HCBP6 upregulates human SREBP1c expression by binding to C/EBPβ-binding site in the SREBP1c promoter

Xueliang Yang1,8, Ming Han2,3,9, Shunai Liu3,4, Xiaoxue Yuan3,4, Xiaojing Liu, Shenghu Feng2,3, Li Zhou2,9, Yaru Li3,9, Hongping Lu3,4, Jun Cheng2,3,8,* & Shumei Lin1,*

1Department of Infectious Diseases, the First Affiliated Hospital of Xi’an Jiaotong University, Xi’an 710061, 2Peking University Ditan Teaching Hospital, Beijing 100015, 3Beijing Key Laboratory of Emerging Infectious Diseases, Beijing 100015, 4Insititute of Infectious Diseases, Beijing Ditan Hospital, Capital Medical University, Beijing 100015, China

Supplementary Table 1 PCR primers, siRNA oligonucleotides and EMSA probes

| Gene name | Sense (5‘-3’) | Anti-sense (5‘-3’) |
|-----------|---------------|-------------------|
| expression plasmid primers | | |
| pcDNA-HCBP6 | GAATTCATGGAAACATCTGCCC GGT | AAGCTTTGCTTCTCCACTCGTTGCC |
| pcDNA-C/EBPβ | GAATTCAATGGCAGCGGCTTCCCG TA | AAGCTTGCAATGGCCAGGAGAGCAG |
| qRT-PCR Primers | | |
| HCBP6 | AGACAAGCTCACCGGAAATGG | CTGGGTGCCCCAGCTATACT |
| SREBP1c | GGAGGGGTAGGGCCAACGGCTTT | CATGTCTTCGAAAGTGTTCAT |
| ACC1 | CCCCTTCTGGGCTTCCAGAG | GGCACAGGAGATGTTCAT |
| FASN | AGCTGCCAGAGTCGGAGAAC | TGTAGCCCACGAGTGTTCG |
| si RNA oligonucleotides | | |
| si-HCBP6 | CUGGGUAACUCAAGAAGUTG | UCAACUUUGAUGUACCGAT |
| si-C/EBPβ | GGCCCUGAGUAAUCGUUATT | UAAAGGAUGUACCGAGCG |
| si-NC | UUUCGUACGUGUGCACGUTT | AGCUGACAGUACCGAGAAT |
| wild SREBP1c promoter cloning primers | | |
| SREBP1c-P | CTCGAGCTAGTCCCCAGCCTGGAT GATC | GATATCCCCTCGTACGGCCTTCTG |
| SREBP1c-P1 | CTCGAGCTAGTCCCCAGCCTGGAT | GATATCCCCTCGTACGGCCTTCTG |
| SREBP1c-P2 | CTCGAGCTAGTCCCCAGCCTGGAT | GATATCCCCTCGTACGGCCTTCTG |
| SREBP1c-P3 | CTCGAGCTAGTCCCCAGCCTGGAT | GATATCCCCTCGTACGGCCTTCTG |
| SREBP1c-P4 | CTCGAGCTAGTCCCCAGCCTGGAT | GATATCCCCTCGTACGGCCTTCTG |
| SREBP1c-P5 | CTCGAGCTAGTCCCCAGCCTGGAT | GATATCCCCTCGTACGGCCTTCTG |
**Supplementary MATERIALS AND METHODS**

**Cell culture, transient transfection, and RNA interference**

HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified chamber with an atmosphere of 5% CO₂. The cells were seeded in different wells and were grown to 80% confluence. Next, the cells were transiently transfected with different plasmids, si-**HCBP6** or si-NC by using jetPRIME (Polyplus Transfection, Strasbourg, France), according to the manufacturer’s instructions and a protocol described in our previous study (Supplementary Table 1) (14).
Cloning of SREBP1c promoter fragments and construction of expression plasmids

Genomic DNA was isolated from HepG2 cells by using Genomic DNA Purification Kit (Promega, USA). Next, a series of 5′-flanking DNA fragments upstream of the transcription initiation site in the SREBP1c promoter (P [-636 to +359 bp], P1 [-636 to -140 bp], P2 [-139 to +359 bp], P3 [-637 to -389 bp], P4 [-390 to -150 bp], P5 [-151 to +98 bp], and P6 [+99 to +359 bp]) were inserted into XhoI and EcoRV sites in the pGL4.10-Basic vector (Promega). PCR primers used in this study are listed in Supplementary Table 1. The pcDNA-HCBP6 and pcDNA-C/EBPβ plasmids were constructed as described previously (Supplementary Table 1) (15) (16). PCR was performed using the following thermocycler conditions: initial denaturation at 94°C for 7 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 1 min, and extension at 72°C for 1 min; and final extension at 72°C for 10 min.

Measurement of total intracellular TG levels

HepG2 cells were transfected with the indicated plasmids or siRNAs. Intracellular TG content was measured using an Adipogenesis Assay Kit (Sigma, USA) after 48 h of transfection, according to the manufacturer’s instructions, and was normalized to the total protein content.

Oil red O staining

After 24 h of cell transfected, HepG2 cells were treated with oleic acid (0.25 mmol/L) for 24 h. After washing with PBS 0.1M pH 7.4, cell cultures were fixed for 20 min with 4% formalin in PBS 0.05M. Cell stained with a filtered 0.35% Oil Red O solution in 60%
isopropanol for 10 min at room temperature. Then, cells were washed with 60% isopropanol for 30 sec and stained with Mayer’s Hematoxylin Bio-Optica ready to use solution for 1 min at room temperature and washed again with PBS. Slides were treated with Dako faramount aqueous mounting medium ready to use and then was applied to the coverslip.

**Total RNA isolation, reverse transcription, and qRT-PCR**

Total RNA was extracted from HepG2 cells by using a total RNA extraction kit (Omega, USA), according to the manufacturer’s instructions. The total RNA obtained was reversed transcribed using PrimeScript1 RT Reagent Kit (TaKaRa, Japan). Next, qRT-PCR was performed using LightCycler Fast Start DNA Master SYBR Green Kit (ABI, USA). Relative mRNA expression was determined using comparative threshold method and was normalized using the mRNA expression of the endogenous control β-actin. Primers used for performing qRT-PCR are listed in Supplementary Table 1.

**Western blotting analysis**

HepG2 cells were lysed using a lysis buffer (Thermo Fisher Scientific, USA) containing a protease inhibitor cocktail (Roche, Switzerland) for 30 min on ice. Protein concentration of the cell lysates was determined using Pierce BCA assay (Thermo Fisher Scientific). Equal amounts of proteins in each sample were resolved by performing SDS-PAGE on a 10% gel (Invitrogen, California, USA) and were transferred onto PVDF membranes (Roche). The membranes were incubated overnight at 4°C with primary antibodies against SREBP1c (Santa Cruz Biotechnology, California, USA),
HCBP6 (Abcam, Cambridge, USA), FASN (Abcam), ACC1 (Cell Signaling Technology, Boston, USA), and phosphorylated ACC1 (p-ACC1) (Cell Signaling Technology), followed by incubation with anti-rabbit or anti-mouse secondary antibodies (ZSGB-Bio, Beijing, China) for 1 h at room temperature. Protein bands obtained were visualized using an enhanced chemiluminescence system (Thermo Fisher Scientific) and were analyzed using Bio1D software (Vilber, Paris, France).

Site-directed mutagenesis

Mutations in the binding sites of different transcription factors in the P2 fragment of the SREBP1c promoter were induced by performing site-directed mutagenesis with Fast Mutagenesis Kit (TransGen Biotech, Beijing, China.). Sequences of sense primers used to generate site-directed mutations are listed in Supplementary Table 1. We mutated the NF-κB-binding site at -75 to -72 bp, the C/EBPβ-binding site at -109 to -106 bp, the E-box-binding site at +31 to +34 bp, the Sp1-binding site at +139 to +142 bp, and the GATA-binding site at +152 to +155 bp in the SREBP1c promoter by using the wild-type P2 promoter fragment (P2) as the template.

Luciferase reporter assay

HepG2 cells were seeded in a 48-well culture plate (density, 5~8 × 10^4 cells/well). For determining the basal activity of the SREBP1c promoter, the cells were transfected with 0.24 μg vectors containing the different SREBP1c promoter derivatives or the pGL4.10-Basic vector. For determining the activity of the SREBP1c promoter after HCBP6 overexpression, the cells were transfected with 60 ng vectors containing the
different SREBP1c promoter derivatives or the pGL4.10-Basic vector with or without 180 ng pcDNA-HCBP6 plasmid. In addition, the cells were cotransfected with 8 ng Renilla luciferase vector (pRL-TK), which served as an internal control. Luciferase activity was measured after 24 h of transfection by using a microplate luminometer (Dual-Luciferase Reporter Assay System; Promega), according to the manufacturer’s protocol.

**Electrophoretic mobility shift assay**

For performing EMSA, double-strand DNA oligonucleotides were labeled with biotin at the 3’ end (Invitrogen, Shanghai). Nuclear extracts were prepared using a Nuclear Extraction Kit (Pierce, USA), and protein content in the nuclear extracts was measured using the BCA protein assay kit, according to the manufacturer’s protocol. EMSA was performed using LightShift chemiluminescent EMSA kit (Thermo Fisher Scientific). Oligonucleotide probes used for performing EMSA are listed in Supplementary Table 1. The cells were transiently transfected with different plasmids, such as pcDNA-HCBP6, pcDNA-C/EBPβ, si-HCBP6 or si-C/EBPβ. Nuclear extracts containing 6 μg proteins were incubated with the oligonucleotide probes for 20 min at 25°C. For performing competition experiments, the nuclear extracts were incubated with 100-fold excess of unlabeled probe for 10 min before adding biotin-labeled probes to the reaction mixture. DNA-protein complexes were separated by performing electrophoresis on a 6.5% non-denaturing polyacrylamide gel.

**Chromatin immunoprecipitation**
ChIP was performed using HepG2 cells (density, $1 \times 10^6$ cells). Chromatin isolation and ChIP assay were performed using EZ-Zyme Chromatin prep kit and EZ-ChIP kit (Millipore, USA), respectively. Chromatin solution was immunoprecipitated using 5 μg anti-His antibody (Abcam), anti-C/EBPβ (Abcam) antibody, or normal anti-IgG antibody, followed by overnight incubation with 20 μl protein A agarose beads at 4°C. Next, the beads were washed multiple times, and antibody-protein-DNA complexes were eluted. Protein and RNA were removed by treatment with proteinase K and RNase, respectively. Next, qPCR was performed using the immunoprecipitated genomic DNA and primers specific for the C/EBPβ-binding site upstream of the transcription start site in the SREBP1c promoter. Primers used in this assay are listed in Supplementary Table 1. PCR products obtained were electrophoresed on a 1% agarose gel.

**Statistical analysis**

All data are expressed as mean ± SEM by using SPSS software version 16.0 (SPSS, Chicago, USA). Statistical significance was determined using analysis of variance (ANOVA), and individual comparisons were made using Student’s $t$-test with GraphPad Prism6 (GraphPad Software Inc., La Jolla, USA). $P < 0.05$ was considered statistically significant.