific knockout mice were generated by crossing floxed mice with an Albumin-Cre line (Jackson Laboratory). Animals were maintained on mixed genetic background: FoxOs floxed mice on C57BL/6J:129/Sv:FVB, Sirt1 floxed mice on C57BL/6J:129/Sv, and Sirt6 floxed mice on NIH Black Swiss:129/Sv:FVB. Genotyping PCR primers and conditions were same as previously described (45, 54, 55). The db/db mice (on C57BL6/J background) were purchased from the Jackson Laboratory. For high-fat diet treatment, a diet containing 60% calories from fat (Harlan Teklad) was used. For adenoviral experiments, overexpression and knockdown adenoviruses were injected at a dose of $5 \times 10^8$ pfu and $1 \times 10^9$ pfu per mouse, respectively; and experiments were performed within 7-14 days after injections. All animal procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Institutional Animal Use and Care Committee of Indiana University School of Medicine.

Plasmid constructs and adenoviruses

For mouse Srebp2 gene promoter analysis, we cloned the short promoter (-309 to +42 bp relative to the transcription start site) into pGL4.10 vector (Promega). IRE mutagenesis was performed using the QuickChange II site-directed mutagenesis kit (Agilent). Mouse FoxO1, Sirt1, and Srebp2 (mature form), and human FOXO3 and SIRT6 coding sequences were cloned into pcDNA3 (Invitrogen) with FLAG or HA tag. Adenoviruses for FoxO1, Sirt1, Sirt6, and FOXO3 overexpression were generated and used in vitro and in vivo as previously described (54, 56). Human SIRT6 cDNA was also cloned into pAdEasy vector (Agilent) following the manufacturer’s manual. shRNAs for mouse Sirt6 gene was designed by the Block-iT RNAi
Designer (Invitrogen) and DNA oligos were cloned into pENTR/U6 vector (Invitrogen). The primers are listed in the Supplemental Table 1.

**Serum and liver cholesterol analysis**

Serum samples were collected from overnight fasted mice. Hepatic lipids were extracted as previously described (54). Total cholesterol concentrations were measured using assay kits from Wako Chemicals USA.

**Luciferase reporter assays**

Mouse *Srebp2* gene promoter was analyzed in HEK293 cells using pGL4.10 luciferase reporter system together with an internal control Renilla luciferase reporter as previously described (54). The primers used for making DNA constructs are listed in the Supplemental Table 1.

**mRNA analysis**

Total RNAs were isolated from cells or tissues using TRI Reagent (Sigma). Reverse transcription was performed using a cDNA synthesis kit (Applied Biosystems). Real-time PCR was performed using GoTaq qPCR Master Mix (Promega). The primers used in PCR reactions are included in the Supplemental Table 1. Real-time PCR data were normalized to an internal control —Ppia and relative fold changes (experimental group/control) were also calculated.

**Protein analysis**

Cell and tissue extract preparation, immunoprecipitation, and immunoblotting were performed as previously described (54). Nuclear extracts were prepared according to manufacturer's manual (Pierce) The following antibodies were used: anti-FLAG (Sigma), anti-Actinin, anti-HA, anti-SIRT1 (Santa Cruz Biotechnology), anti-Lamin A/C, anti-FoxO1, anti-
FoxO3, anti-acetylated lysine (Cell Signaling Technology), anti-SREBP-2 and anti-SIRT6 (Abcam).

**Chromatin immunoprecipitation (ChIP)**

Chromatin crosslinking was performed in mouse primary hepatocytes and mouse liver lysates, followed by chromatin preparation, immunoprecipitation with FLAG (Sigma), HA (Cell Signaling Technology), H3K9Ac, H3K18Ac, H3K56Ac and histone H3 (Millipore) antibodies, and real-time PCR analysis, as previously described (54). ChIP DNA amount for gene promoters of interest was normalized to that of a housekeeping gene—Ppia ChIP or total histone H3 ChIP.

**Statistical analysis**

Quantitative data were presented as mean ± SEM. Significance (p<0.05) was assessed by two-tailed unpaired Student t-test.
RESULTS

Sirt6 regulates hepatic cholesterol homeostasis in vivo

Previously, it has been shown that Sirt6 plays a critical role in hepatic triglyceride metabolism (45). To assess whether hepatic Sirt6 might be involved in cholesterol homeostasis, we analyzed serum and hepatic cholesterol levels in Sirt6 liver-specific knockout mice (LKOT6). The results show that serum cholesterol levels were increased 22% in the LKOT6 mice relative to control littermates (Figures 1A). Hepatic cholesterol levels were also increased 19% in the LKOT6 mice compared to the control mice (Figure 1B). Since SREBP-2 is the key regulator of cholesterol biosynthesis, we analyzed both the precursor and mature nuclear form of SREBP-2 in the control and LKOT6 livers. The results indicate that both forms of SREBP-2 were increased in the Sirt6-deficient livers (Figure 1C), suggesting that the elevated cholesterol levels in the LKOT6 mice may be partly attributed to an increase in SREBP-2 protein in the LKOT6 liver.

Previously, it has been reported that acetylation of SREBP-2 may increase the protein stability and its deacetylation by Sirt1 promotes SREBP-2 degradation (21, 57, 58). Our data suggest that Sirt6 might not directly deacetylate SREBP-2 protein because its acetylation status was not changed in Sirt6-overexpressing HEK293 cells whereas Sirt1 overexpression markedly reduced the acetylation of SREBP-2 (Figure 1D). It is possible that Sirt6 regulates the Srebp2 gene at transcriptional level since Srebp2 mRNA levels were significantly increased (Figure 1E). As expected, SREBP-2 target genes such as Hmgcs1, Hmgcr, and Ldlr (59), were also up-regulated whereas expression of Srebp1a, Srebp1c, Scap, Insig1, and Insig2 genes was not significantly changed (Figure 1E). To examine the feeding effects on SREBP-2 and its target genes, we also analyzed SREBP-2 proteins and Hmgcr and Hmgcs1 mRNAs in the liver of control and LKOT6 mice that were fasted overnight or refed for 4 hours immediately after the fasting. The data
showed that hepatic Sirt6 deficiency only affected cholesterol gene expression in the fasted but not refed livers (Figure 1, F and G).

To further confirm that Sirt6 indeed regulates cholesterol homeostasis in vivo, we also used adenoviral approaches to either knock down or overexpress Sirt6 in the liver of wild-type mice. The results showed that the acute knockdown of Sirt6 not only increased the Srebp2 gene expression at both mRNA and protein levels but also led to elevated levels of hepatic and serum cholesterol (Figure 2, A-D). Conversely, overexpression of Sirt6 in the liver decreased Srebp2 gene expression and reduced hepatic and circulated cholesterol levels (Figure 2, E-H).

**FoxO3 coordinates with Sirt6 for the regulation of Srebp2 gene expression and cholesterol homeostasis**

As Sirt6 is involved in histone deacetylation, we performed chromatin immunoprecipitation (ChIP) analysis of association of Sirt6 to the chromatin and acetylated histone H3 at lysines 9 (H3K9) and 56 (H3K56) in the proximal promoter of the Srebp2 gene in mouse primary hepatocytes that were transduced with either control GFP or Sirt6 adenoviruses. As expected, Sirt6 was significantly enriched in the proximal region of the Srebp2 gene promoter; both H3K9 and H3K56 had reduced acetylation in the same chromatin region (Figure 2I). Conversely, the acetylation levels of H3K9 and H3K56 were elevated in the LKOT6 liver (Figure 2J).

To elucidate the molecular mechanism underlying the Srebp2 gene regulation by Sirt6, we also performed an in-silico analysis of the Srebp2 promoter sequences to identify potential transcription factors that might recruit Sirt6 for the gene regulation. In addition to a previously characterized sterol response element (SRE) (60), we also found two putative insulin-response elements (IRE1 and IRE2). Particularly, the IRE1 sequences are well conserved in human,
mouse, and rat Srebp2 genes (Figure 3A). To test whether these IREs might be indeed involved in the regulation of the Srebp2 gene, we cloned wild-type and mutant mouse Srebp2 gene promoters. Whereas both FoxO1 and FoxO3 could suppress the wild-type and IRE2-mutated Srebp2 promoter activities, IRE1 mutation abolished such an inhibitory effect by FoxO1 or FoxO3 (Figure 3, B-D). To further validate whether the interaction also occurs in vivo, we performed ChIP analysis in mouse primary hepatocytes. The data show that FoxO3 had highly enriched association with the Srebp2 proximal promoter flanking the IRE1 region (Figure 3E).

To further examine whether histone modifications might be involved in the FoxO3-mediated regulation of the Srebp2 gene, we performed ChIP analyses of acetylated histone H3 at lysines 9, 18, and 56 in the proximal Srebp2 gene promoter in mouse primary hepatocytes. Interestingly, H3K9 and H3K56 but not H3K18 had reduced acetylation in the chromatin surrounding the IRE1 sequence when FoxO3 was overexpressed as compared to the GFP control (Figure 3E).

These data suggest that FoxO3 might recruit a histone deacetylase (HDAC) to the Srebp2 gene promoter.

To verify whether FoxOs have a physiological role in cholesterol homeostasis, we analyzed hepatic and serum cholesterol in FoxO1, FoxO3, and FoxO1/3/4 liver-specific knockout mice (LKO1, LKO3, and LTKO, respectively). The results show that FoxO3 indeed had a stronger impact on both liver and serum cholesterol levels because LKO3 but not LKO1 mice had elevated serum and hepatic cholesterol (Figure 4, A-D). The cholesterol data in LTKO mice also indicate some degree of redundancy of FoxOs in cholesterol regulation (Figure 4, E and F). This cholesterol phenotype is further supported by molecular changes in Srebp2 and Hmger mRNAs in the LKO3 and LTKO livers relative to control loxp livers (Figure 5, A and B). We also examined SREBP-2 protein levels and its target genes in the control and LKO3 livers.
under overnight fasting and 4-hour refeeding conditions. The results indicate that hepatic FoxO3 deficiency mainly affected expression of Srebp2, Hmgcr, and Hmgcs1 genes in the fasting condition (Figure 5, C and D). To corroborate the role of FoxO3 in the control of cholesterol homeostasis, we also overexpressed FoxO3 in the liver of HFD-treated wild-type (WT) mice. As compared to chow diet, HFD increased hepatic SREBP-2 protein levels in the WT mice whereas FoxO3 protein levels were not significantly changed (data not shown). In FoxO3 overexpressed HFD mice, hepatic and serum cholesterol levels and hepatic Srebp2 and Hmgcr gene expression were significantly decreased (Figure 6, A-D). In order to verify the role of FoxO3 in the regulation of the Srebp2 gene, we also analyzed histone acetylation and Sirt6 association of the Srebp2 proximal promoter in the LKO3 hepatocytes using ChIP technique. The data indicate that H3K9 and H3K56 acetylation levels were elevated in the LKO3 primary hepatocytes whereas the association of Sirt6 with the Srebp2 gene promoter was dramatically decreased (Figure 6, E and F).

As Sirt1 and Sirt6 are both known to deacetylate H3K9Ac (26, 61) and only Sirt6 was observed to regulate Srebp2 gene expression in this study, it is possible that FoxO3 might coordinate with Sirt6 to modulate Srebp2 gene expression and cholesterol homeostasis. To test this hypothesis, we first examined whether FoxO3 could interact with Sirt6 by co-immunoprecipitation (Co-IP) assays. Both forward and reverse Co-IPs reveal that FoxO1 and FoxO3 could interact with Sirt6 (Figure 7A). To further validate that this interaction also occurs in vivo, we performed Co-IPs in AdSirt6 or AdFoxO3 infected livers from overnight-fasted mice. Indeed, FoxO3 and Sirt6 were found to interact with each other (Figure 7B). To determine whether FoxO3 requires Sirt6 to down-regulate Srebp2 gene expression, we overexpressed or knocked down FoxO3 in LKOT6 primary hepatocytes. The results indicate that the inhibitory
effect of FoxO3 on Srebp2 gene expression was abolished when Sirt6 was deficient (Figure 7C). Moreover, when FoxO3 was overexpressed, nuclear SREBP-2 protein levels were decreased in LKOT1 but not LKOT6 primary hepatocytes (Figure 7D). We also examined the requirement of Sirt6 in FoxO3-controlled Srebp2 expression using shRNAs. While Sirt6 knockdown blocked FoxO3 down-regulation of nSREBP-2, knockdown of Sirt1 could not (Figure 7E). These data suggest that FoxO3 requires Sirt6 for the regulation of Srebp2 gene expression.

To assess whether Sirt6 and Sirt1 are dependent on FoxOs to control SREBP-2 levels, we also overexpressed or knocked down Sirt6 or Sirt1 in LTKO primary hepatocytes. The results indicate that FoxO1/3/4 deficiency only abolished Sirt6 but not Sirt1 effect on Srebp2 protein levels (Figure 7F). To further validate our observation in vivo, we overexpressed GFP or Sirt6 in the liver of control and LTKO mice. Whereas Sirt6 overexpression decreased SREBP-2 protein in the wild-type livers, there was no significant change in the LTKO livers (Figure 7, G and H). Moreover, serum and liver cholesterol levels were not significantly changed in the Sirt6-overexpressing LTKO mice as compared to the control group (Figure 7, I and J). These results suggest that Sirt6 and FoxO3 coordinately regulate cholesterol biosynthesis.

**Sirt6 overexpression improves hypercholesterolemia in mice**

To examine the role of Sirt6 in the development of hypercholesterolemia, we first analyzed Sirt6 gene expression in the liver of wild-type and diabetic db/db mice. The hepatic levels of Sirt6 mRNA and protein were decreased in db/db mice (Figure 8, A and B). To test whether overexpression of Sirt6 can improve hypercholesterolemia, we injected GFP or Sirt6 adenoviruses into WT and db/db mice. Two weeks later, both precursor and nuclear SREBP2 proteins were significantly decreased in the Sirt6 overexpressed db/db livers relative to the control GFP expressed livers (Figure 8B). This is consistent with decreased serum and hepatic cholesterol levels.
cholesterol levels after Sirt6 overexpression (Figure 8, C and D). To verify whether Sirt6 overexpression also affects expression of cholesterol genes, we performed real-time PCR. The results indicate that Srebp2 and Hmgcr mRNAs were significantly decreased by Sirt6 overexpression (Figure 8E). These changes could be attributed to chromatin modification because H3K9 and H3K56 acetylation levels were dramatically decreased in the Sirt6-oeverexpressed db/db livers as compared to GFP controls (Figure 8F). These data reinforce the notion that Sirt6 plays a critical role in cholesterol homeostasis.
DISCUSSION

In this study, we have identified a novel molecular mechanism by which FoxOs recruit Sirt6 to the proximal promoter of the Srebp2 gene and subsequently suppress the gene expression via H3K9 and H3K56 deacetylation. Previously, Sirt6 has been shown to suppress numerous genes through such histone modifications, including glucokinase, pyruvate kinase, fatty acid synthase, and acetyl-CoA carboxylase 1 (45). Hepatic deficiency of Sirt6 also leads to fatty liver disease in mice (45). In addition to transcriptional control of Srebp2 by FoxOs and Sirt6, modulation of SREBP-2 protein stability by Sirt1 may also play a role. It has been reported that SREBP acetylation stabilizes the protein and enhance its transcriptional activity (21, 57, 58).

Since FoxOs regulate the rate-limiting enzyme—nicotinamide phosphoribosyltransferase (Nampt)—in the salvage pathway of NAD⁺ biosynthesis using nicotinamide as substrate, FoxOs deficiency causes a decrease in NAD⁺ levels, therefore resulting in a decrease in sirtuin activity (54). Moreover, Sirt1 can also modulate cholesterol homeostasis via control of ABCA1 gene expression and cholesterol reverse transport (18).

FoxO1/3/4 have been implicated in cholesterol homeostasis in different animal models (13-15, 17, 62, 63). For instance, male transgenic mice that overexpress FoxO1 mutant (TSS-AAA) specifically in the liver have lower total and LDL-cholesterol whereas female transgenic mice are not different from control littermates (15). Although FoxO1 knockdown in the liver of wild-type mice increases plasma cholesterol, liver-specific FoxO1 knockout mice do not exhibit a significant change in circulated cholesterol (14, 62, 63). But under the hyperglycemic condition induced by streptozotocin injections, hepatic FoxO1 deficiency leads to elevated serum cholesterol and a modest increase in Srebp2 mRNA levels in the liver (13). Those results are in line with our observation that FoxO1 is not a major player in hepatic cholesterol biosynthesis.
under physiological conditions. Instead, our data suggest a predominant role of FoxO3 in this process. This may also be the case in rodent ovary granulosa cells because FoxO3 can inhibit follicle-stimulating hormone-induced Srebp2 gene expression in this cell type (16). A recent report suggests a role of FoxO3 in cholesterol homeostasis but no significant change in cholesterol levels is documented in the FoxO3 liver-specific knockout mice, possibly due to the small sample size (14). In our study, both hepatic and serum cholesterol levels are significantly elevated in hepatic FoxO3-deficient mice. With regard to FoxO4, although overexpression in 3T3L1 fibroblasts decreases expression of cholesterol biosynthetic genes (Srebp2 and Cyp51) and cellular cholesterol (17), liver-specific FoxO4 knockout mice do not exhibit any change in serum cholesterol levels (14). Together, these findings suggest that FoxO1/3/4 have some redundant functions in cholesterol regulation, but FoxO3 plays a major role in this process.

Mechanistically, here we have documented a strong coordination between FoxOs and Sirt6 in cholesterol homeostasis. It is believed that Sirt6 may have broad effects on histone H3 modifications; however, our data suggest that specific transcription factors such as FoxOs have the capability to recruit this sirtuin to a subset of target genes, at least for the case of cholesterol regulation. The orchestrated functions of Sirt6 and FoxOs may have implications in the regulation of animal longevity since both have been linked to this phenotype (43, 64-68). Although this study has only investigated hepatic Sirt6 in cholesterol homeostasis, it is possible that such a function may occur in some other organs, for example, brain. Regulation of central cholesterol homeostasis by Sirt6 might also contribute to longevity. Previously, it has been shown that insulin regulates Srebp2 gene expression and cholesterol biosynthesis in the brain (69). It is possible that FoxO3/Sirt6 might be part of the mechanism in that regulation.
In summary, under conditions of fasting or low concentration of insulin/IGF-1, FoxO3 can recruit Sirt6 to the \textit{Srebp2} gene promoter, thereby creating a suppressive epigenetic state to inhibit expression of \textit{Srebp2} and its target genes. In response to insulin and other growth factors that can induce phosphorylation and inhibition of FoxO3, the inhibitory complex of FoxO-Sirt6 may dissociate from chromatin. As a result, H3K9 and K3K56 acetylation levels are increased, and these epigenetic changes set a positive mode for active transcription. Overall, FoxO3 and Sirt6 can coordinate to regulate cholesterol homeostasis and organismal health.
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REFERENCES


FIGURE LEGENDS

Figure 1. Hepatic Sirt6 knockout leads to elevated cholesterol levels and increased cholesterol biosynthetic gene expression. (A, B) Serum and liver cholesterol measurements in 3-month control floxed (LoxpT6) and Sirt6 liver-specific knockout mice (LKOT6, n=10-12). (C) Both cytoplasmic SREBP-2 precursor (Srebp2p) and nuclear mature SREBP-2 (nSrebp2) amounts were increased in the liver of LKOT6 mice, indicated by immunoblot analysis of total liver lysates and nuclear extracts, respectively. (D) Sirt6 might not directly deacetylate SREBP-2 protein. nSrebp2 was cotransfected with GFP, Sirt1, or Sirt6 in HEK293 cells, and acetylation of SREBP-2 was analyzed by immunoprecipitation with anti-FLAG antibody followed by immunoblotting with anti-acetyl lysine antibody. (E) Real-time PCR analysis of mRNAs for cholesterol metabolism genes in the liver of control floxed and LKOT6 mice (n=3-5). (F, G) SREBP-2 and its target genes were analyzed in the liver of control floxed and LKOT6 mice (n=2-3) under overnight fasting or 4-hour refeeding immediately after the fasting. Data are mean ± SEM; *P ≤ 0.05 by t-test.

Figure 2. Sirt6 knockdown and overexpression confirm its critical role in cholesterol homeostasis. (A, B) SREBP-2 mRNA and protein levels were analyzed in the liver of mice that were infected with GFP and Sirt6 shRNA expressing adenoviruses (shGFP and shSirt6, n=5-6, respectively). (C, D) Hepatic and serum total cholesterol levels were measured in shGFP and shSirt6 adenoviruses infected mice (n=5-6). (E-H) Srebp2 gene expression and liver and serum total cholesterol levels were analyzed in GFP and Sirt6 adenoviruses (AdGFP and AdSirt6) injected C57BL6/J mice (n=5-6), respectively. (I, J) Chromatin immunoprecipitation (ChIP) analysis of Sirt6 association and H3K9 and H3K56 acetylations in the Srebp2 proximal gene promoter in mouse primary hepatocytes transduced with GFP and Sirt6 adenoviruses and
isolated from loxpT6 and LKOT6 livers, respectively. ChIP data were normalized to an internal control gene of Ppia or total histone H3 ChIP; Data are mean ± SEM. *P ≤ 0.05 by t-test.

**Figure 3. Transcriptional and epigenetic regulation of the Srebp2 gene.** (A) A schematic diagram of the proximal promoter of the mouse Srebp2 gene, indicating two putative insulin-response elements (IRE1/2) and a sterol-response element (SRE). The conservation of IRE1 sequences among mouse, rat, and human Srebp2 genes is also shown. (B-D) Luciferase reporter assays for wild-type and IRE-mutant Srebp2 promoters were performed in HEK293 cells that were transfected with respective promoter constructs with vector, GFP, FoxO1, or FoxO3. (E) ChIP analysis of FoxO3 association with the mouse Srebp2 gene promoter and histone H3 acetylation in the proximal IRE1 region in mouse primary hepatocytes transduced with GFP (AdGFP) and FoxO3 (AdFoxo3) adenoviruses. DNA copy numbers were normalized to Ppia ChIP or total histone H3 ChIP. Data are mean ± SEM; *P ≤ 0.05 by t-test.

**Figure 4. Hepatic and serum cholesterol levels are elevated in LKO3 and LTKO mice.** (A-D) Serum and hepatic cholesterol levels were measured in 3-5 months FoxO1 and FoxO3 liver-specific knockout mice (LKO1 and LKO3, n=7-12), respectively. (E, F) Serum and hepatic cholesterol levels were analyzed in 3-month FoxO1/3/4 liver-specific triple knockout mice (LTKO) (n=5-7). Data are mean ± SEM; *P ≤ 0.05 by t-test.

**Figure 5. FoxOs regulate expression of cholesterol biosynthetic genes in the liver.** (A, B) Real-time PCR analysis of Srebp2 and Hmgcr mRNAs in the livers of control, LKO1, LKO3, and LTKO mice (n=4-8). Control values were normalized to 1. (C, D) Gene expression analysis of SREBP-2 and its target genes in control and LKO3 livers of overnight fasted and 4-hour refed mice (n=2-3). Data are mean ± SEM; *P ≤ 0.05 by t-test.
Figure 6. FoxO3 overexpression reduces cholesterol levels in high-fat diet treated mice. (A, B) Hepatic and serum cholesterol levels were decreased in hepatic FoxO3 overexpressed mice (n=5) fed a high-fat diet for 2 months. (C) Real-time PCR analysis of Srebp2 and Hmgcr gene expression in GFP or FoxO3 overexpressed livers. (D) Western blot analysis of FoxO3 and Srebp2 proteins in the liver extracts from GFP and FoxO3 adenoviruses infected mice. (E, F) ChIP analysis of histone H3 acetylation and Sirt6 association with the Srebp2 gene promoter in primary hepatocytes from control floxed and LKO3 mice. Data are mean ± SEM; *P ≤ 0.05 by t-test.

Figure 7. Sirt6 interacts with FoxO3 to regulate Srebp2 gene expression and cholesterol homeostasis. (A) Co-immunoprecipitations (Co-IPs) of Sirt6 and FoxO1/3 were performed in transfected HEK293 cells. (B) Co-IP analysis of Sirt6 and FoxO3 interaction in fasted mouse livers transduced with GFP, Sirt6, or FoxO3 adenoviruses. (C) Real-time PCR analysis of Srebp2 mRNAs in loxpT6 and LKOT6 primary hepatocytes that were transduced with GFP or FoxO3 overexpressing or knockdown adenoviruses. (D) Immunoblot analysis of nuclear SREBP-2 in mouse primary hepatocytes that were isolated from LKOT6 and LKOT1 mice and were subsequently transduced with GFP, FoxO1, or FoxO3 adenoviruses. (E) Nuclear SREBP-2 protein was analyzed in wild-type mouse primary hepatocytes transduced with GFP or FoxO3 together with one of the shRNA adenoviruses (shGFP, shSirt6, or shSirt1). (F) Nuclear SREBP-2 protein was analyzed in LTKO mouse primary hepatocytes transduced with GFP, Sirt6, and Sirt1 overexpression or knockdown adenoviruses. (G, H) Immunoblot and quantification analyses of nuclear SREBP-2 protein in the livers of GFP or Sirt6 adenovirus infected Loxp or LTKO mice (n=3), respectively. (I, J) Serum and liver cholesterol levels were measured in 4-month control
Loxp and LTKO mice (n=5-6) that were injected with GFP or Sirt6 adenoviruses, respectively. Data are mean ± SEM; *P ≤ 0.05 by t-test.

**Figure 8. Sirt6 overexpression lowers cholesterol levels in diabetic db/db mice.** (A) Sirt6 mRNA levels in wild-type (WT) and db/db mouse livers (n=5-8) were analyzed by real-time PCR. (B) Immunoblot analysis of SREBP-2 protein in the liver of WT and db/db mice. (C, D) Analysis of serum and hepatic cholesterol levels in WT and db/db mice at age of 2 months (n=5-6) after adenovirus injections. (E) Srebp2 and Hmgcr gene expression in the liver of wild-type and db/db mice (n=5-8) infected with GFP or Sirt6 adenoviruses was analyzed by real-time PCR. (F) H3K9 and H3K56 acetylation levels in the liver of WT and db/db mice (n=3) were assessed by ChIP and data were normalized to total histone H3 ChIP. Data are mean ± SEM; *P ≤ 0.05 by t-test.
Figure 3

A. Srebp2 promoter region with IRE1, IRE2, and SRE binding sites.

B. Wild-type 351 bp promoter

C. IRE1 mutant

D. IRE2 mutant

E. Srebp2 ChIP analysis with AdGFP and AdFoxo3 treatments.
Figure 5

A. Srebp2 mRNA

B. Hmgcr mRNA

C. Western blots showing changes in protein levels in different genotypes: Foxo3, Srebp2p, Actinin, nSrebp2, and Lamin A/C under fasting and refeeding conditions.

D. Relative mRNA values for Hmgcr and Hmgcs1 under fasting and refeeding conditions.
Figure 6

A) Hepatic cholesterol

B) Serum cholesterol

C) mRNA levels

D) Western blots

E) Srebp2_ChIP

F) Sirt6
Figure 8

A. Sirt6 mRNA levels in WT and db mice.

B. Western blot analysis of HA, Sirt6, Srebp2p, Actinin, nSrebp2, and Lamin A/C in WT+GFP, db+GFP, and db+SIRT6 groups.

C. Serum cholesterol levels in WT+GFP, db+GFP, and db+Sirt6 groups.

D. Hepatic cholesterol levels in WT+GFP, db+GFP, and db+SIRT6 groups.

E. mRNA levels of Srebp2 and Hmgcr in WT+GFP, db+GFP, and db+Sirt6 groups.

F. Srebp2_ChIP analysis of H3K9Ac and H3K56Ac in WT+GFP, db+GFP, and db+SIRT6 groups.