A Novel Role for CRTC2 in Hepatic Cholesterol Synthesis Through SREBP-2

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Cholesterol synthesis is regulated by the transcription factor sterol regulatory element binding protein 2 (SREBP-2) and its target gene 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), which is the rate-limiting enzyme in cholesterol synthesis. Cyclic adenosine monophosphate–responsive element (CRE) binding protein–regulated transcription coactivator (CRTC) 2 is the master regulator of glucose metabolism. However, the effect of CRTC2 on cholesterol and its potential molecular mechanism remain unclear. Here, we demonstrated that CRTC2 expression and liver cholesterol content were increased in patients with high serum cholesterol levels who underwent resection of liver hemangiomas, as well as in mice fed a 4% cholesterol diet. Mice with adenovirus-mediated CRTC2 overexpression also showed elevated lipid levels in both serum and liver tissues. Intriguingly, hepatic *de novo* cholesterol synthesis was markedly increased under these conditions. In contrast, CRTC2 ablation in mice fed a 4% cholesterol diet (18 weeks) showed decreased lipid levels in serum and liver tissues compared with those in littermate wild-type mice. The expression of lipogenic genes (SREBP-2 and HMGCR) was consistent with hepatic CRTC2 levels. *In vivo* imaging showed enhanced adenovirus-mediated HMGCR-luciferase activity in adenovirus-mediated CRTC2 mouse livers; however, the activity was attenuated after mutation of CRE or sterol regulatory element sequences in the HMGCR reporter construct. The effect of CRTC2 on HMGCR in mouse livers was alleviated upon SREBP-2 knockdown. CRTC2 modulated SREBP-2 transcription by CRE binding protein, which recognizes the half-site CRE sequence in the SREBP-2 promoter. CRTC2 reduced the nuclear protein expression of forkhead box O1 and subsequently increased SREBP-2 transcription by binding insulin response element 1, rather than insulin response element 2, in the SREBP-2 promoter. *Conclusion:* CRTC2 regulates the transcription of SREBP-2 by interfering with the recognition of insulin response element 1 in the SREBP-2 promoter by forkhead box O1, thus inducing SREBP-2/HMGCR signaling and subsequently facilitating hepatic cholesterol synthesis. (HEPATOLOGY 2017;66:481-497).

Cholesterol plays important roles in animal and cellular membranous structures and serves as a precursor for steroid hormones and bile acids.[1] In addition, it has been identified as a major factor in the pathogenesis of atherosclerosis,[2] myocardial infarction,[3] and strokes.[4] In mammals, both diet and *de novo* biosynthesis are sources for cholesterol,[5] and the liver is an important site for both the synthesis and catabolism of cholesterol.[6]

3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) acts as the key rate-limiting enzyme in cholesterol synthesis and the primary site of feedback

regulation.[1] Several transcription factors, including sterol regulatory element (SRE) binding protein 2 (SREBP-2), SREBP-1a, liver X receptor, and forkhead box O (FoxO) transcription factors, have been linked to cholesterol regulation in the liver.[7-13] SREBP-2 is the master regulator of cholesterol synthesis and can be regulated through transcriptional and posttranscriptional mechanisms.[14] Moreover, FoxO1 modulates the transcription of SREBP-2.[12]

CRTC2 (also referred to as TORC2) is a member of the cyclic adenosine monophosphate–responsive element (CRE) binding protein (CREB)–regulated transcription coactivator (CRTC) superfamily and has been extensively studied in glucose homeostasis.[15,16] In addition, CRTC2 is involved in modulating insulin sensitivity,[17] endoplasmic reticulum stress,[18] virus reactivation,[19] and obesity.[18] CRTC2 acts as a key regulator in fasting glucose metabolism,[18] and studies have suggested roles for CRTC2 also in triglyceride homeostasis.[17,20,21] However, no in-depth study regarding the role of CRTC2 on cholesterol metabolism has been reported to date. This prompted us to investigate whether CRTC2 affects cholesterol levels in the liver, where CRTC2 is highly expressed.[21]

Through a series of studies in mouse and cellular models, we demonstrated here that CRTC2 enhanced SREBP-2 expression, resulting in elevated HMGCR levels and cellular cholesterol synthesis. Remarkably, CRTC2 inhibited the nuclear translocation of FoxO1 and reduced its binding to SREBP-2, resulting in an increase in SREBP-2 transcription. Importantly, knockdown of CRTC2 in mouse livers affected the SREBP-2/HMGCR pathway. Thus, CRTC2 plays a vital role in controlling hepatic cholesterol synthesis.

Materials and Methods

PATIENT TISSUE PREPARATION

Liver samples were obtained from 8 patients who underwent surgical liver resection for liver hemangioma. Each individual underwent serum testing before surgery. We divided the patients into two groups according to serum cholesterol levels[22]: a low total cholesterol group (Low-TC) and a high total cholesterol group (High-TC). Patient liver tissues were biopsied at least 1 cm away from the lesions and confirmed as normal by at least one pathologist. Liver samples were frozen in liquid nitrogen immediately after resection. None of the patients tested positive for hepatitis B virus, hepatitis C virus, or human immunodeficiency virus infection or were treated with a lipid-lowering therapy. The study protocol was approved by the Ethics Committee for Human Studies at the Shandong Provincial Hospital. Written informed consent was obtained from all patients before they entered the study (see Table 1 for patient information).

ASSAY OF HEPATIC CHOLESTEROL SYNTHESIS

Mice were fasted for 4 hours, intraperitoneally injected with 10 μCi [1-^{14}C]-sodium acetate (PerkinElmer, Waltham, MA), and euthanized 30 minutes

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TABLE 1. Characteristics of Patients

Patients	Serum Total Cholesterol Levels (mmol/L)	Sex	Age (Years)	Pathological Diagnosis
Low-TC group				
1	3.55	Male	61	Liver hemangiomas
2	2.87	Female	51	Liver hemangiomas
3	3.19	Male	68	Multiple liver hemangiomas
4	3.74	Female	57	Liver hemangiomas
High-TC group				
5	7.45	Female	51	Liver hemangiomas
6	5.77	Male	60	Liver hemangiomas
7	5.39	Male	40	Liver hemangiomas
8	5.20	Female	63	Liver hemangiomas

after injection. Then, hepatic cholesterol synthesis was assayed as described.[23]

STATISTICAL ANALYSIS

The results are presented as means ± standard deviation for the number of experiments indicated. Statistical significance between two conditions was determined using the Student t test, while one-way analysis of variance (Dunnett t or least significant difference test) was used for multiple comparisons. Data were analyzed using SPSS 11.0 software. Differences were considered significant at $P < 0.05$.

Please see Supporting Information for more detailed information of this section.

Results

HEPATIC CRTC2 EXPRESSION IS INCREASED IN BOTH PATIENTS WITH HIGH SERUM CHOLESTEROL LEVELS AND MICE FED A HIGH-CHOLESTEROL DIET

We collected human liver samples from patients undergoing surgical liver resection and divided them into two groups according to the serum cholesterol levels: Low-TC and High-TC. Subjects in the High-TC group had higher low-density lipoprotein cholesterol (LDL-C) levels, no obvious increases in the serum levels of triglycerides (TG) and high-density lipoprotein cholesterol (HDL-C) (Fig. 1A), and higher free total cholesterol (FTC) and total cholesterol levels in the liver (Fig. 1B); however, there was no difference in hepatic TG between the two groups (Fig. 1B). The High-TC patients also showed a marked increase in the mRNA levels of CRTC2, SREBP-2, and

HMGCR ($P < 0.01$). By contrast, the gene expression of SREBP-1a, liver X receptor, FoxO1, 3-hydroxy-3-methylglutaryl-coenzyme A synthase (HMGCS), and LDL receptor (LDLR) did not change appreciably between the two groups (Fig. 1C).

Mice fed a high-cholesterol (HC) diet showed no significant changes in body weight (Fig. 2A, left). However, the liver-to-body weight ratios (Fig. 2A, right) and serum levels of glucose (Fig. 2B) and lipids (Fig. 2C) were higher in mice fed the HC diet than in mice fed the chow diet. The livers from mice fed the HC diet showed increased fat accumulation as well as higher FTC and total cholesterol contents (Fig. 2D). Moreover, the hepatic mRNA and protein levels of CRTC2 and phosphoenolpyruvate carboxykinase (PEPCK, a downstream gene of CRTC2) were higher in mice fed the HC diet (Fig. 2E,F). These results, which were similar in the liver of patients and mice, indicated that CRTC2 is related to cholesterol levels.

CRTC2 MODULATES CHOLESTEROL BIOSYNTHESIS *IN VIVO* AND *IN VITRO*

Mice with adenovirus-mediated CRTC2 overexpression (Ad-CRTC2) had higher CRTC2 mRNA levels (Fig. 3A, left), body weight (Fig. 3A, middle), and liver-to-body weight ratios (Fig. 3A, right) than controls. Fasting blood glucose levels (Fig. 3B) and impaired glucose tolerance (Fig. 3C) were also increased. CRTC2 overexpression significantly elevated the serum total cholesterol levels (Fig. 3D, left). Moreover, livers from mice overexpressing CRTC2 showed increased fat accumulation (Fig. 3D, right) and FTC contents (Fig. 3E). An approximately 1.8-fold increase in cholesterol levels was observed in the HepG2 cells overexpressing CRTC2 compared with the cells treated with the vector (Fig. 3F, left). Filipin
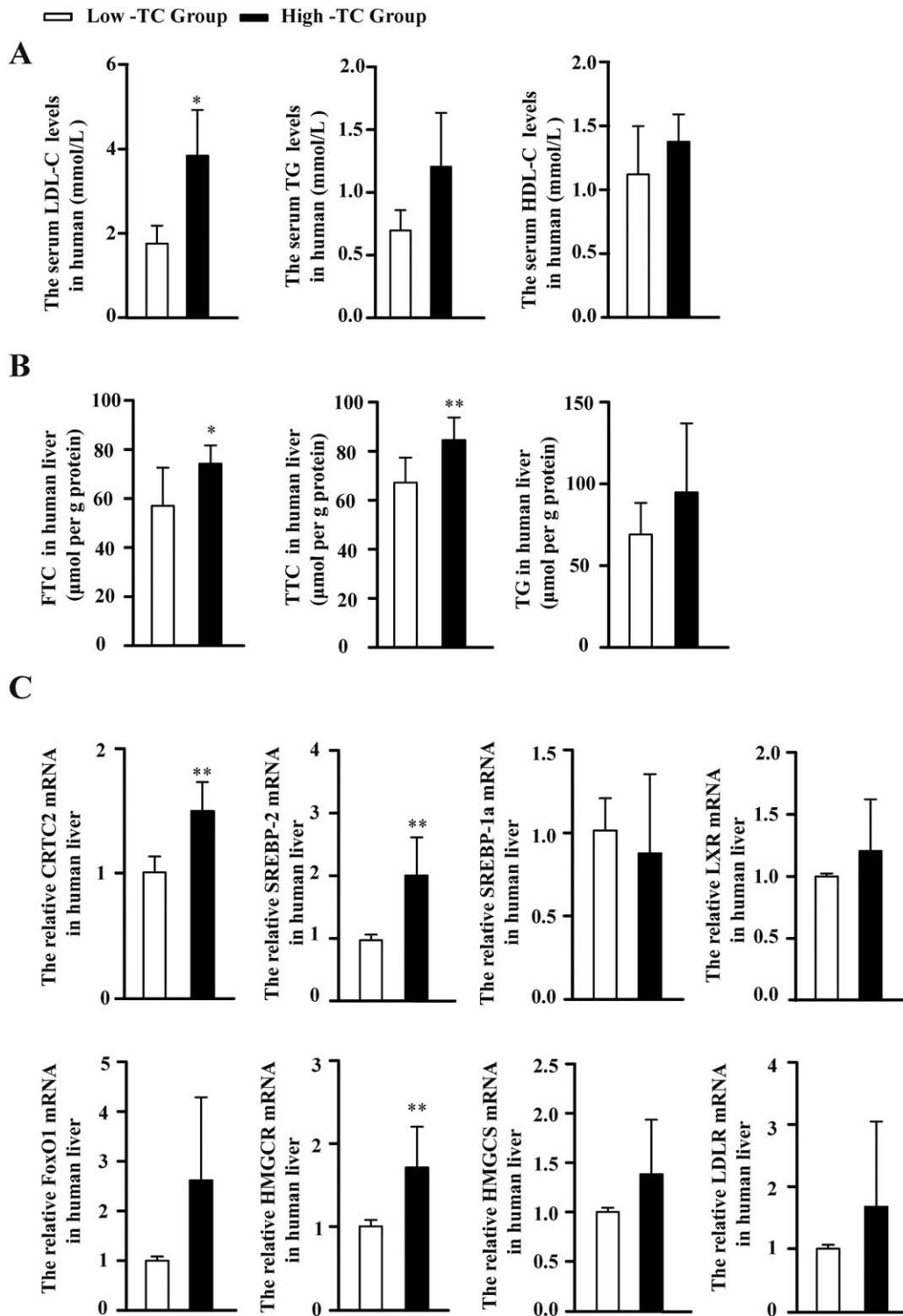
FIG. 1. Hepatic CRTC2 expression is increased in patients with high serum cholesterol levels. Lipid profiles in human serum (A) and liver (B). Expression of cholesterol-related genes in human livers was measured using real-time PCR (C). All values are shown as means ± standard deviation (n = 4). *$P < 0.05$ and **$P < 0.01$ versus the Low-TC group. Abbreviation: LXR, liver X receptor.
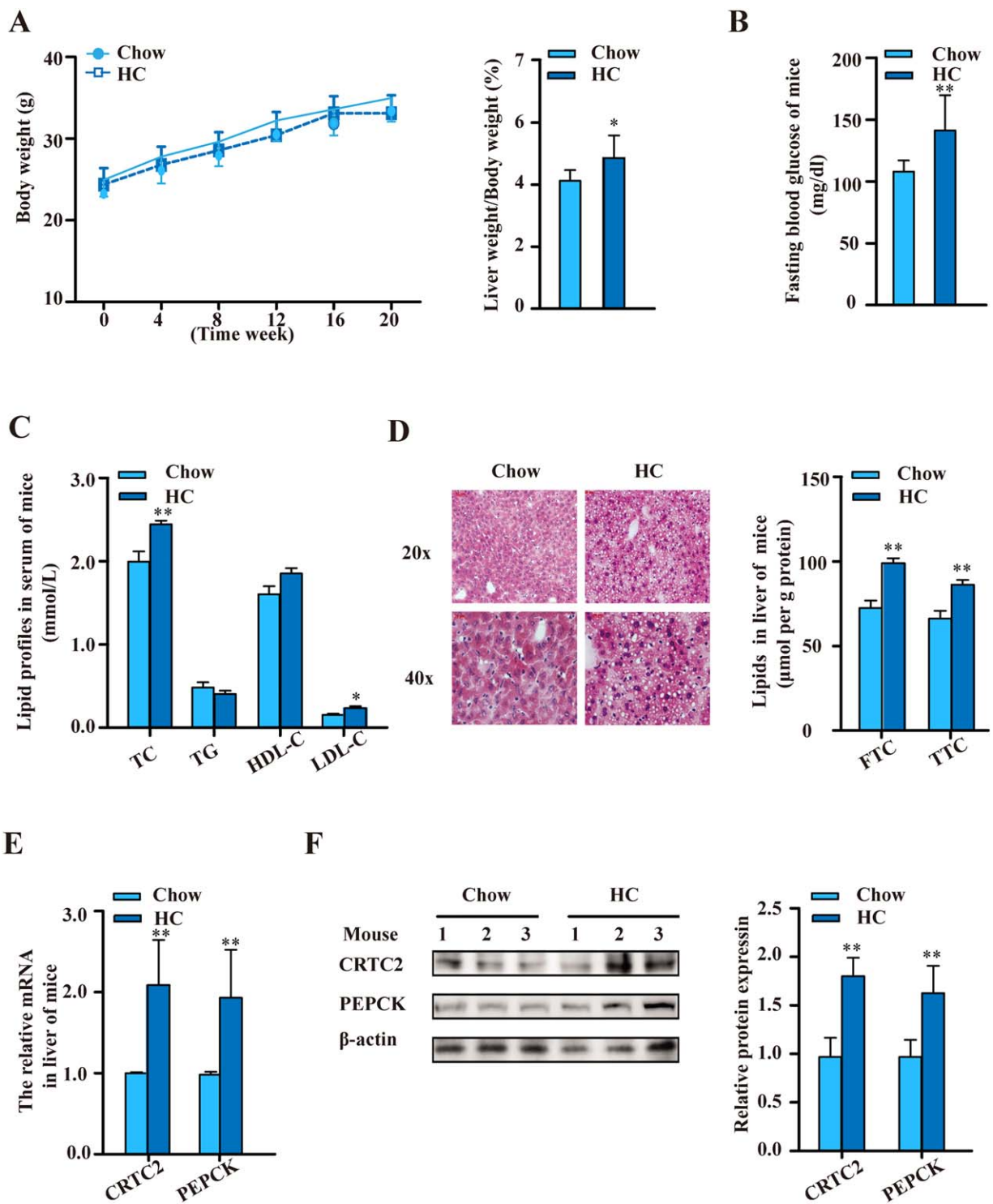
FIG. 2. Hepatic CRTC2 expression is increased in mice fed an HC diet. C57BL/6J mice were fed a 4% cholesterol (HC) diet or a chow diet for 20 weeks. (A) Comparison of body weight gain in mice (left, n = 8-10) and liver-to-body weight ratios (right, n = 6-10). (B,C) Serum glucose and serum lipids levels (n = 5-9). (D) Representative mouse liver sections stained with hematoxylin and eosin (left); hepatic FTC and total cholesterol were measured (right) (n = 5-8). (E,F) Hepatic mRNA and proteins were assessed. CRTC2 and PEPCK were quantified and normalized to β-actin (n = 3). *$P < 0.05$ and **$P < 0.01$ versus mice fed chow. Abbreviations: TC, cholesterol; TTC, total cholesterol.

FIG. 3. Overexpression of CRTC2 elevates cholesterol levels *in vivo* and *in vitro*. (A-E) C57BL/6J mice were injected three times (once every 5 days) with Ad-CRTC2 or Ad-GFP control through the tail vein. Mice were sacrificed for analysis 21 days after the first injection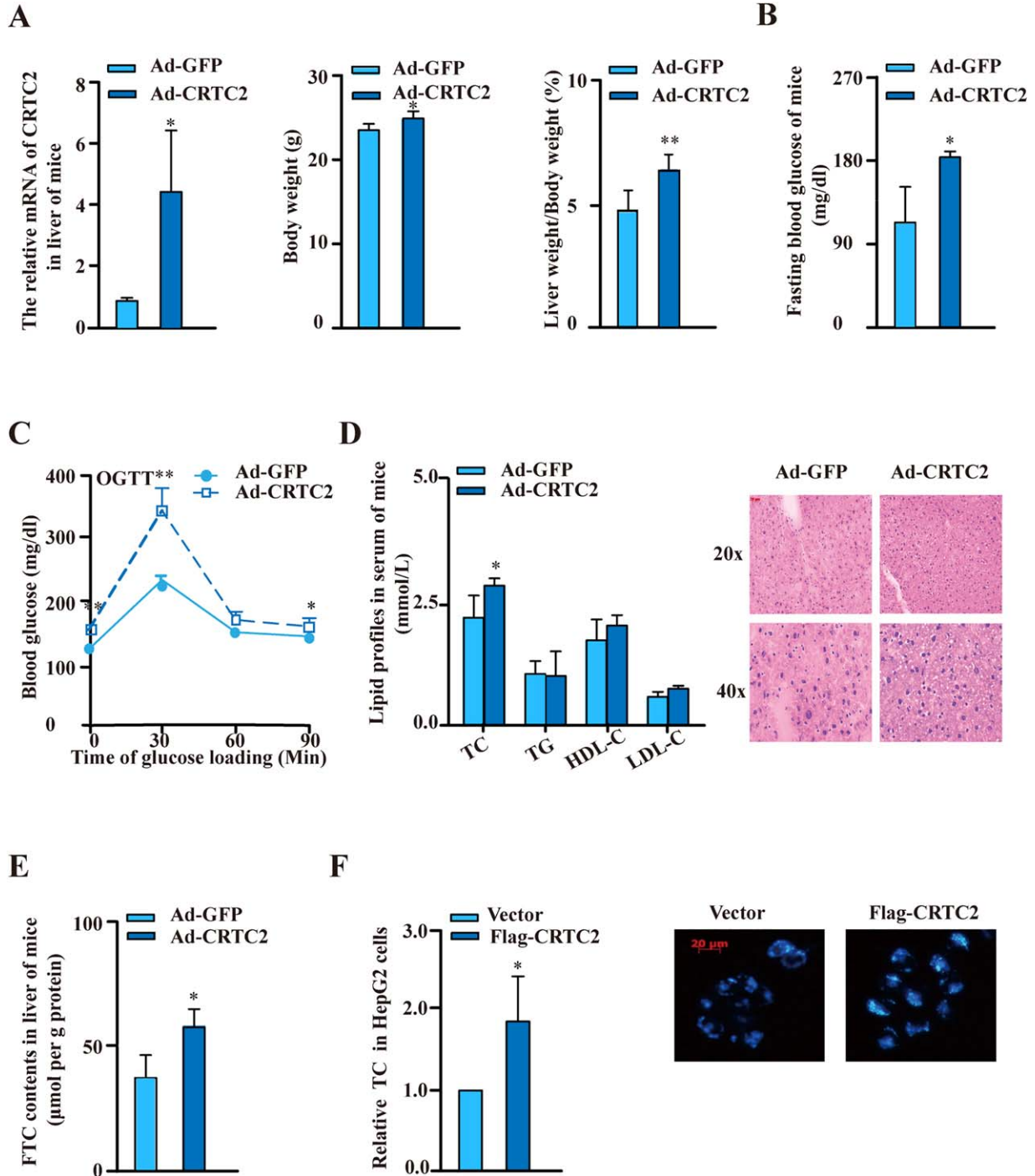. (A) Real-time PCR analysis showing the mRNA of CRTC2 in mouse livers (left). Comparison of body weight gains (middle) and liver-to-body weight ratios (right) (n = 6). (B) Blood glucose levels of mice fasted for 8 hours were measured (n = 6). (C) The oral glucose tolerance test was performed on mice (n = 4-6). (D) Serum cholesterol, TG, HDL-C, and LDL-C were measured (n = 6, left). Representative mouse liver sections stained with hematoxylin and eosin (right). (E) FTC contents in liver tissues were measured and corrected by the total protein content in the same sample (right, n = 4). (F) HepG2 cells were transfected with an empty vector or a Flag-CRTC2 overexpression plasmid for 48 hours. Then, cholesterol levels were measured. The amount of cholesterol is expressed relative to that of the control cells (left) (n = 3). The cellular distribution of free cholesterol was visualized with filipin staining (right). *$P < 0.05$ and **$P < 0.01$ versus Ad-GFP mice or vector. Abbreviations: OGTT, oral glucose tolerance test; TC, cholesterol.

staining, which is commonly used to visualize the cellular distribution of free cholesterol,[24] showed increased cholesterol accumulation in HepG2 cells overexpressing CRTC2 (Fig. 3F, right). In contrast, CRTC2 knockout mice (CRTC2$^{-/-}$) (Fig. 4A) exhibited lower fasting blood glucose concentrations and glucose tolerance than wild-type (Wt) controls (CRTC2$^{+/+}$) (Fig. 4B). Serum total cholesterol and TG levels were also decreased (Fig. 4C), and the livers of CRTC2$^{-/-}$ mice were smaller than those of CRTC2$^{+/+}$ mice (Fig. 4D, left). Hematoxylin and eosin staining showed reduced fat accumulation in the hepatic intracellular vacuoles of CRTC2$^{-/-}$ mice (Fig. 4D, right). Hepatic FTC was also consistently reduced (Fig. 4E, left). However, CRTC2$^{-/-}$ mice injected with Ad-CRTC2 showed higher serum total cholesterol levels (Fig. 4E, middle) and liver FTC contents (Fig. 4E, right) than CRTC2$^{-/-}$ mice injected with adenovirus-mediated green fluorescent protein (Ad-GFP). [1-^{14}C]-Labeled sodium acetate was used to assess *de novo* cholesterol synthesis.[22] Our results showed that hepatic *de novo* cholesterol synthesis was increased by approximately 2.0-fold in Ad-CRTC2 mouse livers (Fig. 4F), indicating that CRTC2 modulates hepatic cholesterol synthesis.

CRTC2 MODULATES HEPATIC HMGCR *IN VIVO* AND *IN VITRO*

Because CRTC2 induced an increase in cholesterol synthesis (Fig. 4F) and because HMGCR is the key rate-limiting enzyme in cholesterol synthesis,[25] we tested whether CRTC2 could affect HMGCR expression. We found that CRTC2 overexpression significantly increased the mRNA levels of HMGCR, farnesyl diphosphate synthase, squalene synthase, adenosine triphosphate–binding cassette transporter A 1 (a gene involved in cholesterol export[26]), and the gluconeogenic gene PEPCK in Ad-CRTC2 mouse livers (Fig. 5A). However, the mRNA levels of cytochrome P450 7A1 (the rate-limiting enzyme in bile acid synthesis[27]) and acetyl-coenzyme A acetyltransferase 2 (ACAT2, a cholesterol-esterifying enzyme[26]) were reduced in the livers of Ad-CRTC2 mice, while the LDLR levels were unchanged (Fig. 5A). Similarly, CRTC2 overexpression consistently increased the mRNA levels of HMGCR and glucogenic genes *in vitro* (Fig. 5B). Moreover, *in vivo* imaging showed that CRTC2 overexpression significantly elevated the luciferase activity of HMGCR-luciferase (Luc) in mouse livers (Fig. 5C). In contrast, the expression of

genes related to cholesterol synthesis and uptake (HMGCR, HMGCS, squalene synthase, and LDLR) and other lipid transport genes (adenosine triphosphate–binding cassette transporter A 1 and ACAT2) was reduced in CRTC2$^{-/-}$ mice (Fig. 5D). As in previous reports,[17] the gluconeogenic gene PEPCK was also reduced in CRTC2$^{-/-}$ mouse livers (Fig. 5D). CRTC2 overexpression consistently increased the protein levels of HMGCR in mouse livers (Fig. 5E), whereas CRTC2 knockout reduced HMGCR protein levels compared to the CRTC2$^{+/+}$ mice (Fig. 5F, left). Similarly, CRTC2 small interfering RNA (siRNA) reduced the expression of HMGCR in HepG2 cells, as shown by immunofluorescence (Fig. 5F, right). These results demonstrated that CRTC2 modulates hepatic HMGCR both *in vivo* and *in vitro*.

SREBP-2 IS INVOLVED IN THE MECHANISM BY WHICH CRTC2 INCREASES HEPATIC HMGCR

Previous studies have reported that the HMGCR promoter contains CRE and SRE sites, which can be recognized by CREB and SREBP-2, respectively.[28] We constructed a recombinant Luc reporter plasmid, pGL4-HMGCR-Luc (Wt), containing the CRE and SRE sequences and then mutated the CRE (HMGCR-Luc [CRE-Mut]) or SRE (HMGCR-Luc [SRE-Mut]) sequences (Fig. 6A). A significant increase of approximately 3.02-fold in the luciferase activity of HMGCR-Luc (Wt) was detected after the overexpression of CRTC2 (Fig. 6B); however, upon mutation of the SRE binding site in the HMGCR promoter (SRE-Mut), the effect of the CRTC2 overexpression from the resulting plasmid, HMGCR-Luc (SRE-Mut), was reduced (Fig. 6B). Moreover, HMGCR-Luc (CRE-Mut), with a mutated CRE binding site, also resulted in reduced Luc activity (Fig. 6B). These data strongly indicated that SREBP-2 and CREB are important for the role of CRTC2 in regulating HMGCR transcription. Nevertheless, in this study, we decided to focus on the modulatory role of CRTC2 on SREBP-2.

SREBP-2 is a master regulator that controls the transcription of critical genes involved in cholesterol synthesis and uptake.[29] Thus, we examined the effect of CRTC2 on HMGCR through SREBP-2. CRTC2 overexpression in mice markedly increased the mRNA levels of SREBP-2 and both the precursor and mature (nuclear) forms of SREBP-2 (SREBP-2 [P] and SREBP-2 [N]) in mouse livers (Fig. 6C, left). Similar
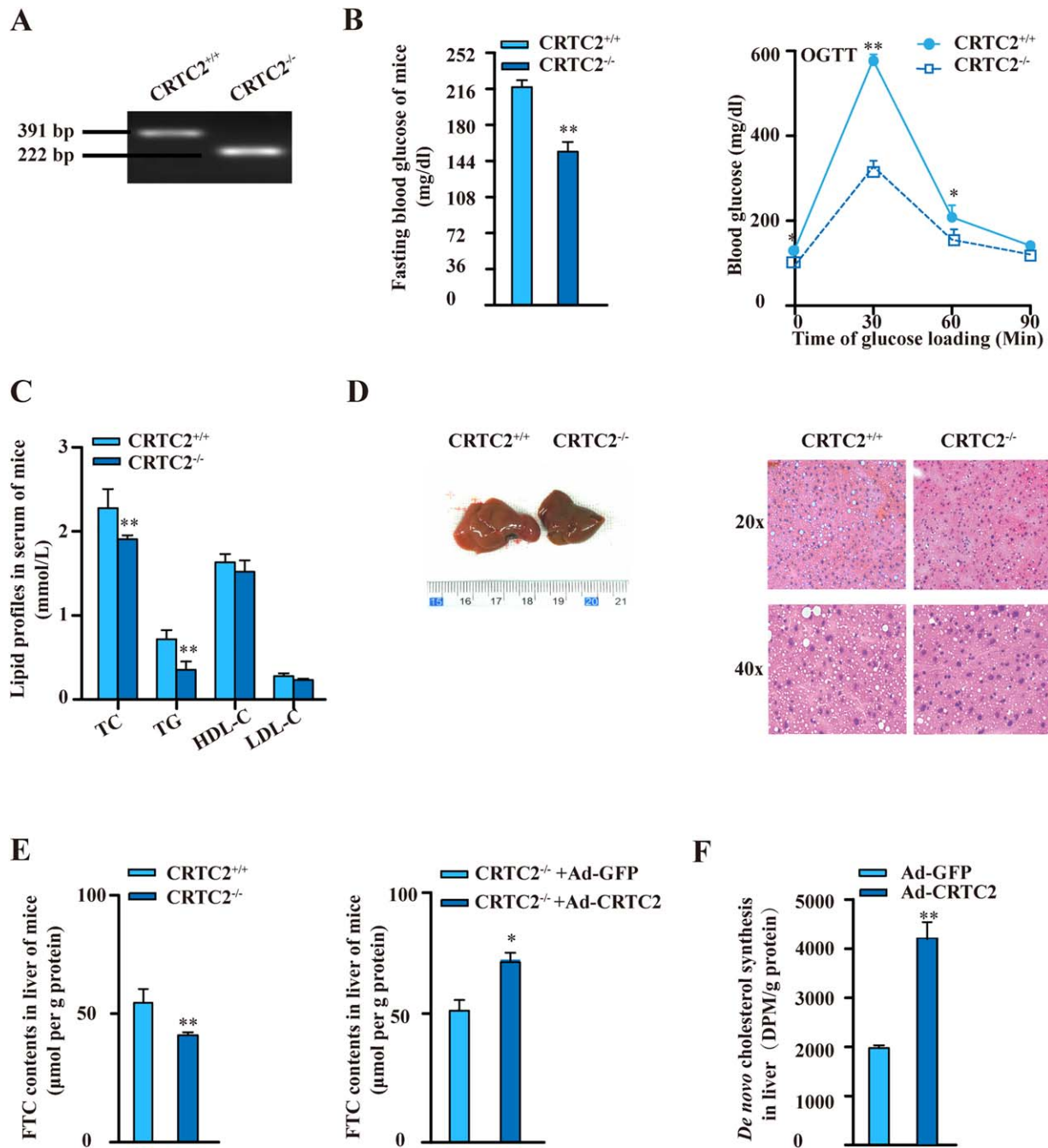
FIG. 4. CRTC2 modulates hepatic cholesterol synthesis *in vivo*. (A-D) CRTC2$^{+/+}$ and CRTC2$^{-/-}$ mice were fed a diet containing 4% cholesterol *ad libitum* for 18 weeks. (A) PCR analysis showing CRTC2 fragments generated from Wt (CRTC2$^{+/+}$) or knockout (CRTC2$^{-/-}$) mice. (B) Blood glucose levels of mice fasted for 8 hours were measured (n = 5). The oral glucose tolerance test was performed on the mice (n = 4-6). (C) Serum lipid profiles were measured (n = 4-6). (D) Macroscopic pictures of mouse livers and representative liver sections stained with hematoxylin and eosin. (E, left) FTC contents in the livers of CRTC2$^{+/+}$ and CRTC2$^{-/-}$ mice fed a 4% cholesterol diet for 18 weeks (n = 5-7 per group). (E, middle and right) Lipids in serum and liver tissues of CRTC2$^{-/-}$ mice fed a 4% cholesterol diet and injected with adenovirus. (F) C57BL/6J mice were injected three times (once every 5 days) with adenovirus for CRTC2 or GFP overexpression through the tail vein. Mice fasted for 4 hours were intraperitoneally injected with 10 μCi [1-^{14}C]-sodium acetate and sacrificed 30 minutes after injection, and the livers were extracted for the analysis of *de novo* cholesterol synthesis (n = 3). *$P < 0.05$ and **$P < 0.01$ versus CRTC2$^{+/+}$ group or Ad-GFP group. Abbreviations: DPM, disintegrations per minute; TC, cholesterol.
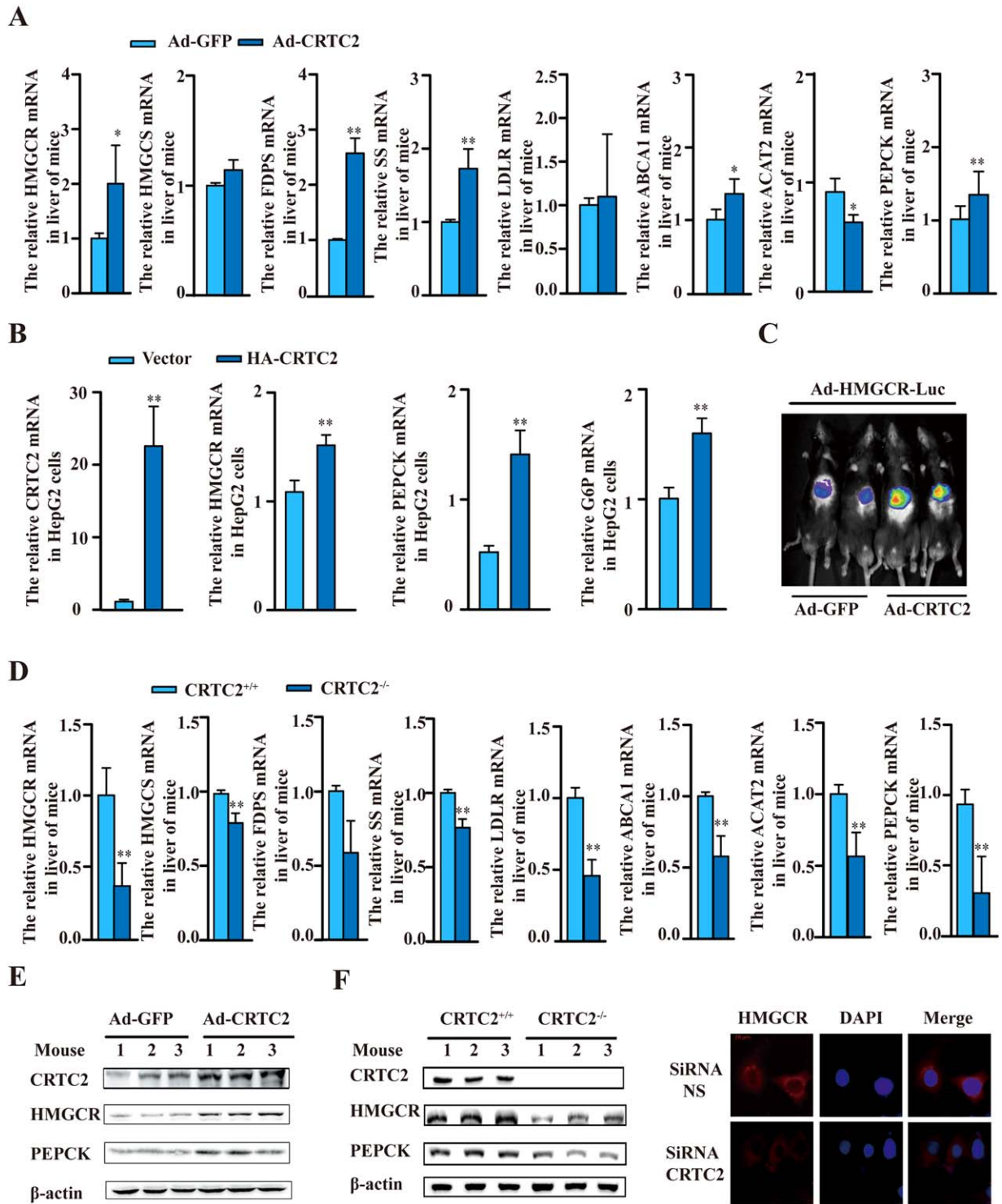
FIG. 5. CRTC2 modulates hepatic HMGCR *in vivo* and *in vitro*. (A) Expression of cholesterol-related and gluconeogenic genes was measured in livers from mice overexpressing Ad-CRTC2 or control Ad-GFP (n = 3-6). *$P < 0.05$ and **$P < 0.01$ versus Ad-GFP group. (B) HepG2 cells were transfected with an empty vector or an hemagglutinin–CRTC2 overexpression plasmid for 48 hours, and mRNA levels were measured. Data are from at least three independent experiments. **$P < 0.01$ versus vector. (C) Live imaging of hepatic HMGCR-luciferase (Ad-HMGCR-Luc) activity in Ad-GFP or Ad-CRTC2-infected mice. (D) Expression of cholesterol-related and gluconeogenic genes was measured in CRTC2$^{+/+}$ and CRTC2$^{-/-}$ mouse livers (n = 3-6). **$P < 0.01$ versus CRTC2$^{+/+}$ group. (E) Western blotting assays were used to evaluate CRTC2, HMGCR, and PEPCK protein expression in Ad-GFP or Ad-CRTC2-infected mouse livers (E). (F) Western blotting assays were used to evaluate protein expression in CRTC2$^{+/+}$ and CRTC2$^{-/-}$ mouse livers (left). After transfection with nontargeting or CRTC2 siRNA for 48 hours, HepG2 cells were stained for immunofluorescence analysis. Blue indicates the DAPI-stained nuclei and red indicates the HMGCR protein (the secondary antibody was tetra methyl rhodamine isothiocyanate–conjugated) (right). Abbreviations: ABCA1, adenosine triphosphate–binding cassette transporter A1; DAPI, 4,6-diamidino-2-phenylindole; FDPS, farnesyl diphosphate synthase; G6P, glucose 6-phosphate; NS, not significant; SS, squalene synthase.
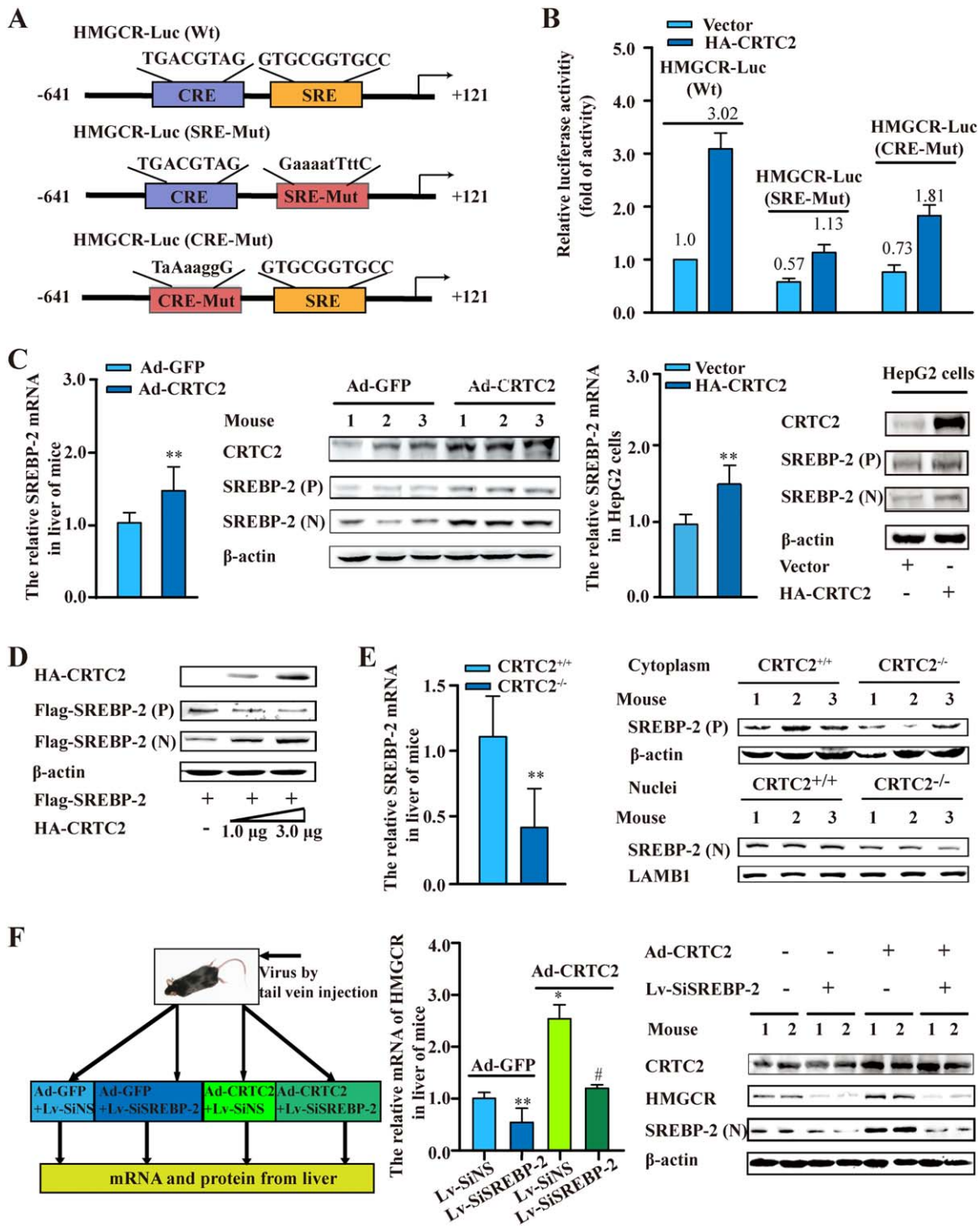
FIG. 6. CRTC2 increases hepatic HMGCR through SREBP-2. (A) The HMGCR reporter vectors containing CRE and SRE sequences, the mutated SRE (SRE-Mut), and the mutated CRE (CRE-Mut). (B) HEK293 cells were transfected for 48 hours with HMGCR-luciferase constructs (Wt, SRE-Mut, or CRE-Mut) with or without control vector or a hemagglutinin–CRTC2 overexpression plasmid. Luciferase activity was assayed in triplicate. (C) The mRNA and protein levels of SREBP-2 were tested in Ad-GFP and Ad-CRTC2 mouse livers (left) and HepG2 cells transfected with or without control vector or a hemagglutinin–CRTC2 overexpression plasmid for 48 hours (right). (D) Chinese hamster ovary cells were transfected with different doses of the indicated plasmids, and the protein levels were assessed by western blotting using anti-Flag or antihemagglutinin antibodies. (E) The mRNA and protein levels of SREBP-2 were assessed in $CRTC2^{+/+}$ and $CRTC2^{-/-}$ mouse livers. (F) C57BL/6J mice were injected three times (once every 5 days) through the tail vein with adenoviruses overexpressing CRTC2 or GFP control and with or without lentivirus-mediated SREBP-2 silencing (Lv-SiSREBP-2) or nontargeting lentivirus (Lv-SiNS) (left). The mRNA and protein levels were measured by real-time PCR (middle) and western blotting assays (right). Precursor SREBP-2 (SREBP-2 [P]), mature (nuclear) SREBP-2 (SREBP-2 [N]). Each sample was analyzed in triplicate, and samples from three different animals were analyzed to determine each value. In (C), the statistical significance is relative to Ad-GFP mice or vector, $**P < 0.01$. In (E), $**P < 0.01$ versus $CRTC2^{+/+}$ mice. In (F) $*P < 0.05$ and $**P < 0.01$ versus the Ad-GFP+Lv-SiNS group. $^{\#}P < 0.05$ versus the Ad-CRTC2+Lv-SiNS group. Abbreviation: HA, hemagglutinin.

results were obtained in HepG2 cells overexpressing CRTC2 (Fig. 6C, right). Furthermore, CRTC2 overexpression in CHO-7 cells, which overexpressed Flag-SREBP-2, markedly increased the proteolytic cleavage of SREBP-2 in a dose-dependent manner, as shown by elevated levels of the mature, nuclear form of SREBP-2 (Flag-SREBP-2 [N]; Fig. 6D). Conversely, in mouse livers, CRTC2 knockout decreased the expression of SREBP-2 mRNA by approximately 60% and both the precursor and mature forms of the protein (Fig. 6E).

Mice were injected with vectors for overexpression of Ad-CRTC2 and lentivirus-mediated SREBP-2 silencing (Lv-SiSREBP-2) to further determine the role of SREBP-2 in the CRTC2-induced regulation of HMGCR (Fig. 6F, left). The results showed that Lv-SiSREBP-2 not only reduced the gene expression of HMGCR but also alleviated the increase in HMGCR gene expression induced by CRTC2 overexpression (Fig. 6F, middle). A similar result was found for HMGCR protein expression in Ad-CRTC2 and Lv-SiSREBP-2 mouse livers (Fig. 6F, right).

CRTC2 REGULATES THE TRANSCRIPTION OF SREBP-2

Previous reports have suggested that the promoter sequence of SREBP-2 is recognized by several potential transcription factors. In addition to the previously characterized SRE[30] and insulin response elements (IRE1 and IRE2),[31] we found a CRE half-site (TGACG-TAA) that can be recognized by CREB.[32] Thus, we constructed pGL4.10-SREBP-2-Luc (Wt), a recombinant luciferase reporter plasmid containing the CRE and IRE sequences (Fig. 7A, upper), and then constructed the Ad-SREBP-2-Luc reporter. CRTC2 knockout reduced the Ad-SREBP-2-Luc activity in mice (Fig. 7A, lower, left). Furthermore, chromatin immunoprecipitation experiments demonstrated that FoxO1 bound specifically to the SREBP-2 promoter. CRTC2 significantly reduced this binding (Fig. 7A, lower, right). We used the transcription inhibitor actinomycin D (Act-D) to further determine the transcriptional effect of CRTC2 on SREBP-2. The results showed that Act-D reduced the transcription of SREBP-2. Similarly, the effect of CRTC2 on SREBP-2 transcription was also inhibited by Act-D (Fig. 7B). Thus, we explored the detailed mechanisms by which CRTC2 can regulate SREBP-2 transcription.

We cloned the mutant CRE half-site in the mouse SREBP-2 gene promoter (Fig. 7C, left) and showed

that the CRE mutation alleviated the effect of CRTC2, although not completely (Fig. 7C, right). This result indicated that CRTC2 might regulate the SREBP-2 gene through other binding sites. FoxO1 is activated after entry into the nucleus[33] and then negatively regulates the transcription of SREBP-2 through IREs.[12,31] We performed experiments using FoxO1 siRNA to confirm these findings (Fig. 7D). Though CRTC2 knockout had no effect on FoxO1 gene expression (Fig. 7E, left), the levels of nuclear FoxO1 were increased in CRTC2 knockout mouse livers (Fig. 7E, middle). Similarly, though CRTC2 overexpression did not change the mRNA levels of FoxO1 in hepatocytes, the levels of nuclear FoxO1 were decreased in BNL cells and HepG2 cells (Fig. 7E, right). This result is similar to those obtained in previous reports.[34] Because there are two IRE sites, we separately cloned mutant IRE1 and IRE2 in the mouse SREBP-2 gene promoter to verify the role of CRTC2 in the regulation of the SREBP-2 gene through FoxO1 (Fig. 7C, left). Compared with the Wt reporter, CRTC2 overexpression significantly increased the activity of the SREBP-2 promoter carrying a mutation in IRE1; by contrast, IRE2 mutation was not associated with notable changes (Fig. 7F).

CRTC2 REGULATES THE ACTIVITY OF SREBP-2 IN MICE FED DIETS CONTAINING VARYING AMOUNTS OF CHOLESTEROL

Dietary absorption and biosynthesis are the two sources for cholesterol acquisition in mammals.[5] We analyzed whether CRTC2 affected lipid synthesis in mice fed diets containing various amounts of cholesterol. CRTC2 knockout mice were fed a diet with 0.02%, 1%, and 4% cholesterol. As expected, SREBP-2 was strongly activated under low-cholesterol conditions and decreased when CRTC2$^{+/+}$ mice were fed a diet containing high cholesterol levels for 3 days (Fig. 8A). CRTC2 knockout greatly reduced SREBP activation in mice fed low-cholesterol diets (0.02% cholesterol diet; Fig. 8A). However, CRTC2 knockout had a minimal effect on SREBP-2 activation in mice fed a 4% cholesterol diet (Fig. 8A). CRTC2 knockout not only strongly reduced the mRNA levels of SREBP-2 target genes (HMGCR, HMGCS, and LDLR) but also ameliorated SREBP-2 gene expression at low cholesterol levels (Fig. 8B). However, CRTC2 knockout
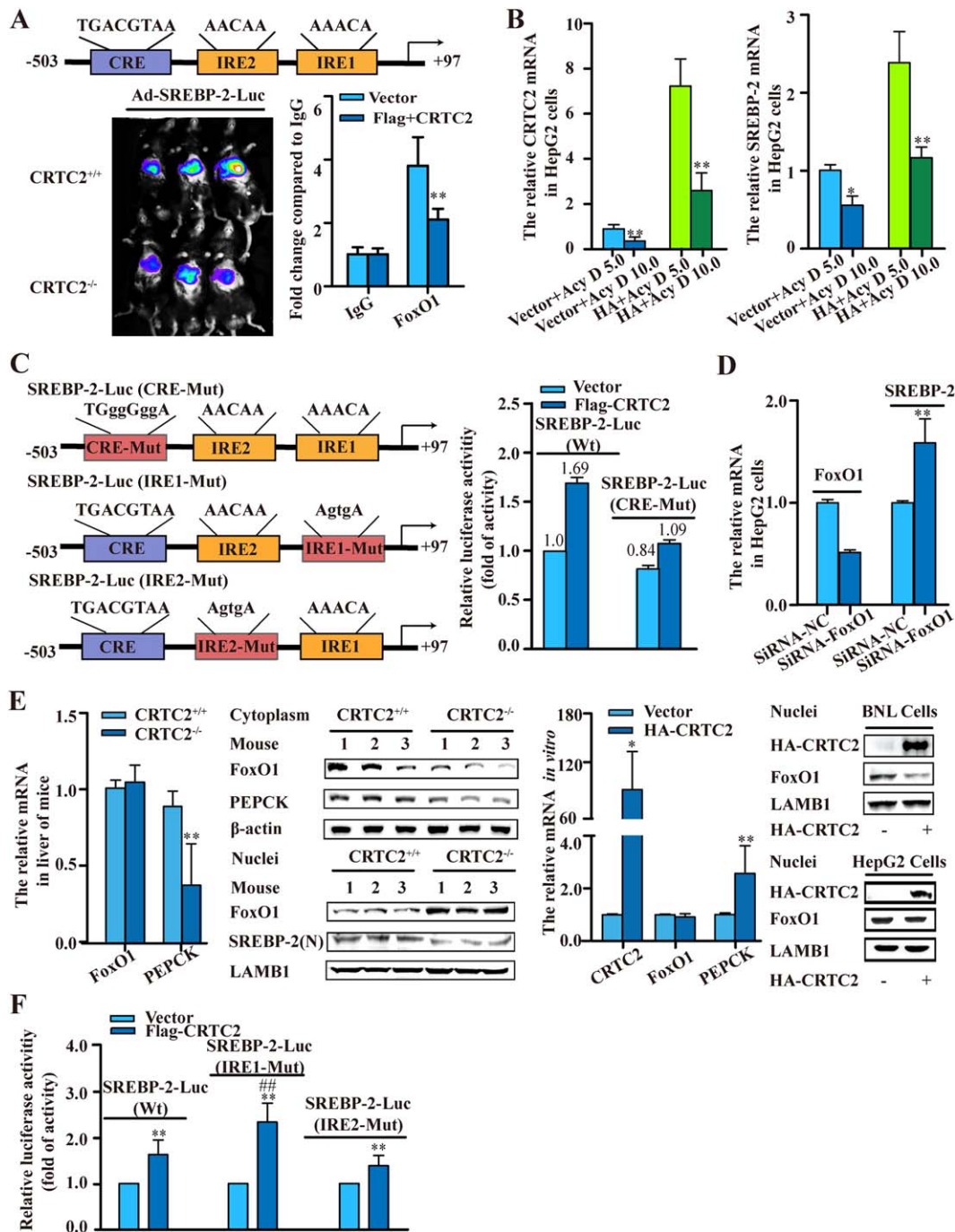
FIG. 7. CRTC2 regulates the transcription of SREBP-2. (A) Schematic diagram of the proximal promoter of the mouse SREBP-2 gene; CRE and the two putative IREs (IRE1 and IRE2) are indicated (upper). Live imaging of hepatic SREBP-2-luciferase (Ad-SREBP-2-Luc) activity in CRTC2$^{+/+}$ and CRTC2$^{-/-}$ mice fed an HC diet (lower, left). Chromatin immunoprecipitation assay histograms representing the relative binding of FoxO1 to the SREBP-2 promoter in cells transfected with Flag-CRTC2 plasmids for 48 hours. The data were normalized to the corresponding immunoglobulin G (lower, right). (B) HepG2 cells were transfected with empty vector or hemagglutinin–CRTC2 overexpression plasmids for 24 hours before actinomycin D (5 or 10 μg/mL) was added for 2 more hours; mRNA levels were then analyzed. (C) SREBP-2 reporter vectors containing the mutated CRE half-site (CRE-Mut), mutated IRE1 (IRE1-Mut), or mutated IRE2 (IRE2-Mut) (left). HEK293 cells were transfected with a Flag-CRTC2 overexpression plasmid or empty vector and SREBP-2-Luc (Wt) or SREBP-2-Luc (CRE-Mut) plasmids for 48 hours. Then, luciferase activity was tested (right). (D) Real-time PCR was used to assess gene expression in HepG2 cells transfected with nontargeting (SiRNA-NC) or FoxO1 siRNA (SiRNA-FoxO1) for 48 hours. (E) mRNA and protein expression levels in the cytoplasm and nuclei were examined in CRTC2$^{+/+}$ and CRTC2$^{-/-}$ mouse livers (left). Real-time PCR for FoxO1 in BNL cells transfected with vector or a hemagglutinin–CRTC2 overexpression plasmid for 48 hours (middle). Western blotting for nuclear FoxO1 in BNC cells and HepG2 cells (right). (F) HEK293 cells were transfected with SREBP-2-luciferase constructs (Wt, IRE1-Mut, or IRE2-Mut), along with control vector or a Flag-CRTC2 overexpression plasmid for 48 hours. Luciferase activity was assayed. Each sample was analyzed in triplicate, and samples from three different animals were analyzed to determine each value. In (B-F), *$P < 0.05$ versus "Vector+Act-D 5.0" or vector; **$P < 0.01$ versus actinomycin D 5.0, siRNA NC, CRTC2$^{+/+}$ mice, or vector; ##$P < 0.01$ versus "Flag-CRTC2+SREBP-2-Luc (Wt)." Abbreviations: Acy D, actinomycin D; HA, hemagglutinin; IgG, immunoglobulin G.
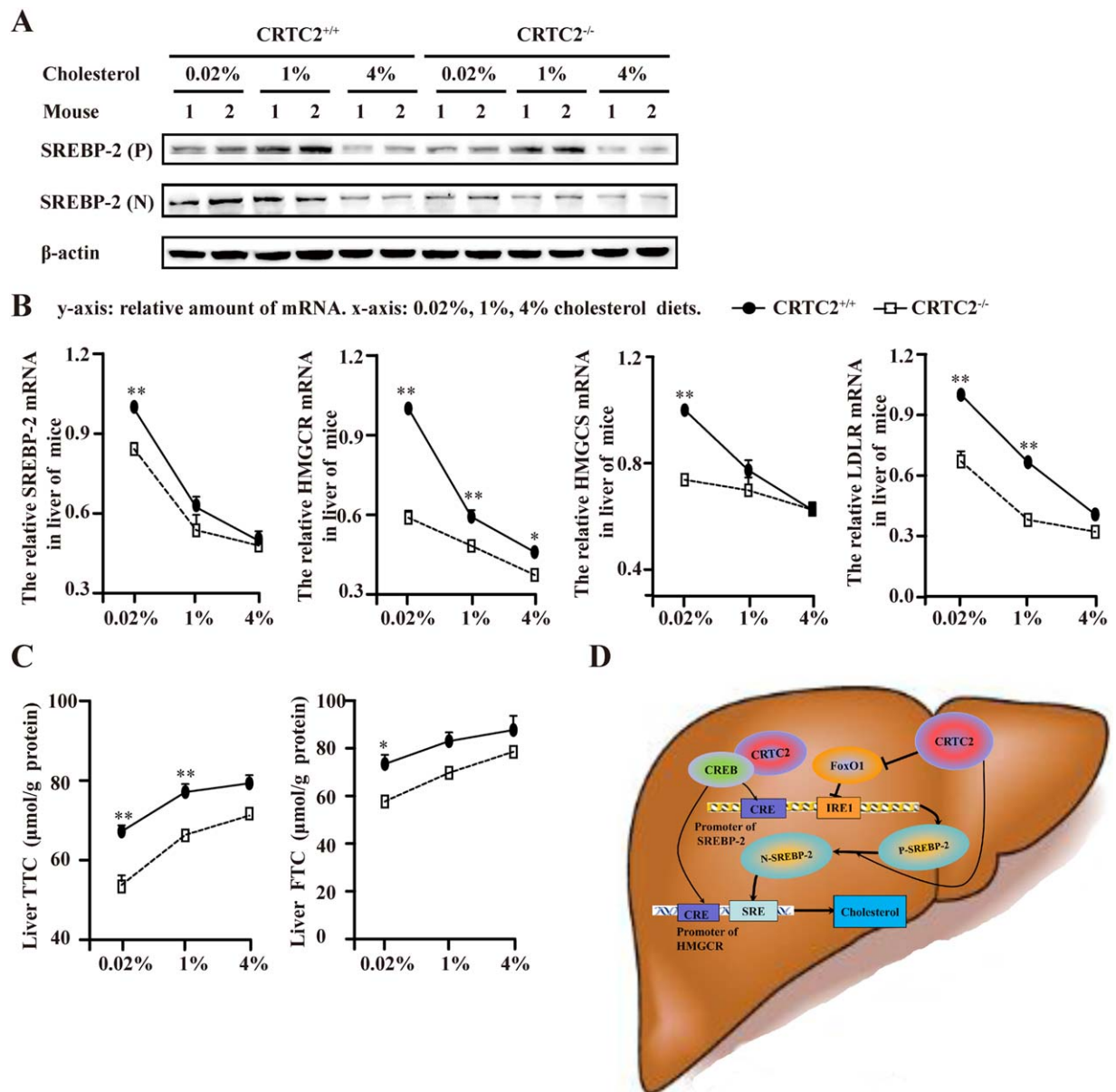
FIG. 8. CRTC2 regulates SREBP-2 activity in mice fed diets with varying amounts of cholesterol. (A-C) Seven-week-old male CRTC2$^{-/-}$ mice and littermate CRTC2$^{+/+}$ mice were fed diets containing different cholesterol levels *ad libitum* for 3 days. Mice were then sacrificed, and liver tissues were collected for analysis. Western blotting (A), mRNA (B), and lipid (C) analysis in mouse livers (n = 4). Lipid content was normalized by total protein content. *$P < 0.05$ and **$P < 0.01$ versus CRTC2$^{+/+}$ mice. (D) Schematic diagram: CRTC2 activates SREBP-2/HMGCR signaling, resulting in hepatic cholesterol synthesis. CRTC2 inhibits nuclear FoxO1 expression in the liver, blocking the negative effect of FoxO1 on SREBP-2 transcription by binding the IRE1 sequence located in promoter of SREBP-2. Thus, the levels of the SREBP-2 precursor (P-SREBP-2) and mature (nuclear) SREBP-2 (N-SREBP-2) increase. Mature SREBP-2 up-regulates the transcription of HMGCR by recognizing the SRE sequence in the HMGCR promoter. In addition, CRTC2 promotes the proteolytic cleavage of the SREBP-2 precursor, yielding mature SREBP-2. Moreover, as a coactivator of CREB, CRTC2 also increases the transcription of HMGCR by enhancing the effect of CREB on the CRE sequence within the HMGCR promoter region. In this way, CRTC2 induces cholesterol synthesis. Abbreviation: TTC, total cholesterol.

had a minimal effect on SREBP-2 function in mice fed a 4% cholesterol diet (Fig. 8B). In addition, the effects of CRTC2 knockout on the liver FTC and total cholesterol levels were maximal at low cholesterol concentrations (Fig. 8C). Collectively, these data indicated that CRTC2 exerts its effect on lipids under

physiological conditions of low cholesterol, in which cholesterol synthesis is required.

Discussion

In this study, we confirmed that CRTC2 is a crucial controller of cholesterol homeostasis. The expression level of CRTC2 is important for the activities of liver SREBP-2: CRTC2 overexpression stimulates SREBP-2 activation, whereas CRTC2 knockout reduces SREBP-2 activation. These changes are also accompanied by changes in the expression of the SREBP-2 target genes HMGCR and LDLR. Furthermore, CRTC2 overexpression reduces the nuclear localization of FoxO1, whereas CRTC2 knockout elevates it. Importantly, CRTC2 increases the transcription of SREBP-2 through FoxO1 recognition of the sequence of IRE1, rather than IRE2, in the SREBP-2 promoter. Therefore, CRTC2 plays an active role in hepatic cholesterol synthesis by facilitating SREBP-2 transcription.

A key goal of this study was to determine whether there is an association between CRTC2 and cholesterol levels in the liver. The data from clinic samples chosen in our study provided a positive, though indirect, answer to this question. We employed various tests to verify this finding *in vivo* and *in vitro*. CRTC2 overexpression in mice markedly increased cholesterol levels in both the liver and serum, whereas the cholesterol levels decreased in CRTC2 knockout mice. In addition, CRTC2 initiated cholesterol synthesis, particularly in mice fed low-cholesterol diets. The amount of cellular free cholesterol is dependent on the synthesis and hydrolysis of cholesteryl ester.[35-37] The levels of hepatic free cholesterol were increased in Ad-CRTC2 mice and decreased in CRTC2 knockout mice. [1-^{14}C]-Labeled acetate is often used to assess *de novo* cholesterol synthesis.[23] To further determine the effect of CRTC2 on *de novo* cholesterol synthesis, we injected [1-^{14}C]-labeled acetate into Ad-CRTC2 mice and verified that *de novo* cholesterol synthesis was elevated in the liver.

The HMGCR gene promoter contains CRE and SRE binding sites, which are the targets of the CREB and SREBP-2 proteins, respectively.[27] CRTC2, a coactivator of CREB, increases CREB activity following its association with residues in the carboxy-terminal basic Leu zipper domain of CREB.[38] However, mutation of CRE attenuated the activity of HMGCR-Luc, though not completely. Importantly, we observed a marked reduction in CRTC2-induced

HMGCR expression with HMGCR-Luc (mutation of SRE) and with lentivirus-mediated knockdown of SREBP-2. This result confirmed that CRTC2 has beneficial effects on the transcription of HMGCR by SREBP-2 over CREB.

SREBPs are regulated at the transcriptional level, and they are also regulated by the proteolytic cleavage of SREBP precursors and by the posttranslational modification of mature SREBP proteins.[39] Expression of the precursor and mature nuclear forms of SREBP-2 in mouse livers was shown to be dependent on CRTC2 levels. Furthermore, CRTC2 induced the activity of SREBP-2-Luc *in vivo* and *in vitro*, prompting us to focus on the transcription of SREBP-2. The mechanism involved in this regulation remains unclear. FoxO1 is currently considered an important factor controlling SREBP-2 transcription.[12,40] A previous report showed that the liver-specific deletion of FoxO1 induced a 60% increase in SREBP-2 mRNA, suggesting that FoxO1 negatively regulates the transcription of SREBP-2.[12] Similarly, we found that FoxO1 gene silencing increased the transcription of SREBP-2. The process of CRTC2-mediated SREBP-2 transcription was associated with the decreased translocation of FoxO1 into the nucleus. Tao et al. identified the IRE (IRE1 and IRE2) sequences as the sites where the FoxO1 protein binds to the SREBP-2 promoter.[31] Intriguingly, in our model, CRTC2 performed its regulatory function on SREBP-2 transcription exclusively through IRE1. Previous reports showed that CRTC2 is a coactivator of CREB, which promotes the activity of CREB through the CRTC2/CREB complex.[17] CREB stimulates targeted gene expression at its promoters that contain CREs. These typically appear as either palindromic (TGACGTCA) or half-site (TGACG or CGTCA) sequences.[32] In this study, we also observed that CRTC2 regulated the transcription of SREBP-2 through the CRE half-site located in the SREBP-2 promoter. Thus, the mechanism of CRTC2 transcriptional regulation of SREBP-2 is complicated.

The regulation of posttranslational proteolysis is also important for SREBP-2 activation.[41] We introduced a model involving transfection with the exogenous full SREBP-2 sequence[1] in combination with CRTC2 overexpression to confirm whether CRTC2 also affects the proteolysis of SREBP-2. We determined whether there was a decrease in the precursor or an increase in the mature form of SREBP-2, finding that CRTC2 modulates SREBP-2, at least to a certain extent, by promoting proteolysis. Further investigation will improve our understanding of this process.

Previous reports have shown that CRTC2 has different effects on lipid metabolism under different conditions.[20,34] For example, on a chow diet, the chronic overexpression of CRTC2 activated CRTC2-induced hepatic steatosis and higher levels of hepatic TG, as expected in hyperglycemia.[33] After being fed a high-fat diet (60% of calories from fat) for 12 weeks, liver-specific CRTC2 siRNA mice showed reduced TG in the liver and muscles as well as hyperglycemia.[20] However, on the same diet for 18 weeks, CRTC2 knockout mice showed higher liver TG levels and related gene expression but no notable changes in liver cholesterol levels.[21] When we fed CRTC2 knockout mice an HC diet (4% cholesterol) for 18 weeks, they exhibited decreased serum TG levels and, in particular, decreased cholesterol levels in the serum and liver. Aside from the liver and muscle, CRTC2 also affected adipocytes, including differentiated preadipocytes in mice and the 3T3-L1 cell line,[42] in which ablation of CRTC2 induced a decrease in the expression of TG-related genes, such as SREBP-1C, fatty acid synthase, and acetyl-coenzyme A carboxylase 1. Thus, CRTC2 is a coregulator of glucose, TG, and cholesterol metabolism, with different roles in different tissues, at different feeding times, and with different diets.

Our study has some limitations. A previous study showed that mice fed a high-fat diet exhibited an increase in hepatic CRTC2 in a time-dependent manner.[21] In addition, db/db and ob/ob mice exhibited higher hepatic CRTC2 levels than lean mice.[21] The current study provides evidence that clinical subjects with elevated serum total cholesterol levels exhibit higher levels of CRTC2 in the liver. This finding indicated that CRTC2 is associated with cholesterol levels. However, the analysis of human tissues did not permit intervention experiments aimed at defining the specific role of CRTC2 in the accumulation of cholesterol or the role of cholesterol in the increase of CRTC2 expression. Another limitation of this study was the relatively small sample size of patients due to the difficulty in obtaining liver specimens; therefore, we could not analyze the relevance of CRTC2 and lipid levels in humans. However, we included age-matched and sex-matched patients with liver hemangiomas and excluded patients with hepatitis B virus, hepatitis C virus, or human immunodeficiency virus or those receiving a lipid-lowering therapy. Thus, our data indicated that in humans cholesterol levels are related, at least partially, to CRTC2 expression in the liver. It would be beneficial to observe the effect of CRTC2 in mice with CRTC2 liver-specific overexpression or deletion. Nevertheless, we generated several *in vivo* and *in vitro* models, including CRTC2 overexpression or knockout mice and SREBP-2 knockdown mice, to observe hepatic cholesterogenesis. Together with marked changes in cholesterol levels and related gene expression in mouse livers, we also observed changes in the gene expression of adenosine triphosphate–binding cassette transporter A1, cytochrome P450 7A1, and ACAT2. These results suggested that CRTC2 may also be involved in cholesterol efflux, conversion, and esterification. We also noted that the expression of ACAT2 was similar in the livers of Ad-CRTC2 mice and CRTC2$^{-/-}$ mice. It has been reported that hepatocyte nuclear factor 4α up-regulates the expression and activity of ACAT2 in hepatocytes.[43] Our previous study showed that SREBP-2 reduced the expression of hepatocyte nuclear factor 4α in the liver.[27] In this study, CRTC2 increased the expression of hepatic SREBP-2. Thus, we suggest that the overexpression of CRTC2 reduced the expression of ACAT2 by increasing the levels of SREBP-2 in the liver. Furthermore, FTC, LDL, and HDL also regulated the expression of ACAT2 in hepatocytes.[44] FTC and LDL increased the expression of ACAT2 in hepatocytes in a dose-dependent manner. In contrast, HDL had the opposite effect on ACAT2. Our results showed that Ad-CRTC2 mice exhibited high hepatic FTC contents and HDL and LDL-C levels in serum. CRTC2$^{-/-}$ mice exhibited decreased hepatic FTC contents and HDL-C and LDL-C levels in serum. Therefore, the changes in ACAT2 in Ad-CRTC2 and CRTC2$^{-/-}$ mice were comprehensive.

This study suggests a potential role for CRTC2 in cholesterol homeostasis. Specifically, we revealed that CRTC2 can act as a regulator by decreasing the levels of nuclear FoxO1 in the liver, resulting in an increase in SREBP-2 transcription and, consequently, up-regulation of hepatic HMGCR and cholesterol synthesis (Fig. 8D). Combined with previous reports on the role of CRTC2 in the regulation of glucose, we suggest that CRTC2 is a coregulator of both glucose and lipid metabolism. These results might partially explain the pathophysiological mechanisms of lipid metabolism in diabetes.

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Supporting Information

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