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HCBP6 Modulates Triglyceride Homeostasis in Hepatocytes Via the SREBP1c/FASN Pathway

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ABSTRACT

Hypertriglyceridemia leads to liver steatosis, cardiovascular disease, and type 2 diabetes. Although HCBP6 (hepatitis C virus core-binding protein 6) was previously shown to be an HCV (hepatitis C virus) core-binding protein, its biological function remains unclear. Here, we demonstrate that HCBP6 negatively regulates intracellular triglyceride (TG) levels in hepatocytes. We found that bidirectional manipulation of hepatocyte HCBP6 expression by knockdown or overexpression results in increased or decreased TG accumulation, respectively. In addition, HCBP6 mRNA and protein levels exhibited significant time- and dose-dependent increases in a cellular model of lipid-overload hepatic steatosis. Furthermore, TG levels are regulated by HCBP6-sterol regulatory element binding protein 1c (SREBP1c)-mediated fatty acid synthase (FASN) expression. We also demonstrate that HCBP6 mRNA and protein expression is inhibited by microRNA-122 (miR-122), and miR-122 overexpression elicited more robust translational repression of luciferase activity driven by the full 3′-UTR of HCBP6. Taken together, our results provide new evidence that miR-122-regulated HCBP6 functions as a sensor protein to maintain intrahepatocyte TG levels. J. Cell. Biochem. 116: 2375–2384, 2015.

KEY WORDS: FATTY ACID; GENE EXPRESSION; LIPASE/HEPATIC; NUCLEAR RECEPTORS/SREBP; TRIGLYCERIDES; MicroRNA-122

Triglycerides (TGs) constitute the main and most reliable source of energy in the body, participating in key metabolic functions, such as thermal insulation and fatty acid deposition. Pathophysiological serum TGs levels are involved in the development of hepatic steatosis [Browning and Horton, 2004; Fabbrini et al., 2010; Anstee et al., 2013; Koo, 2013], metabolic syndrome [Borena et al., 2011], type 2 diabetes [Goldberg, 2001], and cardiovascular disease [Sone et al., 2012].

Hepatic steatosis occurs when the production and removal of intrahepatic TGs become unbalanced due to reduced fatty acid oxidation as well as increased de novo lipogenesis, fatty acid uptake and very low-density lipoprotein (VLDL) export [Browning and Horton, 2004; Fabbrini et al., 2010; Anstee et al., 2013; Koo, 2013]. Maintaining TG homeostasis is essential for normal liver function. Many risk factors can promote hepatic steatosis through different mechanisms, such as metabolic diseases, alcohol, virus, drugs, and nutritional disorders, with insulin resistance (IR) being the most common feature leading to hepatic lipid accumulation [Choi and Ginsberg, 2011]. Multiple lines of evidence have demonstrated that various genes, including sterol regulatory element binding protein 1c (SREBP1c), glycerol-3-phosphate acyltransferase (GPAT), liver X receptor (LXR), fatty acid synthase (FASN), carbohydrate-responsive element-binding protein (ChREBP), forkhead box protein O1 (FoxO1), AKT2, and peroxisome proliferator-activated receptor gamma (PPARγ), are involved in IR-induced hepatic steatosis [Siri et al., 2001; Horton et al., 2002; Matsumoto et al., 2002; Ono et al., 2003; Cha and Repa, 2007; Postic and Girard, 2008]. Clinical studies have identified drugs that decrease TG levels, such as farnesol [Duncan and Archer, 2008], n-3 fatty acids, and apolipoprotein C3 inhibitors [Nordestgaard and Varbo, 2014].

SREBP1c belongs to the basic helix-loop-helix-leucine zipper family of transcription factors [Brown and Goldstein, 1997] and plays a key role in regulating fatty acid synthesis and TG accumulation in the liver. FASN is a major enzyme in lipid

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metabolism. In hepatocytes, FASN catalyzes the reaction of acetyl-CoA and malonyl-CoA into palmitate, which is then esterified into TGs [Semenkovich, 1997]. FASN expression is regulated by SREBP1c at the transcriptional level [Shimano, 2001].

MicroRNA-122 (miR-122), a liver specific microRNA, plays an important role in lipid metabolism by regulating hepatic sterol, TG and fatty acid synthesis [Krutzfeldt et al., 2005; Esau et al., 2006]. However, its direct target gene is unknown.

HCBP6, also known as FUNDC2, HCC3, DC44, or PD03104, was originally identified in our laboratory as a hepatitis C virus (HCV) core-binding protein via yeast two-hybrid assays [Li et al., 2002]. Although, the HCV core protein plays an important role in hepatic steatosis [Barba et al., 1997], its biological function is not fully understood. Although the HCV core protein induces TG accumulation by stimulating SREBP1c gene expression [Kim et al., 2007] and HCBP6 binds the HCV core protein, whether HCBP6 is involved in SREBP1c-FASN-mediated TG accumulation has yet to be determined. Here, we demonstrate that HCBP6 is a negative regulator and sensor of intracellular TG levels. HCPB6 expression was induced by TG in a time- and dose-dependent manner. Furthermore, we demonstrate that miR-122 inhibits both HCBP6 mRNA and protein expression, therefore ultimately regulating SREBP1c/FASN expression. These results offer new insights into the maintenance of intracellular TG homeostasis.

MATERIALS AND METHODS

CELL CULTURE

The hepatoma cell lines HepG2, Huh7 and hepatocyte cell line L02 were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Gibco, Life Technologies, USA), 100 U/ml of penicillin G, and 100 μg/ml of streptomycin (SW30010, BD, USA) at 37°C in a 5% CO_2 atmosphere. HepG2 and L02 cells that were exposed to oleate and palmitate (FFA) were maintained in DMEM containing 10% fatty acid free heat shock bovine serum albumin (BSA; Equitech-Bio, USA), 100 U/ml of penicillin G, and 100 μg/ml of streptomycin (SW30010, BD) at 37°C in a 5% CO_2 atmosphere.

PLASMIDS

The construction of pcDNA3.1/myc-His(−)-HCBP6 was described previously [Yang et al., 2003], and pcDNA3.1/myc-His(−)-SREBP1c was a kind gift of Dr. Yongsheng Chang [Wang et al., 2010]. The 5′-flanking region (from nt −1005 to +24) of SREBP1c was polymerase chain reaction (PCR)-amplified from the genomic DNA of HepG2 cells using the following primers: sense, 5′-GAGCTCCAAAAGAACCCTA-GAGCCTGT-3′; antisense, 5′-GCTAGCAATACCTTCGAAAGTG-CAATC-3′. The PCR product was sequenced and inserted into the pGL4.10 vector (Promega, Madison, WI). The HCBP6 3′-UTR were cloned into the pmir-GLO vector (Promega), with the following primers: sense, 5′-GCTAGCCTAGTAACTGGGGGATTTTTCGG-3′; antisence, 5′-CTCGAGAACCATGACCCTCACAGTCG-3′. The mutational HCBP6 3′-UTR were cloned into the pmir-GLO vector (Promega), with the following primers: sense, 5′-CGTCGGAGATCTCACGTAGTATCCTG-3′; antisence, 5′-GCAGCCTG-TAGAGTGCATCATAGGAC-3′.

MicroRNA OLIGONUCLEOTIDES AND siRNA OLIGONUCLEOTIDES

The chemically synthesized miR-122 mimic and inhibitor were purchased from RiboBio Co, Ltd. (Guangzhou, China). HCBP6 siRNA (sc-90945) SREBP1c siRNA (sc-36557) and negative-control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology (CA, USA).

DETERMINATION OF TOTAL INTRACELLULAR TG

Hepatoma and hepatocyte cell lines were treated with plasmids and/or FFA at various time points and dosages as indicated in the text. Intracellular TG content was measured using an Adipogenesis Assay Kit (MAK040, Sigma, USA) according to the manufacturer's instructions and normalized to total protein concentrations. The intracellular TG content was expressed as nmol/μg protein.

RNA ISOLATION, REVERSE TRANSCRIPTION AND QUANTITATIVE REAL-TIME PCR (qPCR)

Total RNA was prepared from hematoma cell lines using the Total RNA Kit (R6834, Omega, USA) according to the manufacturer's instructions. Total RNA was reverse transcribed into single-strand cDNA using the PrimeScript® RT reagent Kit (DRR037A, TaKaRa, China). A 1:4 dilution of the cDNA was subjected to qPCR (4367659, ABI, USA) amplification using gene-specific primers as indicated below. The relative amounts of SREBP1c, FASN and HCBP6 miRNA were calculated using the comparative Ct method (ΔΔCt) and normalized to the endogenous levels of GAPDH. The SREBP1c sense and antisense primers were 5′-GGAGGGGTAGGGCCAACGGCCT-3′ and 5′-CATGTCTTCGAAAGTGCAATCC-3′, respectively. The FASN sense and antisense primers were 5′-AGCTGCCAGAGTCGGAGAAC-3′ and 5′-TGTAGCCCACGAGTGTCTCG-3′, respectively. The HCBP6 sense and antisense primers were 5′-AGACAAGCTCACCGAAATGG-3′ and 5′-CTGGGTTGCCACGCTATACT-3′, respectively. The GAPDH sense and antisense primers were 5′-GCACCGTCAAGGCTGAGAAC-3′ and 5′-TGGTGAAGACGCCAGTGGA-3′, respectively.

WESTERN BLOTTING

Cells were lysed in lysis buffer (Thermo, 78501, USA) containing a protease inhibitor cocktail (5872s, CST, USA). Protein concentrations were determined via the Pierce BCA assay (23225, Thermo Scientific, USA) according to the manufacturer's protocol. A total of 60 μg of protein from whole cell lysates was subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (ISEQ00010, Millipore, USA). After blocking with 5% nonfat dry milk (2321000, BD), the membranes were incubated with primary antibodies, including anti-FASN antibody (ab128870, Abcam, USA), anti-SREBP1c antibody (SC-8984, Santa Cruz), anti-HCBP6 (LS-C249022, LS Bio, USA), and anti-GAPDH (5174, CST). The positive bands were detected using an enhanced chemiluminescence system (32209, Thermo Scientific) and analyzed with Bio1D software (VILBER, S: 11. 640150, France).

LUCIFERASE REPORTER ASSAY

Transfection assays were performed using jetPRIME™ (Polyplus-transfection, Inc., France) in 48-well culture dishes with 250 ng of SREBP1c promoter reporter plasmids (pGL4.10-SREBP1c) with or without 250 ng of the pcDNA3.1/myc-His(−)-HCBP6 plasmid. Renilla luciferase vector (pRL-TK) plasmid served as control. Twenty four hours after transfection, cells were lysed in the passive lysis buffer (E1941, Promega). SREBP1c promoter luciferase activity was measured in a microplate luminometer according to the protocol for the Dual-Luciferase Reporter Assay System (E1910, Promega).

Transfection assays were performed using jetPRIME™ (Polyplus-transfection, Inc.) in 48-well culture dishes with 250 ng of wild-type (HCBP6-wt) or mutant-type reporter plasmids (HCBP6-mut) with 100 mM of miR-122 mimics. Forty eight hours after transfection, cells were lysed in the passive lysis buffer (E1941, Promega). HCBP6-3′UTR luciferase activity were measured in a microplate luminometer according to the protocol for the Dual-Luciferase Reporter Assay System (E1910, Promega).

STATISTICAL ANALYSIS

Each experiment was performed in triplicate. Statistic differences were determined using the paired Student's t test. All data are presented as the mean ± standard error of the mean (SEM). Statistical significance was defined as $P < 0.05$.

RESULTS

HCBP6 PROTEIN DECREASES TG ACCUMULATION

To examine the potential role of HCBP6 in TG accumulation, we used gain and/or loss of function studies to assess the effects on intracellular TG content. HepG2, Huh7 hematoma cell lines and L02 hepatocyte cell lines were transiently transfected with the pcDNA3.1/myc-His(−)-HCBP6 overexpression plasmid or the control vector pcDNA3.1/myc-His(−). Western blotting confirmed successful HCBP6 protein overexpression in HepG2, Huh7 and L02 cell lines (Fig. 1A–C). Interestingly, intracellular TG content was significantly decreased upon HCBP6 overexpression compared with the control group (Fig. 1D). Next, we successfully silenced endogenous HCBP6 expression in HepG2, Huh7, and L02 cells using HCBP6 siRNA (Fig. 1E–G). Notably, intracellular TG content was significantly increased upon HCBP6 knockdown (Fig. 1H). Taken together, these results strongly indicated that HCBP6 expression modulates intracellular TG biosynthesis.

TIME- AND DOSE-DEPENDENT INDUCTION OF HCBP6 BY TG

We used a lipid-overload hepatic steatosis cellular model to examine whether intracellular TG levels influences HCBP6 expression. Intracellular TG content and HCBP6 mRNA and protein levels were measured after HepG2 cells were incubated with 0.25, 0.5, 1.0, and 2.0 mM of oleate and palmitate at a 2:1 ratio (FFA) for 14, 24, and 36 h (Fig. 2 and Supplementary Fig. S2A–O). As expected, a clear dose- and time-dependent increase in intracellular TG accumulation was observed after exposure to FFA, with the most effective time and dose being 24 h and 0.5 mM, respectively (Fig. 2A and B). Similarly, HCBP6 mRNA and protein levels both exhibited time- and dose-dependent increases after lipid treatment (Fig. 2C–F). Then we incubated L02 hepatocyte cell line with 0.25, 0.5, 1.0, and 2.0 mM of oleate and palmitate at a 2:1 ratio (FFA) for 24 h. And L02 cells were incubated with 0.5 mM of oleate and palmitate at a 2:1 ratio (FFA) for 14, 24, and 36 h. We observed similar results (Fig. 2G–L).

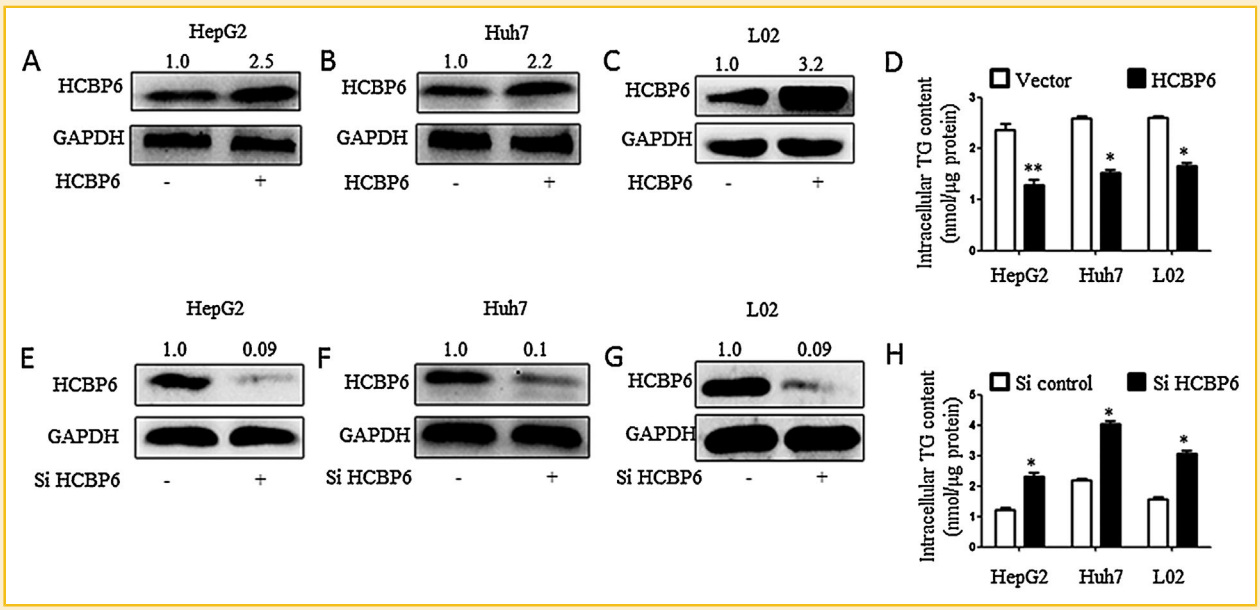

Fig. 1. HCBP6 decreases TG accumulation. HCBP6 protein was successfully overexpressed in HepG2, Huh7, and L02 cells transiently transfected with pcDNA3.1/myc-His (−)-HCBP6 (A–C). After 48 h of transfection with an HBCP6-expressing vector, intracellular TG levels were significantly decreased in these cell lines (D). Data presented are means ± SD of three independent experiments (n = 3, $^{*}P < 0.05$, $^{**}P < 0.005$). On the other hand, after successfully knocking down HCBP6 gene expression by siRNA (E, F, G), intracellular TG levels were significantly increased in these cell lines (H). Data shown are means ± SD of three independent experiments (n = 3, $^{*}P < 0.05$).

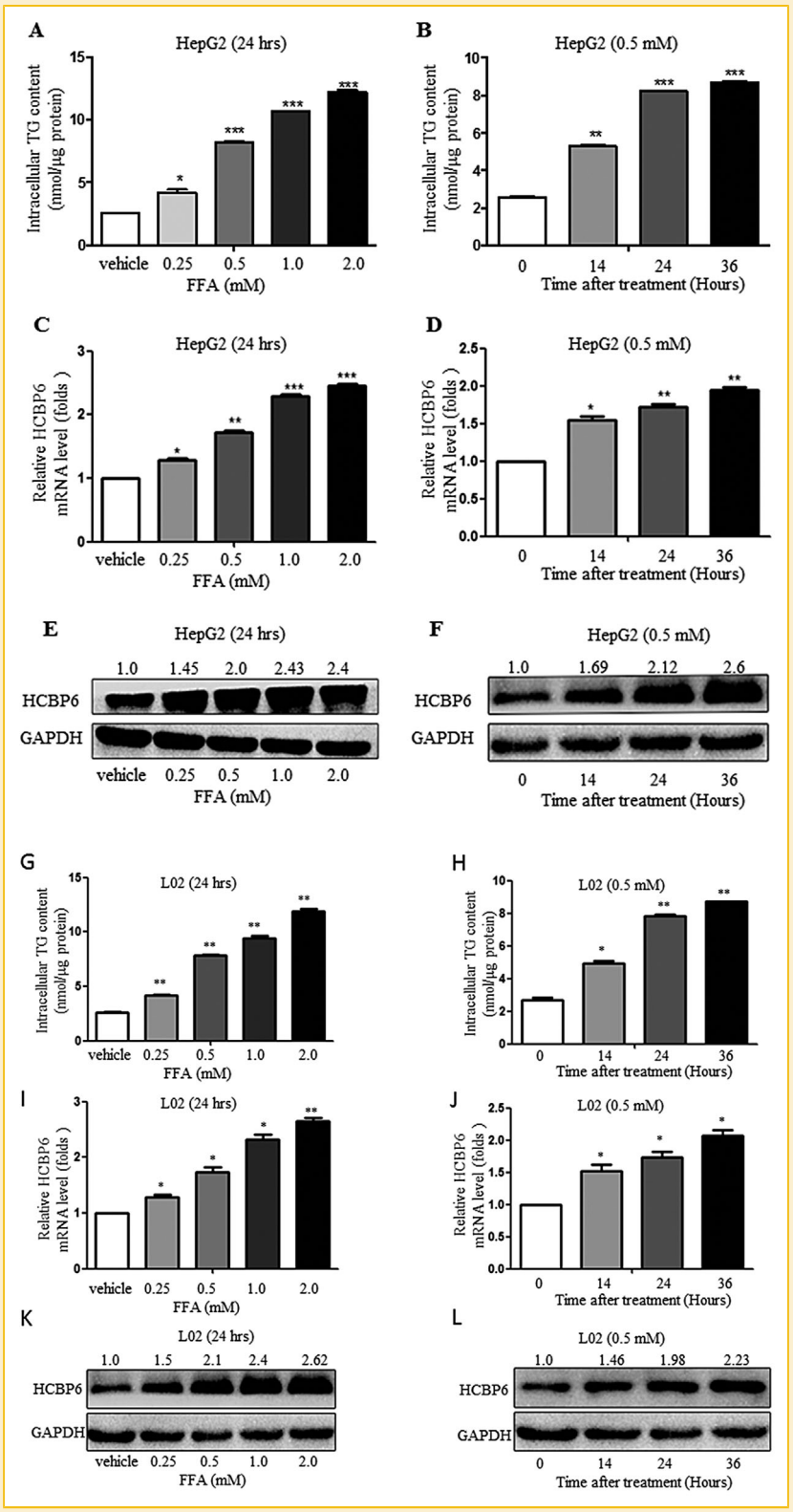

Fig. 2. TG increases HCBP6 expression in a time- and dose-dependent manner. To generate a cellular model of lipid-overload hepatic steatosis, HepG2, and L02 cells were incubated with 0.25, 0.5, 1.0, 1.5, and 2.0 mM of oleate and palmitate at a 2:1 ratio (FFA). The intracellular TG content exhibited a dose-dependent increased 24 h after lipid incubation (A, G). In parallel, HCBP6 mRNA and protein expression also increased in a dose-dependent manner (C, E, I, K, respectively). Upon treatment with 0.5 mM lipid, the intracellular TG content and HCBP6 mRNA and protein expression all increased in a time-dependent manner (B, D, F, H, J, L). Data shown are means ± SD of three independent experiments ($n = 3$, $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$).

These results clearly indicate that intracellular lipid accumulation affects HCBP6 expression.

HCBP6 DECREASES SREBP1c AND FASN EXPRESSION

Next, we investigated the potential regulatory mechanism of HCBP6 during TG accumulation. To this end, we screened fifteen genes that are involved in TG and cholesterol biosynthesis and metabolism, including SREBP1c, FASN, acetyl-CoA carboxylase (ACC), stearoyl-CoA desaturase (SCD), GPAT, SREBP2 and 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), following HCBP6 overexpression in HepG2 cells (Supplementary Fig. S1A). SRBEP1c and FASN expression were dramatically reduced after HCBP6 overexpression (Supplementary Fig. S1A). SREBP1c and FASN are involved in TG synthesis. Therefore, we focused on these two target genes in the present study. We also found HCBP6 can down-regulate SREBP2 and HMGCR, the detailed mechanism will be published in another paper. SREBP1c and FASN mRNA expression was significantly reduced after HCBP6 overexpression compared with the empty vector control (Fig. 3A and B). In parallel, premature/mature SREBP1c (pSREBP1c/mSREBP1c) and FASN protein expression was significantly reduced after HCBP6 over-expression (Fig. 3C and D). Conversely, knocking down HCBP6 expression led to the up-regulation of both SREBP1c and FASN mRNA and protein expression (Fig. 3E and H). Taken together, these results indicate that HCBP6 may directly regulate SREBP1c and FASN expression.

HCBP6 DECREASES FASN EXPRESSION AND TG ACCUMULATION IN A SREBP1c-DEPENDENT MANNER

To investigate whether HCBP6 down-regulates FASN directly or via a SREBP1c-dependent manner, we analyzed HCBP6, SREBP1c and FASN mRNA and protein expression in hepatic cell lines over-expressing both HCBP6 and SREBP1c. Interestingly, the HCBP6-induced down-regulation of FASN is rescued by SREBP1c over-expression (Fig. 4A–D). Consistent with this observation, decreased intracellular TG content caused by HCBP6 overexpression is also rescued by SREBP1c overexpression (Fig. 4J and K). Then HCBP6 were overexpressed in SREBP1c-silenced HepG2 and L02 cells, intracellular TG content and HCBP6, SREBP1c, FASN mRNA and protein expression were analyzed. SREBP1c siRNA significantly shielded the HCBP6-induced down-regulation of FASN (Fig. 4E–H). Consistent with this decreased intracellular TG content caused by HCBP6 overexpression is also shielded by SREBP1c siRNA. These results indicated that HCBP6 directly regulates intracellular TG content by down-regulating SREBP1c-dependent FASN expression.

HCBP6 DECREASED SREBP1c AT THE TRANSCRIPTIONAL LEVEL

We determined whether HCBP6 directly regulates the transcriptional activity of the SREBP1c promoter. To test this hypothesis, we cloned the SREBP1c promoter region (nt-1005 to +24) into a pGL4.10 vector and investigated whether HCBP6 affects promoter activity. Compared to pGL4.10-basic vector, pGL4.10-SRBEP1c has promoter activity (Fig. 5A). HCBP6 overexpression significantly decreased

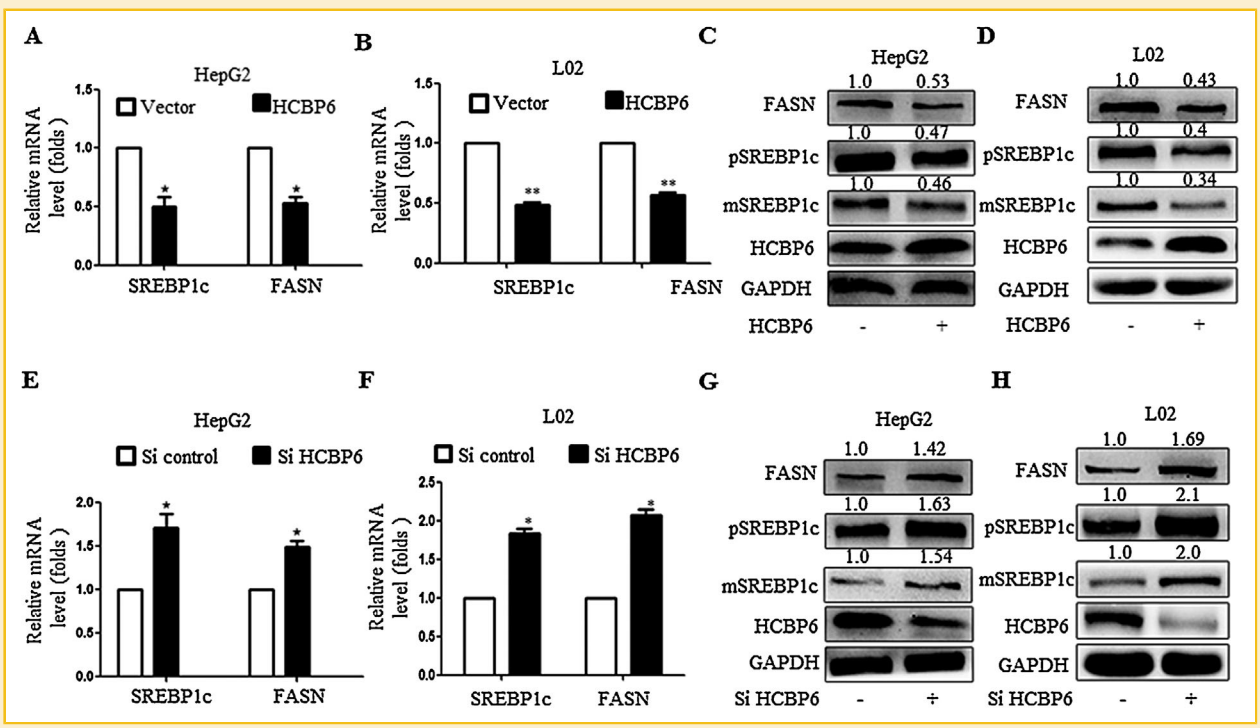

Fig. 3. HCBP6 down-regulates SREBP1c and FASN expression. SREBP1c and FASN mRNA and protein expression were significantly down-regulated after HCBP6 overexpression (A–D) and significantly up-regulated after HCBP6 siRNA treatment (E–H) in HepG2 (A, C, E, G) and L02 cells (B, D, F, H). Data shown are means ± SD of three independent experiments ($n = 3$, $^{*}P < 0.05$, $^{**}P < 0.005$).

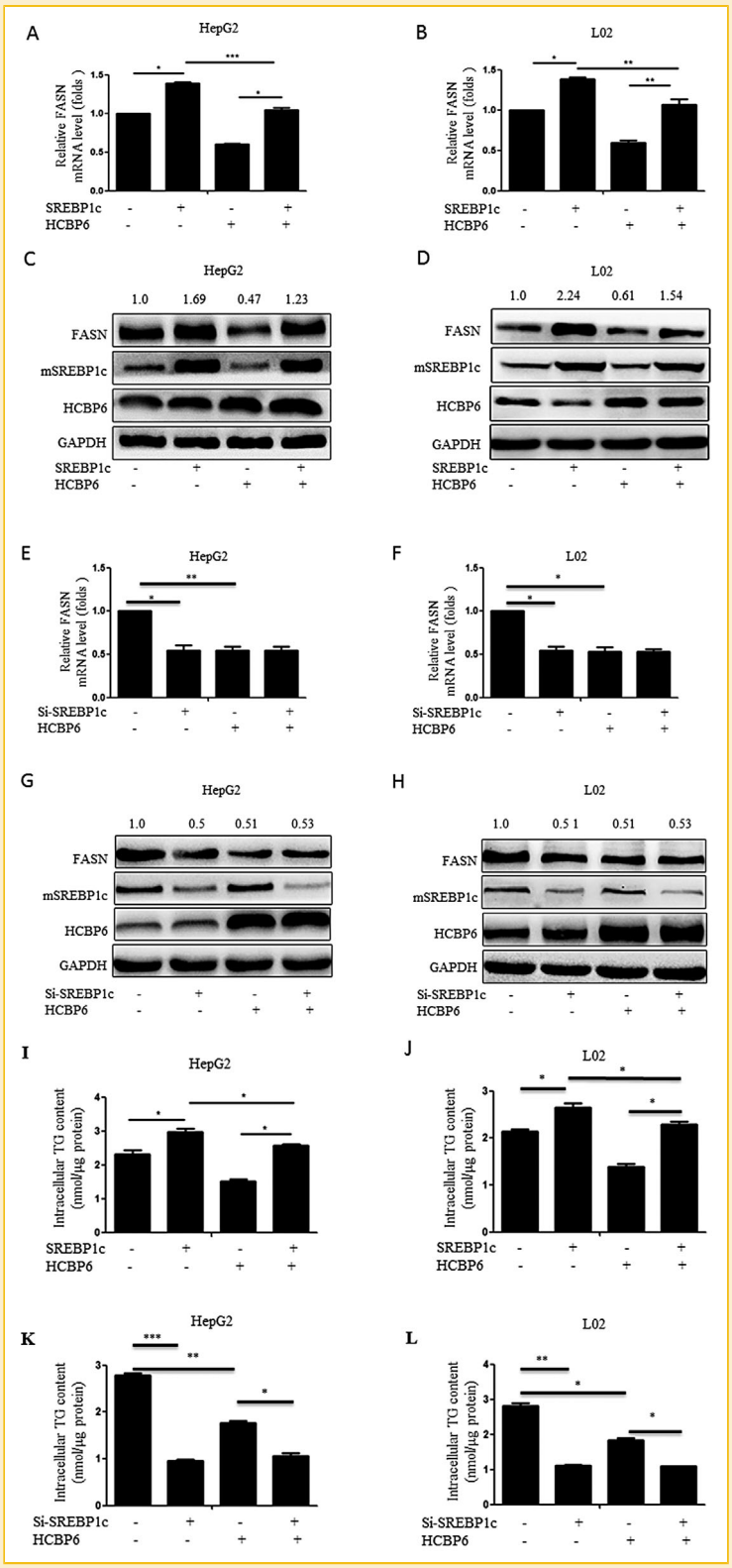

Fig. 4. HCBP6 decreases FASN expression and TG accumulation in a SREBP1c-dependent manner. HCBP6-induced down-regulation of FASN mRNA and protein expression is rescued by SREBP1c overexpression (A–D). Similarly, the decrease in intracellular TG content mediated by HCBP6 overexpression is rescued by SREBP1c overexpression (I, J). HCBP6-induced down-regulation of FASN mRNA and protein expression is shielded by SREBP1c siRNA (E–H). The decrease in intracellular TG content mediated by HCBP6 overexpression is shielded by SREBP1c siRNA (K, L). Data shown are means ± SD of three independent experiments ($n = 3$, $^*P < 0.05$).

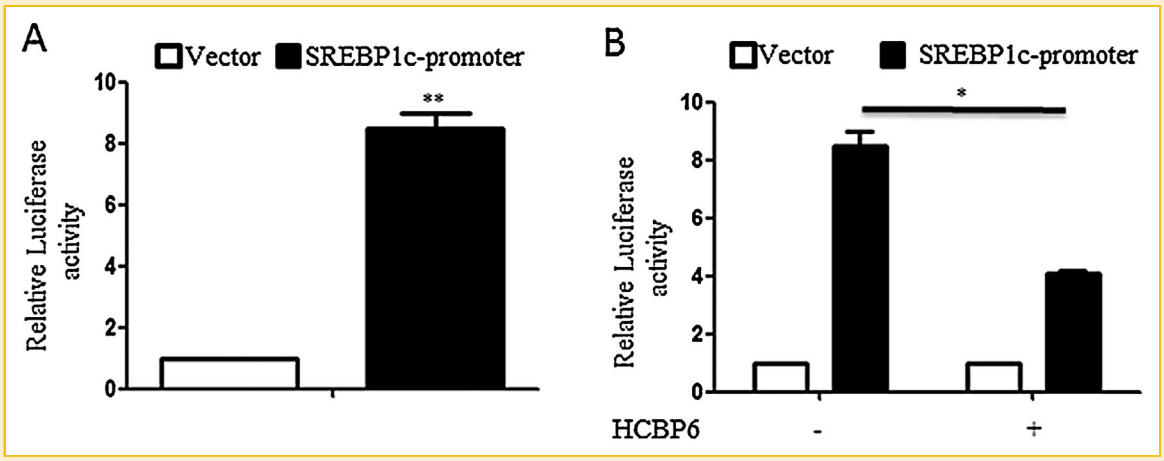

Fig. 5. HCBP6 decreased SREBP1c at the transcriptional level. Compared to pGL4.10-basic, pGL4.10-SREBP1c-promoter has promoter activity (A). HepG2 cells were co-transfected with pGL4.10-SREBP1c-promoter and pcDNA3.1/myc-His(−)-HCBP6. SREBP1c promoter activity was determined at 24 h after co-transfection (B). Data shown are means ± SD of three independent experiments ($n = 3$, $^*P < 0.05$).

SREBP1c promoter activity compared with the empty vector (Fig. 5B). Collectively, these results indicate that SREBP1c expression is primarily regulated by HCBP6 at the transcriptional level.

miR-122 DIRECTLY REGULATES HCBP6 EXPRESSION

miR-122 plays important roles in lipid metabolism. No direct target genes in lipid metabolism have thus far been reported, although previous studies indicated that HCBP6 may be a target of miR-122 [Tsai et al., 2009; Androsavich and Chau, 2014]. To determine whether HCBP6 is a direct target of miR-122 in lipid metabolism, we performed bidirectional manipulation of miR-122 expression by overexpressing a miR-122 mimic or a miR-122 inhibitor. Compared with the control group, the miR-122 mimic significantly reduced HCBP6 mRNA and protein levels (Fig. 6A and C), whereas inhibiting miR-122 expression significantly increased HCBP6 mRNA and protein expression (Fig. 6B and D). SREBP1c and FASN mRNA and protein expression were significantly increased after treatment with the miR-122 mimic (Fig. 6A and C). In contrast, SREBP1c and FASN mRNA and protein expression were significantly reduced after miR-122 inhibitor treatment (Fig. 6B and D). Treatment with miR-122 mimic significantly reduced the activity of firefly luciferase with the wild-type but not mutant 3′-UTR of HCBP6 (Fig. 6E and F). These results strongly indicate that HCBP6 is a direct target of miR-122.

DISCUSSION

In the current study, we demonstrated that HCBP6 modulates TG homeostasis via SREBP1c/FASN regulation in hepatocytes. HCBP6, which is overexpressed by lipid overload, directly regulates intracellular TG content by down-regulating SREBP1c-dependent FASN expression in HepG2, Huh7, and L02 cells. Furthermore, we also demonstrated that miR-122 participates in TG metabolism by inhibiting both HCBP6 mRNA and protein expression. To our knowledge, this is the first report to demonstrate that HCBP6 plays an important role in intracellular lipid sensing and regulation.

HCBP6 was initially identified from yeast two-hybrid assay screens of liver cDNA libraries as a HCV core-binding protein in our laboratory [Li et al., 2002]. Our previous studies demonstrated that HCBP6 regulates the transcriptional activity of the alpha chain of nascent polypeptide-associated complex (NACA) promoter [Yang et al., 2003] and the expression of genes involved in cell proliferation, signal transduction, growth, and differentiation [Wang et al., 2006]. In addition, HCBP6 inhibits HCV core protein transactivation effects [Liu et al., 2004]. Numerous studies suggest that the HCV core protein plays an important role in HCV-induced liver steatosis [Barba et al., 1997], suggesting that HCBP6, as an HCV core-binding protein, impacts lipid metabolism. Here, we provide evidence that HCBP6 functions as a sensor of intrahepatocyte TG levels. We demonstrate that bidirectional manipulation of HCBP6 gene expression by knockdown or overexpression decreased or increased intracellular TG content, respectively. These results imply that HCBP6 is an important regulator of TG synthesis.

Furthermore, we investigated whether intracellular TG content influences HCBP6 expression. Indeed, HCBP6 expression was largely dependent on FFA accumulation in a cellular model of hepatic steatosis, consistent with a previous report [Gomez-Lechon et al., 2007]. These results revealed a clear time-dependent increase in intracellular TG accumulation with 0.5 mM FFA in HepG2 and L02 cells. Interestingly, HCBP6 mRNA and protein levels also exhibited a notable time-dependent increase. HepG2 and L02 cells incubated for 24 h exhibited a clear dose-dependent accumulation of intracellular TG. Notably, HCBP6 mRNA and protein levels also exhibited clear dose-dependent increases. These results indicate that HCBP6, which increased in parallel with TG content, senses alterations in intracellular TG.

The HCV core protein induces liver steatosis through multiple mechanisms, including the induction of insulin resistance [Shintani et al., 2004], the reduction of VLDL secretion [Perlemuter et al., 2002],

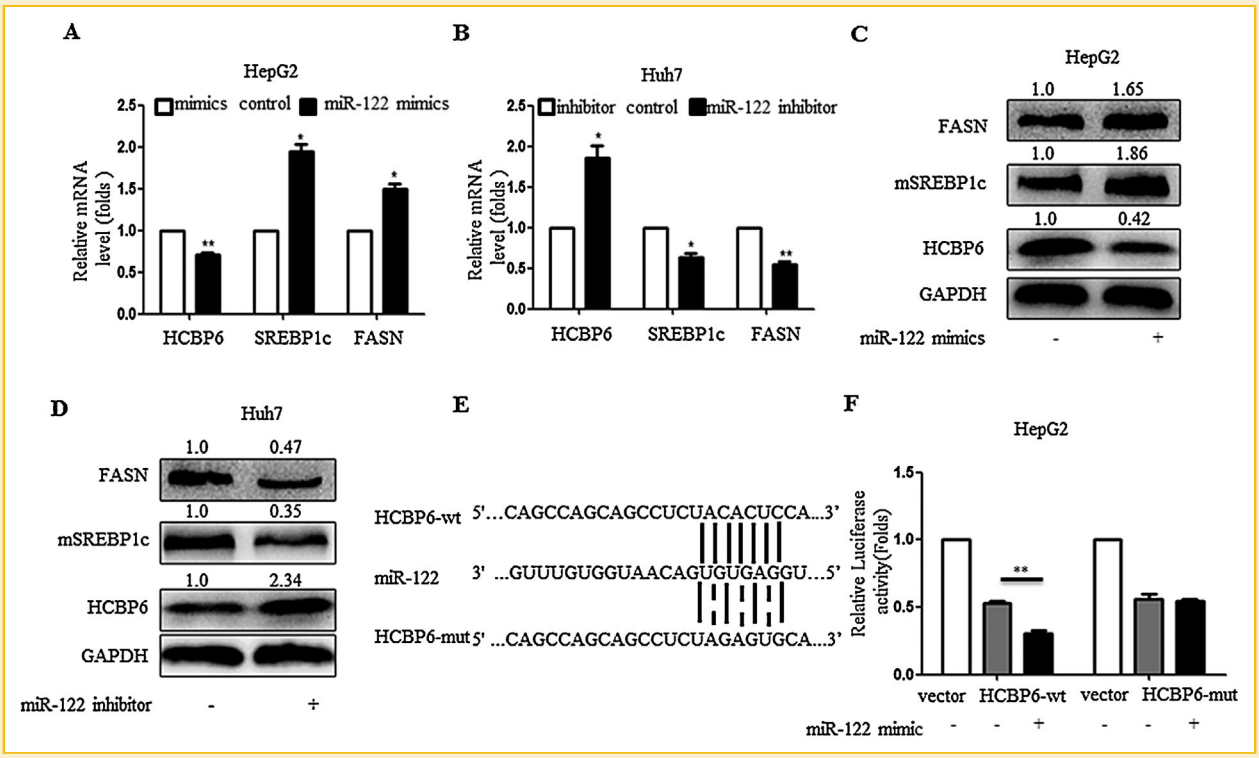

Fig. 6. HCBP6 is involved in the regulation of lipid metabolism mediated by miR-122s. HepG2 and Huh7 cells were transiently transfected with miR-122 mimics or controls (A, B). SREBP1c, FASN and HCBP6 mRNA expression was quantified by qPCR using GAPDH as an internal control. Cell lysates were analyzed for SREBP1c, FASN, HCBP6 and GAPDH expression by Western blot (C, D). The predicted miR-122 binding site located in the 3′-UTR of HCBP6 mRNA. Mutations are made to the seed region of the miR-122 binding site for the reporter gene assay (E). HepG2 cells were co-transfected with miR-122 mimics or controls and wild type pmir-GLO-HCBP6-3′UTR (HCBP6-wt) or mutation-type pmir-GLO-HCBP6-3′UTR (HCBP6-mut) or pmir-GLO-basic (vector), HCBP6-3′UTR activity were detected at 48 h after co-transfected (F). Data shown are means ± SD of three independent experiments ($n = 3$, $^{*}P < 0.05$, $^{**}P < 0.005$).

the inhibition of fatty acid oxidation [Dharancy et al., 2005], and increased fatty acid synthesis [Moriishi et al., 2007]. Numerous genes have been implicated in these processes, including SREBP1c, FASN, LXR, PCG-1, SIRT1, LDLR, PA28-γ, PPARα, and FOXOL-1. Among these genes, we found that SREBP1c and FASN mRNA and protein levels were significantly reduced upon HCBP6 overexpression (Supplementary Fig. S1A and Fig. 3). These results again imply that HCBP6 impacts TG metabolism through the regulation of SREBP1c and FASN, which are involved in fatty acid and TG synthesis.

The SREBP pathway had been delineated by the work of Brown and Goldstein [2009] [Goldstein et al., 2006]. Three mammalian SREBP isoforms (SREBP-1a, SREBP-1c, and SREBP-2) are encoded by two genes, SREBF1 and SREBF2, and have distinct but overlapping function in lipogenic gene regulation. SREBP-1a activates fatty acid and cholesterol synthesis. SREBP-1c activates fatty acid and TG synthesis, and SREBP-2 activates cholesterol synthesis and uptake [Goldstein et al., 2006]. SREBP1c increases the transcriptional activity of genes involved in hepatic fatty acid synthesis (including FASN, ACC, and SCD) [Shimano et al., 1997]. Alterations to the SREBP1c-FASN pathway can result in liver steatosis and type 2 diabetes [Horton et al., 2002]. Based on our results demonstrating that HCBP6 down-regulates the SREBP1c-FASN pathway, we hypothesize that HCBP6 is involved in liver steatosis and type 2 diabetes.

In addition to lipid metabolism, a growing body of evidence suggests that SREBP pathways play important roles in cancer, neuroprotection, autophagy (mainly SREBP2), and immune function (mainly SREBP1a) [Shao and Espenshade, 2012]. Numerous studies have observed accelerated TG synthesis in various tumor tissues, including lung, breast, colon, prostate, and liver [Yahagi et al., 2005], suggesting that increased de novo lipid synthesis is a hallmark of cancer [Krycer et al., 2010]. Although the precise mechanisms are not fully understood, the increased expression of lipogenic enzymes during cancer development is thought to be mediated at least in part by the activation of the SREBP pathway. A previous study found that SREBP1c may provide a link between lipid synthesis, proliferation, and cell growth [Bengoechea-Alonso et al., 2005]. Considering these findings, it is possible that HCBP6-mediated regulation of SREBP1c and TG synthesis plays a protective role in the process of cancer development; however, further studies are needed to clarify this issue. The overactivation of N-methyl-D-aspartate receptors (NMDARs) can cause neuronal excitotoxicity after stroke and brain trauma, and the activation of SREBP-1 is an essential step in NMDAR-mediated excitotoxic neuronal death [Taghibiglou et al., 2009]. Given that HCBP6 decreases SREBP1c expression, HCBP6 might also be involved in neuroprotection.

SREBP transcription factors are regulated at three major levels: (1) transcriptionally; (2) via the proteolytic cleavage of SREBP

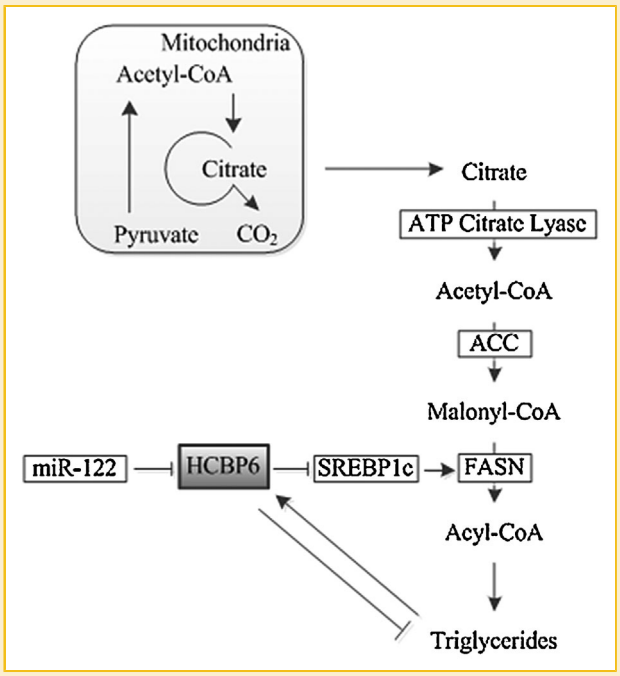

Fig. 7. Schematic image. HCBP6, a direct target of miR-122, functions as a sensor for intracellular TG content via the SREBP1c-dependent regulation of FASN expression.

precursors; and (3) via post-translational modification of nSREBPs [Eberle et al., 2004]. Numerous studies have demonstrated that SREBP-1c is mainly regulated at the transcriptional level [Eberl et al., 2004; Nakakuki et al., 2014]. Therefore, we asked whether HCBP6 down-regulates SREBP1c at the transcriptional level. Indeed, transcriptional analyses revealed that HCBP6 overexpression decreased SREBP1c promoter activity. Does HCBP6 modulates transcription directly or indirectly? We found that HCBP6 protein exclusively co-localized in cytoplasma with mitochondria in HepG2 cells (Supplementary Fig. S3). We conjecture HCBP6 may decrease SREBP1c promoter activity through other transcription factor. While which is the particular transcription factor? And how is the detail mechanism? This will be our major job on the next step.

miRNAs have also recently received substantial attention, and increasing evidence suggests that they play important roles in lipid metabolism [Novak et al., 2014]. As a liver specific miRNA, miR-122 plays an important role in hepatocyte differentiation [Lagos-Quintana et al., 2002] by enhancing the replication of HCV [Jopling et al., 2005], inhibiting the replication of HBV [Wang et al., 2012], and suppressing hepatocellular carcinoma [Tsai et al., 2009]. miR-122 increases hepatic sterol, TG, and fatty acid synthesis through the increased expression of various lipid metabolic genes, including SREBP1c, FASN, ACC, SCD, SREBP2, and HMGCR [Krutzfeldt et al., 2005; Esau et al., 2006]. miRNAs participate in gene regulation mainly through miRNA-directed translational repression and mRNA destabilization [Bartel, 2009]. In the current study, we demonstrated that miR-122 down-regulates HCBP6 and HCBP6 simultaneously up-regulates genes involved in lipid metabolism. Consistent with these observations, we demonstrated that miR-122 overexpression elicited more robust translational repression of luciferase activity driven by the full 3′-UTR of HCBP6. These results indicate that HCBP6 is the direct target of miR-122.

In conclusion, we demonstrate that miR-122-mediated HCBP6 protein expression is involved in sensing TG levels and down-regulating SREBP1c/FASN expression in hepatocytes, thereby modulating intracellular TG synthesis (Fig. 7). Thus, our results suggest that HCBP6 represents a new potential therapeutic target in lipid metabolism.

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