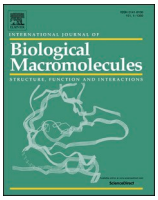
International Journal of Biological Macromolecules 256 (2024) 128414

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac



HBP1 promotes chicken preadipocyte proliferation via directly repressing *SOCS3* transcription

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ARTICLE INFO

Keywords:
Chicken
HBP1
SOCS3
ChIP-seq
Preadipocytes proliferation

ABSTRACT

Preadipocyte proliferation is an essential process in adipose development. During proliferation of preadipocytes, transcription factors play crucial roles. HMG-box protein 1 (HBP1) is an important transcription factor of cellular proliferation. However, the function and underlying mechanisms of HBP1 in the proliferation of preadipocytes remain unclear. Here, we found that the expression level of HBP1 decreased first and then increased during the proliferation of chicken preadipocytes. Knockout of HBP1 could inhibit the proliferation of preadipocytes, while overexpression of HBP1 could promote the proliferation of preadipocytes. ChIP-seq data showed that HBP1 had the unique DNA binding motif in chicken preadipocytes. By integrating ChIP-Seq and RNA-Seq, we revealed a total of 3 candidate target genes of HBP1. Furthermore, the results of ChIP-qPCR, RT-qPCR, luciferase reporter assay and EMSA showed that HBP1 could inhibit the transcription of suppressor of cytokine signaling 3 (*SOCS3*) by binding to its promoter. Moreover, we confirmed that SOCS3 can mediate the regulation of HBP1 on the proliferation of preadipocytes through RNAi and rescue experiments. Altogether, these data demonstrated that HBP1 directly targets *SOCS3* to regulate chicken preadipocyte proliferation. Our findings expand the transcriptional regulatory network of preadipocyte proliferation, and they will be helpful in formulating a molecular breeding scheme to control excessive abdominal fat deposition and to improve meat quality in chickens.

1. Introduction

Chicken is an important agricultural animal and a popular vertebrate research model [1]. Broiler chicken is one of the most efficient animal production systems after intensive selection based on growth rate and feed efficiency for about a half of a century [2,3]. However, with fast growth comes excessive body fat (mainly abdominal fat) deposition, which has adverse effects on the broiler performance and health, such as reduction of feed efficiency, muscle yield, meat quality, and reproductive performance, as well as consequent metabolic syndrome [4,5]. Thus, controlling excessive fat deposition and further improving the feed efficiency, carcass quality, and reproductive performance are major issues that need to be solved urgently in the broiler industry.

To solve the problem of excessive fat deposition in broilers, it is necessary to understand the molecular genetic basis of chicken adipose tissue growth and development. Adipose tissue is an important energy storage and endocrine organ [6]. Adipose tissue expansion is a result of the increase in the number and size of adipocytes. The number of adipocytes is mainly determined by the proliferation of preadipocytes [7]. Studies have shown that transcription factors play the important regulatory roles in the proliferation of preadipocytes. SREBP-1 can promotes human preadipocyte proliferation by targeting *RBBP8* and *CDKN3* [8]. Foxc2 promotes the proliferation of mouse preadipocytes by upregulating the expression of *Cyclin E* [9]. C/EBPβ can promotes mouse preadipocyte proliferation by upregulating the transcription of *Banp* and *Trim35* [10]. Overexpression of FoxO6 inhibits the proliferation of

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https://doi.org/10.1016/j.ijbiomac.2023.128414
Received 11 July 2023; Received in revised form 13 November 2023; Accepted 22 November 2023
Available online 28 November 2023

chicken preadipocytes by upregulating the transcription of *CCND2* [11]. The previous research of our group found that overexpression of KLF7 promotes the proliferation of chicken preadipocytes [12], while overexpression of PPARγ1 and PPARγ2 markedly suppressed the proliferation of chicken preadipocytes [13]. Although several transcription factors have been found to affect chicken preadipocyte proliferation, the transcriptional regulation network of preadipocyte proliferation, especially the identification of targets of transcription factors, remains to be improved.

The HMG-box protein 1 (HBP1) is a transcription factor belonging to the high-mobility-group (HMG) family, which has an HMG box DNA-binding domain [14]. HBP1 could bind to a variety of transcription factors to form a complex via the HMG box domain and regulate the transcription of its downstream targets [15]. Emerging evidences have shown that HBP1 is involved in regulating a variety of biological processes, including cell proliferation, differentiation, senescence, and tumor suppression, by directly or indirectly binding to the promoter region of its target genes [16,17]. A number of studies have shown that HBP1 plays a negative regulatory role in the process of cell proliferation. Knockout of HBP1 can shorten the cell cycle of neural stem cells during the development of cerebral cortex in mouse and promotes the proliferation of neuronal cells [18]. HBP1 represses the expression of the *p47phox* gene by binding to the *p47phox* promoter region, which in turn inhibits the proliferation of human 293T cells [16]. On the contrary, some studies have found that HBP1 can also promote cell proliferation. Overexpression of HBP1 promotes the proliferation of neural stem cells and pulmonary artery smooth muscle cells in rats [19,20]. Overexpression of HBP1 can promote the proliferation of human hepatic stellate cells and accelerate the development of liver fibrosis [21]. A recent study has shown that knockdown of HBP1 inhibits the proliferation of nasopharyngeal carcinoma cells by reducing the expression of cyclin D1 and cyclin D3 in nude mice in vivo [22]. The above studies indicate that the regulatory effect of HBP1 on proliferation is different in various cell types. Therefore, the role of HBP1 in the proliferation of different types of cells is worth studying separately. More importantly, it is unclear whether HBP1 is involved in the regulation of preadipocyte proliferation so far.

In our previous study, bulked segregation analysis of pooled-DNA sequencing using 729 male chickens (382 lean chickens and 347 fat chickens) from the 4th to 18th generations of the Northeast Agricultural University High- and Low-fat lines (NEAUHLF) showed that *HBP1* is an important candidate gene that affects chicken abdominal fat content [5]. In addition, our previous RNA-seq data showed that *HBP1* was differentially expressed in abdominal fat tissue between the two chicken lines [5]. Recently, we have demonstrated that HBP1 inhibited chicken preadipocyte differentiation by activating the STAT3 signaling via directly enhancing *JAK2* expression [23]. These results suggest that HBP1 plays an important role in adipose deposition in chickens. Considering that besides preadipocyte differentiation, the proliferation of preadipocyte is also an important biological process in the growth and development of adipose tissue, and HBP1 plays a critical role in mammalian cell proliferation, we hypothesized that HBP1 may be involved in the regulation of preadipocyte proliferation. The aim of the current study was to investigate the function and regulatory mechanism of HBP1 in chicken preadipocyte proliferation. Here, we demonstrated that HBP1 was a positive regulator of preadipocyte proliferation, and confirmed that HBP1 downregulated SOCS3 expression by directly binding and inhibiting *SOCS3* promoter.

2. Materials and methods

2.1. Experimental animals and management

Forty-two male chickens (lean line, $n = 21$; fat line, $n = 21$) from the 19th generation (G_{19}) and three male chickens (fat line) from the 23rd generation (G_{23}) of the Northeast Agricultural University High and Low Fat Lines (NEAUHLF) were used. NEAUHLF lines have been chosen since 1996 by using plasma very-low-density lipoprotein concentration and abdominal fat percentage (AFP) as the selection criteria: AFP (%) = AFW/BW7 × 100 %, where AFW is abdominal fat weight, and BW7 is body weight at 7 weeks of age. The breeding procedure was described in a previous study [24]. All chickens used in this study were kept under similar environmental conditions and given free access to food and water. All chickens were given the starter feed [metabolizable energy (ME): 3000 kcal/kg; crude protein (CP): 210 g/kg] from hatching to 3 weeks of age, and then fed with a grower diet (ME: 3100 kcal/kg; CP: 190 g/kg) from 4 to 7 weeks of age. Details of the chickens used in this study are shown in Additional file 1: Table S1.

2.2. Tissue collection

In total, 42 male chickens (three chickens for each broiler line, aged 1–7 weeks) from G_{19} and three male chickens (all for the fat broiler line, aged 7 weeks) from G_{23} were sacrificed after fasting for 10 h, and the AFP was calculated in G_{19} (Additional file 1: Table S1). At the end of each week, the abdominal fat tissue was collected. For chickens sacrificed at 7 weeks of age from G_{23}, 12 other tissue samples, namely, cerebrum (Cr), pectoral muscle (PM), leg muscles (LM), liver (L), spleen (Sp), gizzard (G), proventriculus (P), duodenum (D), jejunum (J), ileum (I), pancreas (Pa), and heart (H), were also collected. After washing with 0.75 % NaCl, all of the tissues were collected, snap-frozen in liquid nitrogen, and stored at −80 °C until further use.

2.3. Cell culture

The immortalized chicken preadipocytes (ICP2), HBP1 knockout ICP2 cells ($HBP1^{-/-}$), and HBP1 overexpression ICP2 cells (HBLV-HA-HBP1) and their control cells (HBLV-HA) used in this study were constructed previously in our laboratory [25,26]. The cells were cultured in DMEM/F12 (Gibco, New York, NY, USA) medium containing 10 % fetal bovine serum (Biological Industries, Kibbutz Beit HaEmek, Israel). The cells were grown in a standard humidified incubator at 37 °C with 5 % CO_2.

2.4. Cell transfection

ICP2 and HBP1 knockout ICP2 cells were plated on a 12-well cell culture plate. After growth to 60 % confluence, SOCS3-siRNA or the negative control (NC)-siRNA were transfected into the cells using Liposome 2000 reagent (Invitrogen, CA, USA). The siRNAs were synthesized commercially (Gene pharma, Shanghai, China). The siRNA sequences are shown in Additional file 1: Table S2.

2.5. RT-qPCR

Total RNA was extracted using a Trizol reagent kit (Invitrogen, CA, USA) following the manufacturer's protocol. First strand cDNA synthesis was performed with 1 μg of total RNA (Takara, Beijing, China). We performed qPCR using the FastStart Universal SYBR Green Master kit (Roche, Switzerland), and reaction conditions were as follows: 95 °C for 10 min followed by 40 cycles for 95 °C for 1 min, and 60 °C for 1 min. The results were analyzed using the mean of threshold cycle (Ct) for each sample. Relative expression level was calculated using the $2^{-\triangle Ct}$ method, and TATA-box-binding protein (*TBP*) was used as the reference gene [27]. The primers used for qPCR are shown in Additional file 1: Table S3.

2.6. Western blot

Total proteins were extracted from the tissues and cells with RIPA lysis buffer (Beyotime, Nanjing, China). The protein samples were then separated by SDS/PAGE on a 12 % gel and transferred to polyvinylidene

fluoride membranes (PVDF) (Millipore, Bedford, MA, USA) and blocked with 5 % bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) for 2 h. Membranes were incubated with HBP1 rabbit polyclonal antibody (Abcam, Cambridge, UK), PCNA mouse monoclonal antibody (Abcam, Cambridge, UK), SOCS3 rabbit polyclonal antibody (Abcam, Cambridge, UK) and beta-actin (β-actin) mouse monoclonal antibody (Beyotime, Nanjing, China) overnight at 4 °C. The membranes were then incubated with the HRP-labeled corresponding IgG at room temperature for 1 h. Finally, the target protein bands were imaged and analyzed using the Tanon-5200 automated chemiluminescence analyzer (Tanon Science and Technology Co., Ltd., Shanghai, China). Immunoreactive protein levels were determined semi-quantitatively by densitometric analysis, using Image J 1.46Rsoftware (NIH, Bethesda, MD, USA).

2.7. Cell proliferation assay

Cell proliferation was detected using the Cell CountingKit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan) and EdU incorporation assay (Ribobio, Guangzhou, China). For the CCK-8 assay, the cells were plated onto 96-well plates at a density of 1×10^4 cells/well in 100 μL of culture medium per well, and for each transfection group, three replicates were performed. At each designated time point after transfection, 10 μL of CCK-8 reagent was added to each well and incubated at 37 °C for 2 h. The absorbance of each sample at a wavelength of 450 nm was detected using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). For the EdU incorporation assay, the cells were plated onto 24-well plates at a density of 5×10^4 cells/well in 500 μL of culture medium per well, and for each transfection group, three replicates were performed. The staining of cells was performed using the EdU cell proliferation detection kit according to the manufacturer's instructions. The results of cell staining were observed under a fluorescence microscope (Leica, Wetzlar, Germany). Three fields were randomly selected from each well, and the ratio of EdU positive cells was calculated by Image J 1.46R software (NIH, Bethesda, MD, USA). The ratio of EdU positive cells (%) equals the number of red fluorescent cells/the number of blue fluorescent cells ×100 %.

2.8. Cell cycle assay

The cell cycle was assessed with a Cell Cycle Staining Kit (Multi-sciences, Hangzhou, China). The cells in six-well plates were harvested and centrifuged at 800*g* for 5 min. The supernatant was discarded, and the cells were washed once with cold phosphate-buffered saline (PBS). The cells were resuspended in 1 mL of kit reagent A and 10 μL of reagent B, followed by vortexing for 10 s and incubation for 30 min at room temperature, after which the cell suspension was used for flow cytometry (FACS Canto™ II, BD BioSciences, San Jose, CA, USA).

2.9. ChIP-seq and RNA-seq

ChIP-Seq and RNA-Seq were performed by SeqHealth Company (Wuhan, China). HBLV-HA-HBP1 preadipocytes ($n = 3$) were used for ChIP assays. ChIP was conducted using a ChIP assay kit (Cell Signaling Technology, Boston, MA, USA) as described previously [28]. The obtained ChIP DNA libraries were sequenced on a Hiseq X Ten system (Illumina, CA, USA). After quality control and data filtering, reads were mapped to the chicken genome (*Gallus_gallus 6.0*). MACS2 software was used for peak calling with the following parameters: macs2 callpeak -t ip.bam -c input.bam -g Gg -n wildcards.sample –outdir f BAM —B –bw 300 –mfold 5 50 –slocal = 1000 –llocal = 10,000 –keep-dup = 1 -p 0.01. Already known motifs and de novo motifs were identified by Homer software (version V4.10) [29]. The ChIP-seq results were validated by ChIP-qPCR, and the data were calculated as previously detailed [23]. The primers used in ChIP-qPCR are listed in Additional file 1: Table S3.

For RNA-Seq, total RNA of the HBLV-HA-HBP1 ($n = 3$) and HBLV-HA ($n = 3$) preadipocytes was extracted using TRIzol (Invitrogen, CA, USA). This was used to prepare the mRNA sequencing library, which was sequenced on a Hiseq X Ten system (Illumina, CA, USA). After quality control and data filtering, reads were mapped to the chicken genome (*Gallus_gallus 6.0*). Significant differences in gene expressions were filtered with the criteria of P value <0.05 and fold change >1.5.

2.10. Promoter luciferase reporter assay

The effect of over-expressed HBP1 on the promoter activities of candidate target genes identified by ChIP-seq and RNA-Seq were investigated by luciferase reporter assay. For that, promoter reporter constructs with wild or mutated HBP1 binding sites of the candidate target genes (*TSC22D3* and *SOCS3*) were constructed by Genewiz (Suzhou, China). The promoter reporter vector or pGL3-basic empty vector was co-transfected with pRL-TK Renilla luciferase vector into HBLV-HA and HBLV-HA-HBP1 preadipocytes, which were plated in 24-well plates. After 48 h of transfection, the luciferase activities were detected by the Dual-Luciferase Assay System (Promega, WI, USA).

2.11. Electrophoretic mobility shift assay

The nuclear extracts were isolated from HBLV-HA-HBP1 pre-adipocytes using the nuclear extraction kit (Thermo Fisher, MA, USA). The binding of nuclear extracts to the DNA probes was detected by an EMSA Kit (Thermo Fisher, MA, USA). Biotin-labeled probes harboring the potential HBP1 binding site and the corresponding cold and mutant probes containing the *SOCS3* promoter sequence were synthesized by Genewiz (Suzhou, China). The sequences of the probes are shown in Additional file 1: Table S3. The labeled double-stranded probes were incubated with nuclear extracts for 30 min. For competition assays, a 50-fold molar excess of unlabeled specific probes (cold probes) was added to the binding reactions before the labeled probes were added. For the supershift assay, 1 μg of HA-tag specific antibody (Abcam, Cambridge, UK) or normal rabbit IgG (Abcam, Cambridge, UK), used as a negative control, was added to the reactions. The protein-DNA complexes were separated on a 6 % polyacrylamide gel and observed with ECL reagent.

2.12. Statistical analysis

SPSS 16.0 software and GraphPad 5.0 software were used for statistical analyses. All experiments were repeated three times. Data are shown as the mean ± standard deviation (SD). Comparisons between the two groups were performed using Student's *t*-tests. Comparisons among multiple groups were performed using two-way ANOVA followed by Duncan's test. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. The temporal and spatial expression of HBP1

RT-qPCR results showed that *HBP1* mRNA was expressed in all of the tissues tested from 7-week-old fat male chickens of G_{23} (Fig. 1A), and that the expression level of *HBP1* transcript was relatively higher in pectoral muscle, leg muscle, heart, liver, and abdominal fat (Fig. 1A). Subsequently, we investigated the expression pattern of HBP1 during the proliferation of chicken preadipocytes. When ICP2 cells reached 60 % confluency in the normal medium (10 % FBS), the cells were washed twice with PBS, then serum-free medium added. After 48 h, serum-free medium was replaced by the normal medium (this time point was defined as 0 h), and the cells were continued cultured in the normal medium until 96 h. We chose to starve ICP2 cells for 48 h because the effect of cell cycle synchronization was the best (the proportion of cells in the G1 phase was the highest) at this time point (Additional file 2: Fig. S1). The results of a CCK-8 assay showed that ICP2 cell number increased from 0 to 96 h (Fig. 1B), which indicated that the cells were

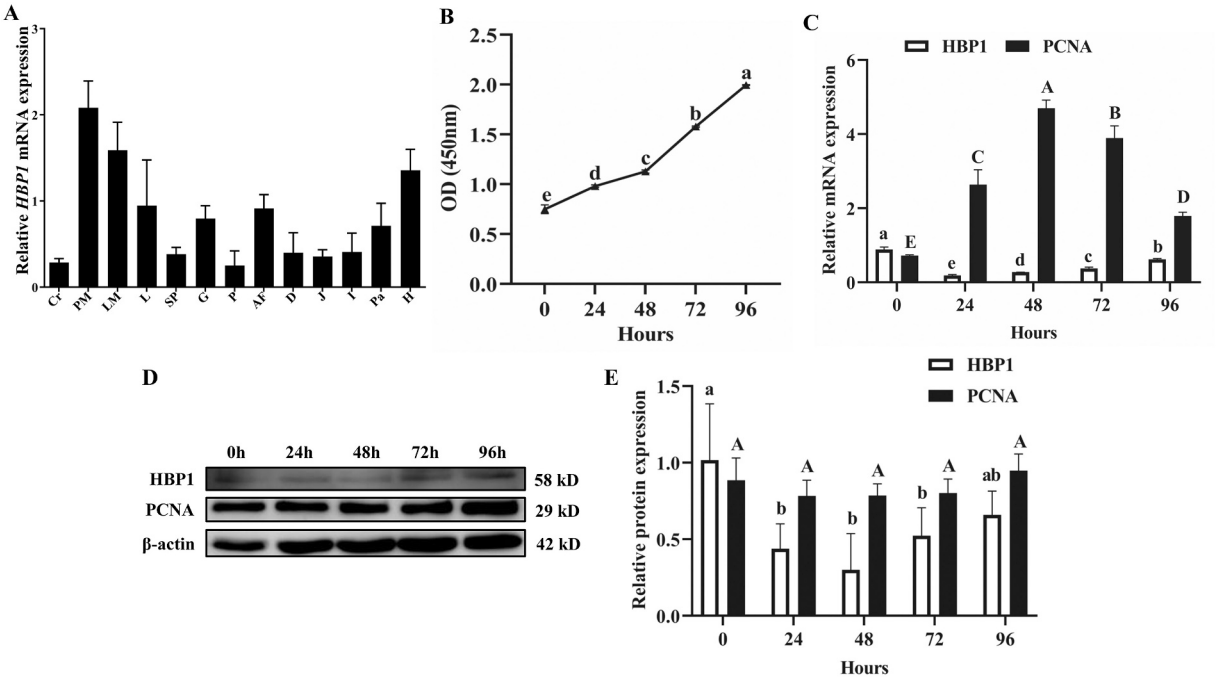

Fig. 1. The temporal and spatial expression of chicken HBP1. (A) The mRNA levels of *HBP1* in different tissues of fat chickens were assessed by RT-qPCR. AF, abdominal fat; Cr, cerebrum; PM, pectoral muscle; LM, leg muscles; L, liver; Sp, spleen; G, gizzard; P, proventriculus; D, duodenum, J, jejunum; I, ileum; Pa, pancreas; H, heart. (B) The proliferation activity of ICP2 cells was detected by CCK-8. [a–e]The different lowercase letters represent a significant difference ($P < 0.05$). (C) The mRNA levels of *HBP1* and *PCNA* during the proliferation of ICP2 cells were assessed by RT-qPCR. (D, E) The protein levels of HBP1 and PCNA during the proliferation of ICP2 cells were assessed by western blot. [a–e]The different lowercase letters above the columns indicate significant differences in the expression of HBP1 among various time points ($P < 0.05$). [A-E]The different uppercase letters above columns indicate significant differences in the expression of PCNA among various time points ($P < 0.05$). Data are presented as the mean ± SD, $n = 3$.

proliferating as normal. RT-qPCR and western blotting results showed that the expression level of HBP1 was decreased first and then increased during the proliferation of ICP2 cells (Fig. 1C-E). These results suggested that HBP1 may play a critical role in adipose tissue and preadipocyte proliferation.

3.2. *HBP1 is a positive regulator of preadipocyte proliferation*

Next, we want to know whether HBP1 plays an important role in preadipocyte proliferation. First, we analyzed the effect of HBP1 knock out on the proliferation of preadipocytes. The CCK8 results showed that knock out of HBP1 inhibited the cell viability ($P < 0.01$; Fig. 2A). RT-qPCR and western blotting results showed that knock out of HBP1 inhibited the expression of PCNA and Ki67 ($P < 0.05$ or $P < 0.01$; Fig. 2B-C). EdU staining results showed that knock out of HBP1 inhibited DNA synthesis in the cells ($P < 0.001$; Fig. 2D-E). The flow cytometry results showed that knock out of HBP1 significantly reduced the proportion of cells in the G1 phase and significantly increased the proportion of cells in the S phase ($P < 0.05$; Fig. 2F-G). These results indicated that knock out of HBP1 could inhibit the proliferation of preadipocytes.

Next, we analyzed the effect of overexpression of HBP1 on the proliferation of preadipocytes. The CCK8 results showed that over expression of HBP1 promoted the cell viability ($P < 0.05$ or $P < 0.01$; Fig. 3A). RT-qPCR and western blotting results showed that overexpression of HBP1 promoted the expression of PCNA and Ki67($P < 0.05$ or $P < 0.01$; Fig. 3B-C). EdU staining results showed that overexpression of HBP1 promoted DNA synthesis in cells ($P < 0.01$; Fig. 3D-E). The flow cytometry results showed that overexpression of HBP1 significantly reduced the proportion of cells in the G1 phase, as well as significantly increased the proportion of cells in the S and G2 phases ($P < 0.05$ or $P < 0.01$; Fig. 3F-G). These results indicate that overexpression HBP1 can promote the proliferation of preadipocytes. In summary, we demonstrated that HBP1 is a positive regulator of preadipocyte proliferation.

3.3. *SOCS3 is the direct target of HBP1*

To identify the target genes of HBP1, ChIP-Seq was performed with HBLV-HA-HBP1 preadipocytes. A total of 5195 common peaks (Fig. 4A) corresponding to 1778 common genes (Additional file 3: Data S1) were obtained, of which 62.29 % were located in the promoter region (Fig. 4B). The results of motif analysis of 5195 HBP1 binding peaks showed that YY1 motif (AAnATGGC) [30] was the most significant enriched motif (17.64 % of the binding peaks contain this motif), which was similar to the known HBP1 motif (T/A)(T/A)CAA(A/T)GG (Fig. 4C) [31]. To examine the HBP1-mediated gene expression regulation, we employed RNA-Seq for HBLV-HA and HBLV-HA-HBP1 preadipocytes. We identified a total of 102 differentially expressed genes (DEGs), including 21 upregulated and 81 downregulated genes (Fig. 4D-E; Additional file 2: Fig. S2; Additional file 3: Data S2). In order to further screen the candidate target genes bound and regulated by HBP1, we intersected the 1778 genes identified in the ChIP-seq with the 102 DEGs. We found a total of 3 candidate HBP1 target genes at the intersection, including *CC2D2A*, *TSC22D3* and *SOCS3*.

Next, we intend to conduct ChIP-qPCR assay on the 3 candidate target genes of HBP1 to validate the ChIP-seq data. When designing ChIP-qPCR primers, we found that only two of the 3 genes (*TSC22D3* and *SOCS3*) contained potential HBP1 binding sites in their promoter regions. Therefore, we carried out ChIP-qPCR assay for these two genes, and they were successfully verified (Fig. 5A). Next, we performed RT-qPCR assay on the two genes to validate the RNA-seq data. RT-qPCR results showed that the expressions of these two genes were consistent with the RNA-seq data (Fig. 5B). The above results confirmed the reliability of ChIP-seq and RNA-seq data.

To further verify the HBP1-mediated transcriptional regulation of the candidate target genes, we cloned the promoter sequences of the two candidate target genes harboring the wild-type or mutated HBP1 binding motifs upstream of the firefly luciferase reporter gene into the pGL3-

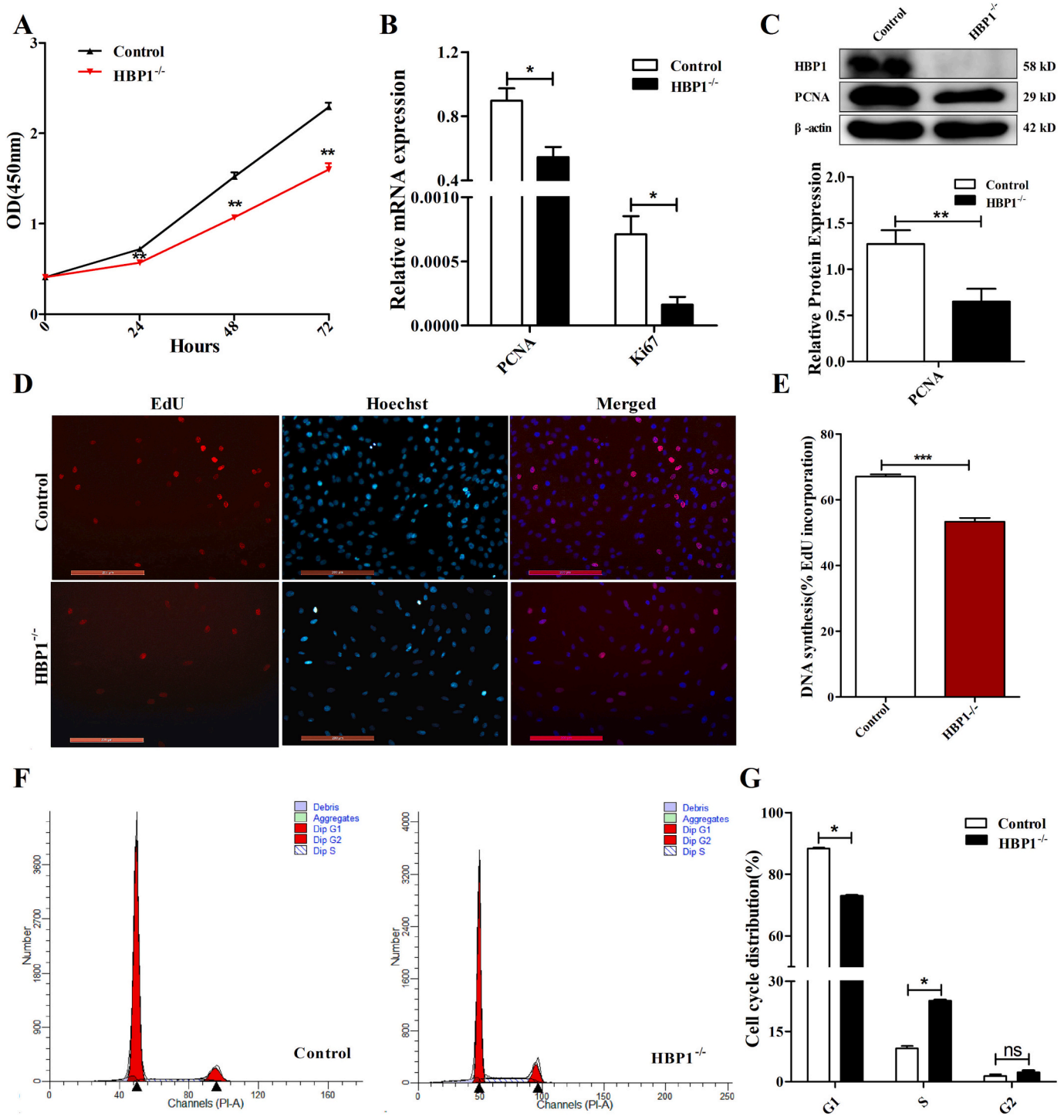

Fig. 2. Knockout of HBP1 inhibited the proliferation of preadipocytes. (A) Cell viability was analyzed using the CCK-8 assay. (B) The mRNA levels of *PCNA* and *Ki67*were assessed by RT-qPCR. (C) The protein levels of PCNA were assessed by western blot. (D, E) The DNA synthesis in the cells was assessed by EdU staining (scale bars: 200 μm). (F, G) The distribution of cell cycle was assessed by flow cytometry. Data are presented as the mean ± SD, $n = 3$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

basic vector. Luciferase reporter assays showed that overexpression of HBP1 had no effect on the activity of wild-type promoter of *TSC22D3* (Fig. 5C), but it caused inhibition of wild-type promoter activity of *SOCS3* ($P < 0.001$; Fig. 5D). When the HBP1 binding site (−1808 to −1792 bp; Additional file 2: Fig. S3) within the context of the −2000 bp Luc-SOCS3-promoter was mutated, the inhibitory effect of overexpression of HBP1 on *SOCS3* promoter activity was significantly attenuated ($P < 0.05$; Fig. 5D), suggesting that this site is important for HBP1-mediated inhibition of the *SOCS3* promoter. To determine whether HBP1 was able to bind this site, electrophoretic mobility gel shift studies were performed. As shown in Fig. 5E, HBP1 is able to bind to this site (Fig. 5E, lane 2); the specificity of this interaction was confirmed by competition with an identical but not mutated oligomer (Fig. 5E, lane 4). Furthermore, this DNA-protein complex was supershifted in the presence of an anti-HA antibody (Fig. 5E, lane 7), but not IgG (Fig. 5E, lane 6). Thus, we demonstrated that *SOCS3* is a direct target

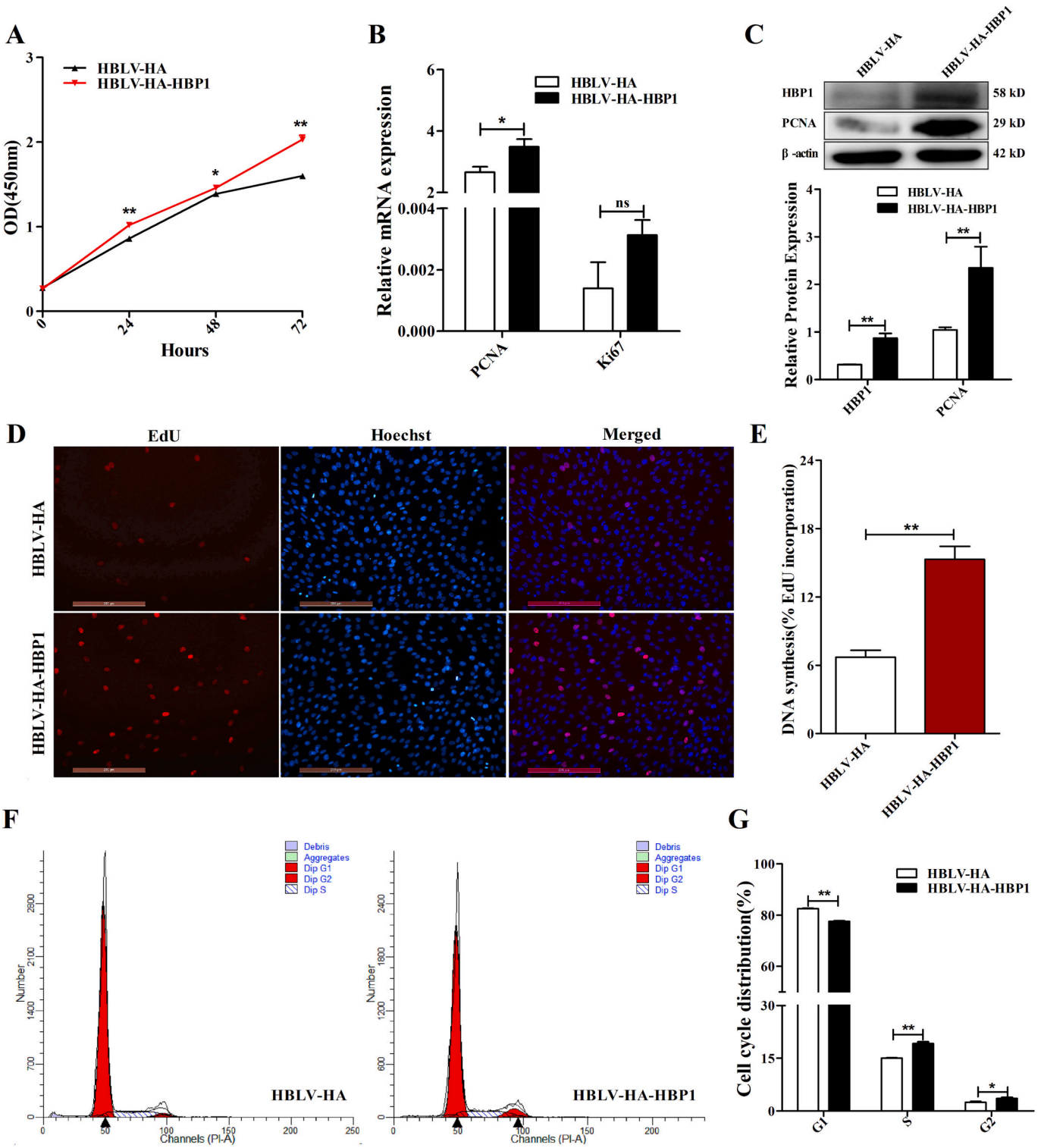

Fig. 3. Overexpression of HBP1 promoted the proliferation of preadipocytes. (A) Cell viability was analyzed using the CCK-8 assay. (B) The mRNA levels of *PCNA* and *Ki67* were assessed by RT-qPCR. (C) The protein levels of HBP1 and PCNA were assessed by western blot. (D, E) The DNA synthesis in the cells was assessed by EdU staining (scale bars: 200 μm). (F, G) The distribution of the cell cycle was assessed by flow cytometry. Data are presented as the mean ± SD, $n = 3$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

of HBP1.

3.4. The temporal and spatial expression of SOCS3

RT-qPCR results showed that *SOCS3* mRNA was expressed in all of the tissues tested from 7-week-old fat male chickens of G_{23} (Fig. 6A), and the expression level of *SOCS3* transcript was relatively higher in pectoral muscle, leg muscle, ileum, and abdominal fat (Fig. 6A). In addition, the mRNA expression levels of *SOCS3* in the abdominal adipose tissue of fat broilers were significantly higher than those of the lean broilers at 5, 6, and 7 weeks of age ($P < 0.05$; Fig. 6B). Because *SOCS3* is a target gene that is negatively regulated by HBP1, we speculated that the expression

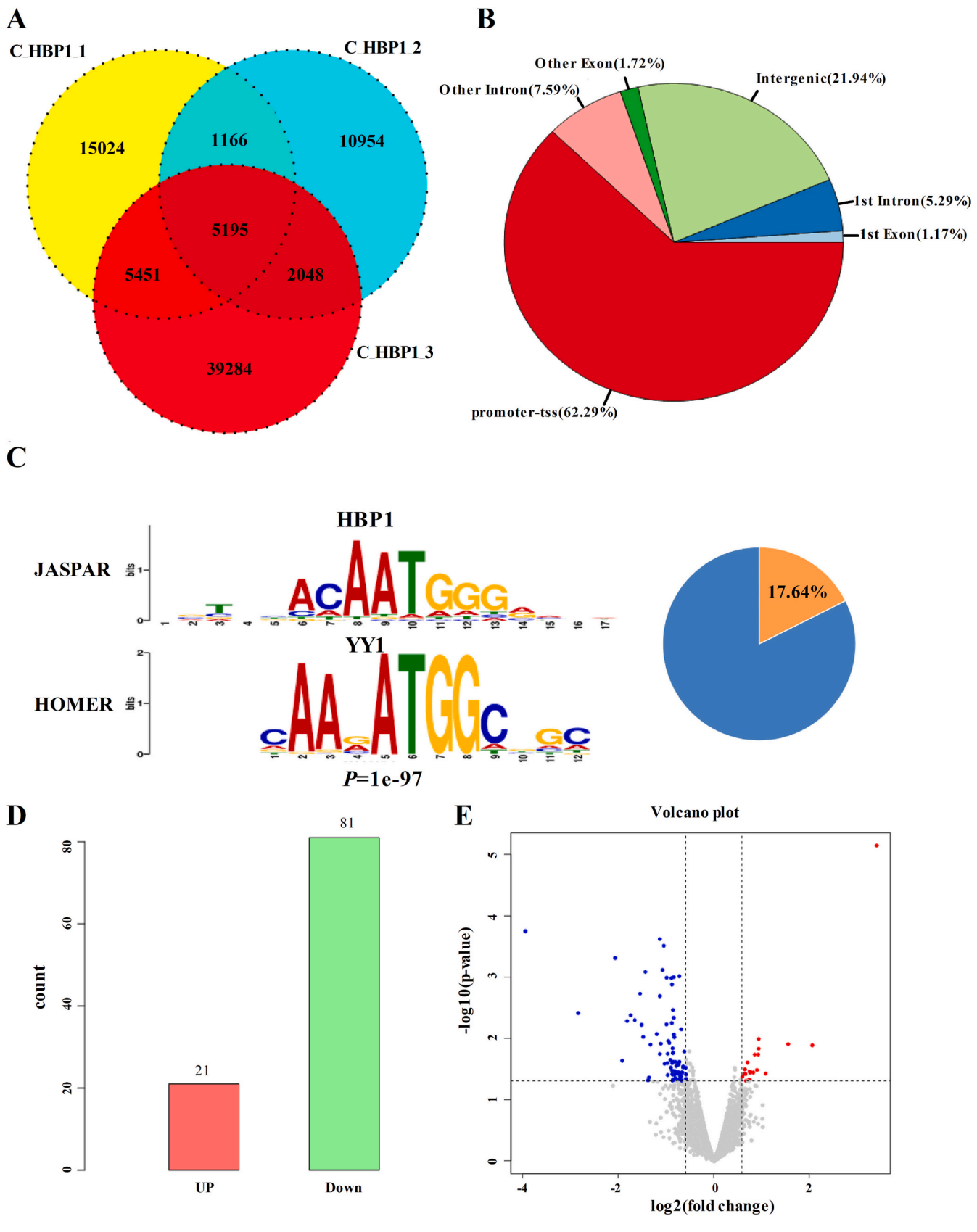

Fig. 4. ChIP-seq and RNA-seq of HBP1 in chicken preadipocytes. (A) Venn diagram of the number of peaks in the three independent samples (C-HBP1–1, C-HBP1–2, and C-HBP1–3). (B) Peak distribution for the genomic functional area of ChIP-seq. (C) De novo motif prediction by HOMER of DNA sequences enriched in HBP1 binding regions. HBP1 JASPAR matrix is presented for comparison. (D, E) Bar and volcano plots of differentially expressed genes in RNA-seq.

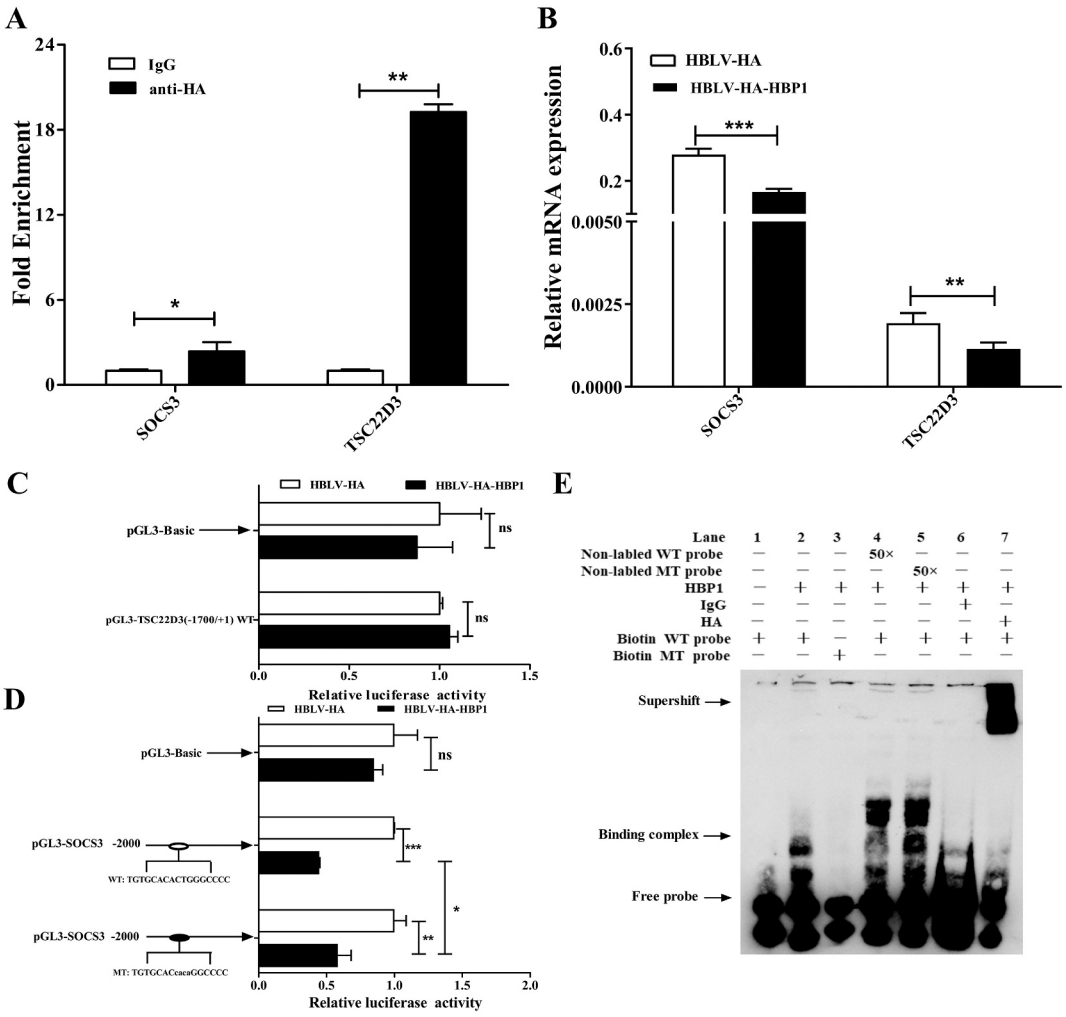

Fig. 5. *SOCS3* is a direct target of HBP1. (A) The enrichment of HBP1in the promoter regions of *TSC22D3* and *SOCS3* was detected by ChIP-qPCR. (B) The mRNA levels of *TSC22D3* and *SOCS3* in the HBLV-HA and HBLV-HA-HBP1 cells were assessed by RT-qPCR. (C) The effect of HBP1 overexpression on the transcriptional activity of the *TSC22D3* promoter harboring wild HBP1 binding site. (D) The effect of HBP1 overexpression on the transcriptional activity of the *SOCS3* promoter harboring wild or mutant HBP1 binding site. (E) The interaction between HBP1 and the promoter fragment of *SOCS3* (−1811 to −1788 bp) was detected by EMSA. Lanes 1–3: Biotinylated wild or mutant oligo was used to determine their interactions with nuclear extract from HBLV-HA-HBP1 preadipocytes. Lanes 4 and 5: Excess unlabeled wild or mutant oligo was added for competitive experiment. Lanes 6 and 7: IgG or anti-HA antibody was added for supershift assay. Data are presented as the mean ± SD, $n = 3$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

level of *SOCS3* may be negatively correlated with the expression level of *HBP1* in adipose tissue. As we expected, the expression levels of *SOCS3* and *HBP1* in chicken adipose tissue had significant negative correlation ($r = -0.5363$, $P = 0.0011$; Fig. 6C). Subsequently, we investigated the expression pattern of SCOS3 during the proliferation of chicken preadipocytes. When ICP2 cells reached 60 % confluency in the normal medium (10 % FBS), the cells were washed twice with PBS, then serum-free medium added. After 48 h, serum-free medium was replaced by the normal medium (this time point was defined as 0 h), and the cells were continued cultured in the normal medium until 96 h. We found the expression level of SOCS3 was decreased first and then increased during the proliferation of ICP2 cells (Fig. 6D-G). Taken together, these data suggested that SOCS3 may be involved in adipose deposition and preadipocyte proliferation.

3.5. *HBP1 promotes the proliferation of preadipocytes by inhibiting the expression of SOCS3*

To validate the functional role of SOCS3 in preadipocyte proliferation, the ICP2 cells were transfected with effective SOCS3 interference fragment (SOCS3-siRNA 1; Additional file 2: Fig. S4). The CCK8 results showed that knockdown of SOCS3 promoted the cell viability ($P < 0.05$; Fig. 7A). RT-qPCR and western blotting results showed that knockdown of SOCS3 promoted the expression of PCNA and Ki67 ($P < 0.05$ or $P < 0.01$; Fig. 7B-D). EdU staining results showed that knockdown of SOCS3 promoted DNA synthesis in the cells ($P < 0.05$; Fig. 7E-F). These results indicate that knockdown of SOCS3 could promote the proliferation of preadipocytes.

Subsequently, in order to determine whether SOCS3 could mediate the regulatory effect of HBP1 on the proliferation of preadipocytes, we carried out functional rescue experiments. The results showed that knockdown of SOCS3 attenuated the inhibitory effects of knockout of HBP1 on the cell viability ($P < 0.05$; Fig. 8A), the expression of PCNA and Ki67 ($P < 0.05$ or $P < 0.01$; Fig. 8B-D), and DNA synthesis ($P < 0.05$; Fig. 8E-F). Taken together, these data indicate that HBP1 promotes the proliferation of preadipocytes by inhibiting the expression of SOCS3.

4. Discussion

The proliferation of preadipocytes is an important biological process in the growth and development of adipose tissue, which is regulated by a network of transcription factors, such as SREBP-1, C/EBPβ and FoxO6

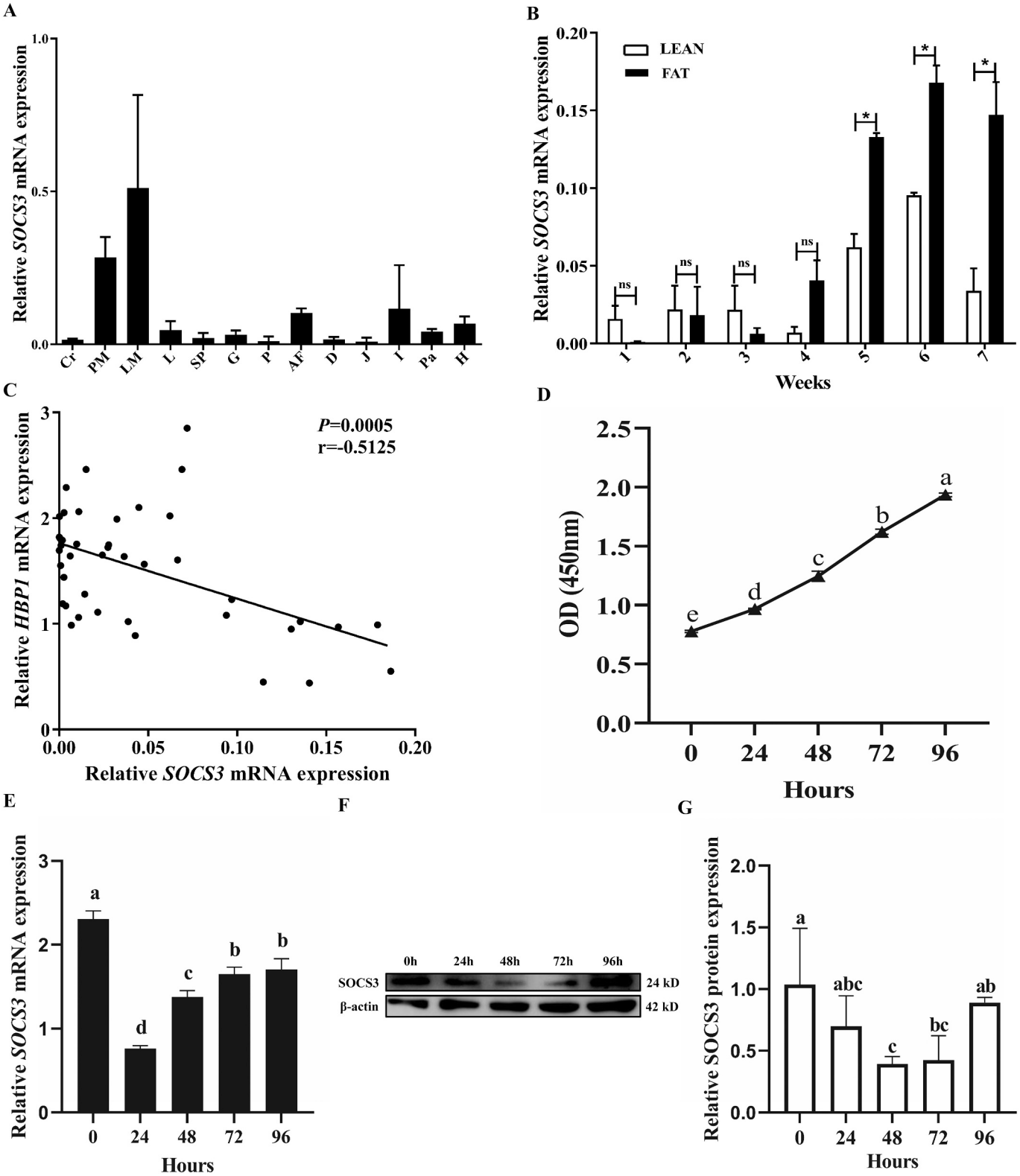

Fig. 6. The temporal and spatial expression of SOCS3. (A) The mRNA levels of *SOCS3* in different tissues of fat chickens were assessed by RT-qPCR. (B) The mRNA levels of *SOCS3* in the abdominal fat tissues of lean and fat chickens were assessed by RT-qPCR. (C) The correlation between *HBP1* mRNA expression level and *SOCS3* mRNA expression level. (D) The proliferation activity of ICP2 cells was detected by CCK-8. (E) The mRNA levels of *SOCS3* during the proliferation of ICP2 cells were assessed by RT-qPCR. (F, G) The protein levels of SOCS3 during the proliferation of ICP2 cells were assessed by western blot. Data are presented as the mean ± SD, $n = 3$. The different lowercase letters represent a significant difference ($P < 0.05$).

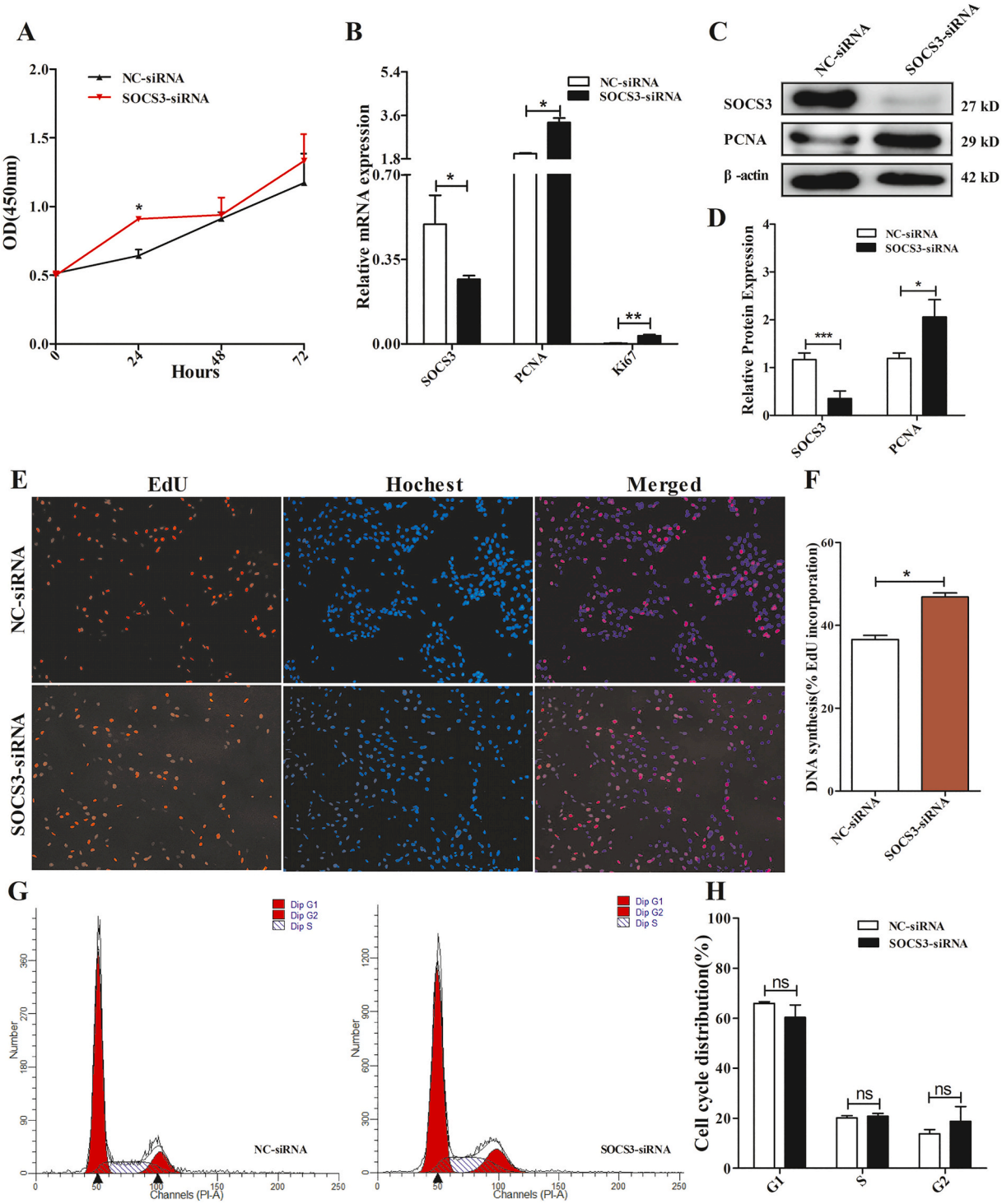

Fig. 7. Knockdown of SOCS3 promotes the proliferation of preadipocytes. (A) Cell viability was analyzed using the CCK-8 assay. (B) The mRNA levels of *SOCS3*, *PCNA*, and *Ki67* were assessed by RT-qPCR. (C, D) The protein levels of SOCS3 and PCNA were assessed by western blot. (E, F) The DNA synthesis in the cells was assessed by EdU staining (scale bars: 200 μm). (G, H) The distribution of the cell cycle was assessed by flow cytometry. Data were presented as the mean ± SD, $n = 3$. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

[8,10,11]. Previous studies have shown that transcription factor HBP1 plays different roles in the regulation of proliferation in different cell types, such as 293 T cells, neural stem cells, hepatic stellate cells, and nasopharyngeal carcinoma cells [16,18,21,22]. However, whether HBP1 is involved in the regulation of preadipocyte proliferation remains unclear. Here, we for the first time demonstrated that HBP1 was a positive regulator of preadipocyte proliferation, and confirmed that HBP1 downregulated SOCS3 expression by directly binding and inhibiting *SOCS3* promoter (Additional file 2: Fig. S5).

Firstly, we studied the temporal and spatial expression pattern of HBP1. The results showed that *HBP1* mRNA was widely expressed in chicken tissues, with relatively high expression levels in pectoral muscle, leg muscle, heart, liver, and abdominal fat (Fig. 1A), consistent with previous studies in rats [32,33]. Meanwhile, we found that the

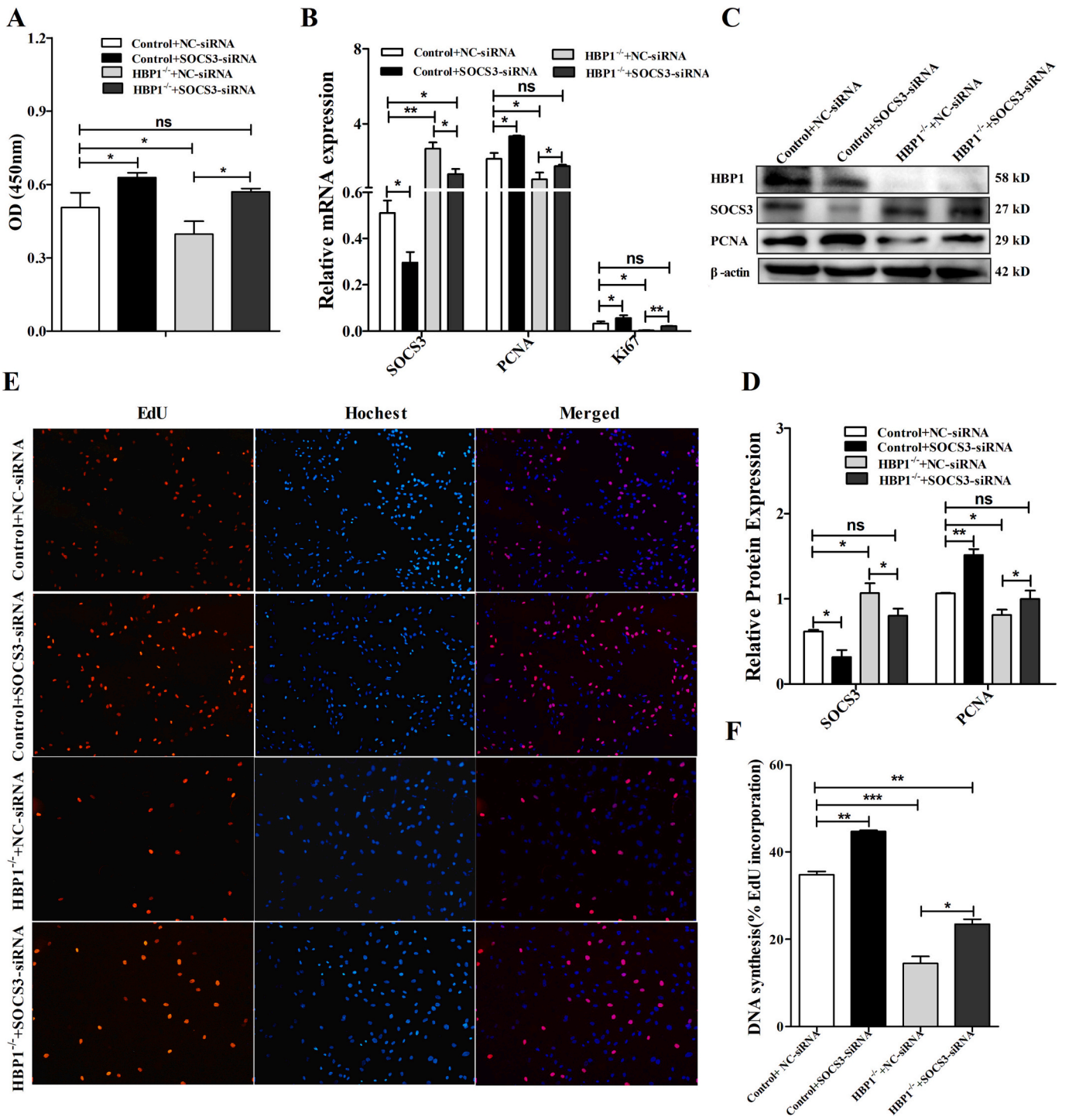

Fig. 8. SOCS3 mediates the regulatory effect of HBP1 on the proliferation of preadipocytes. (A) Cell viability was analyzed using the CCK-8 assay. (B) The mRNA levels of SOCS3, PCNA, and Ki67 were assessed by RT-qPCR. (C, D) The protein levels of HBP1, SOCS3 and PCNA was assessed by western blot. (E, F) The DNA synthesis in the cells was assessed by EdU staining (scale bars: 200 μm). Data were presented as the mean ± SD, n = 3. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

expression level of HBP1 decreased first and then increased during the proliferation of chicken preadipocytes (Fig. 1B-E). These results suggested that HBP1 may play a critical role in regulating the proliferation of preadipocytes.

To confirm the above hypotheses, we used gain- and loss-of-function strategies to study the role of HBP1 in the proliferation of preadipocytes. The results showed that knockout of HBP1 inhibited the cell viability and DNA synthesis of chicken ICP2 cells (Fig. 2A-E), while overexpression had the opposite result (Fig. 3A-E). These results indicate that HBP1 is a positive regulator of the proliferation of chicken preadipocytes, consistent with previous studies in rats and humans showing that overexpression of HBP1 can increase the cell viability and DNA synthesis ability of rat hematopoietic stem cells [21], promote the proliferation of primary pulmonary artery smooth muscle cells in rats [19], as well as promote the proliferation of human vascular smooth muscle cells [34]. Cell cycle plays an important role in the regulation of cell

proliferation [35]. In nasopharyngeal carcinoma, HBP1 has been found to act as a positive regulator of G1/S conversion in the cyclin CDK-CDKI network. Overexpression of HBP1 promotes the proliferation and G1/S transition of human nasopharyngeal carcinoma cells, while knockdown of HBP1 inhibits cell proliferation and arrests the cell in the G1 phase [22]. In this study, we also found that overexpression of HBP1 promotes proliferation and G1/S transition of chicken preadipocytes, but knockout of HBP1 leads to cell cycle arrest in the S phase. Our results were similar to those reported for the above human nasopharyngeal carcinoma cells, but were not completely consistent. A previous study showed that there is a cell cycle checkpoint in the S phase, which regulates the speed of DNA replication through a series of cell-cycle regulatory proteins to reduce the probability of errors in the replication process and maintain the stability of the genome [36]. Studies have shown that HBP1 plays an important role in DNA replication and DNA repair [37]. Therefore, we speculated that knockout of HBP1 may cause DNA damage in cells, hinder the DNA replication process, and thus, arrest the cell cycle in S phase.

Transcription factors regulate the transcription of target genes by binding to specific DNA sequences in its promoter region [28]. Studies on mammals have found that there were three binding motifs with high affinity for HBP1, which are (T/A)(T/A)CAA(A/T)GG [32], TTCATTCATTCA [38], and GGGTAGGG [39]. In this study, we performed motif analysis of 5195 HBP1 binding peaks obtained by ChIP-seq via Homer software, which mainly contains the binding motifs of transcription factors in mammals. We found that the top 1 motif was not HBP1, but YY1 motif (AAnATGGC) [30], which is similar to HBP1 motif (T/A)(T/A)CAA(A/T)GG. This phenomenon might be due to two reasons: (1) Compared with mammalian HBP1 motif, the HBP1 motif in birds might be more similar to the mammalian YY1 motif; (2) HBP1 might have a unique binding motif in preadipocytes. In addition, we found some motifs of other transcription factors in the HBP1 binding peaks, such as Gabpa, RDR1, and ZNF711D (Additional file 1: Table S4), suggesting that HBP1 would interact with these transcription factors to indirectly regulate the transcription of downstream target genes.

Next, we validated the sequencing results by ChIP-qPCR and RT-qPCR and found that *TSC22D3* and *SOCS3* were the target genes of HBP1. It is worth noting that when analyzing the regulatory effect of HBP1 on the promoter activity of these two target genes, we found that overexpression of HBP1 did not affect the promoter activity of *TSC22D3* (Fig. 5C). This phenomenon might be due to the overexpression of HBP1, which induced the expression of some unknown transcription factors. These factors may compete with HBP1 to bind the promoter region of *TSC22D3*, which in turn hinder the regulation of HBP1 on the promoter activities of *TSC22D3*. Consistent with our expectation, overexpression of HBP1 significantly inhibited *SOCS3* promoter activity (Fig. 5D). The ChIP-seq data combined with the bioinformatics analysis by JASPAR tool (http://jaspar.genereg.net/) revealed the presence of a putative HBP1 binding site (−1808 to −1792 bp) within the HBP1 binding peak on the *SOCS3* promoter (Additional file 2: Fig. S3). Gel shift studies demonstrated that HBP1 can bind to this site (Fig. 5E). It was worth noting that the band of binding complex weakened upon the addition of IgG (lane 6, Fig. 5E), consistent with a previous study [40]. We speculated that the addition of IgG might affect the stability of protein-DNA complex in the gel or in free solution, leading to weakened binding band. In addition, we observed a strong band introduced above the binding complex by adding non-labeled probes (lanes 4 and 5, Fig. 5E), consistent with the previous research [41]. This phenomenon might be due to the complex components contained in the nuclear extracts, resulting in the non-specific binding of the proteins with biotin-labeled probes after adding non-labeled probes. Anyhow, our EMSA results indicated that HBP1 could bind to −1808 to −1792 bp within the promoter of *SOCS3*. Further promoter mutation analyses supported a role for this site in mediating the inhibitory effect of HBP1 (Fig. 5D). Together, the above results demonstrated that *SOCS3* was a direct target of HBP1.

SOCS3 is a member of the suppressor of cytokine signaling (SOCS) family, which is involved in cell proliferation, differentiation, and apoptosis by regulating the activities of the MAPK/ERK and JAK-STAT3 signaling pathways [42]. SOCS3 also plays an important role in regulating leptin resistance and maintaining body energy homeostasis [43]. Studies in mice have found that the lack of SOCS3 promotes liver lipid accumulation, leading to the development of non-alcoholic fatty liver and obesity [44]. In rats, resveratrol inhibits dyslipidemia and liver steatosis by inhibiting the expression of SOCS3 [45]. Studies have found that SOCS3 is highly expressed in human adipose tissue [46]. The above studies suggested that SOCS3 may be associated with obesity, but the biological function of SOCS3 in preadipocyte proliferation has not been reported. To explore the role of SOCS3 in the proliferation of preadipocytes, we first analyzed the tissue expression pattern of *SOCS3*. We found that *SOCS3* was widely expressed in a variety of chicken tissues and highly expressed in abdominal adipose tissue (Fig. 6A), consistent with the study in humans [46]. Many studies have shown that there exists a correlation of expression level in the tissues or cells between the transcription factors and their target genes [47]. In the current study, we also found that the expression levels of *SOCS3* and *HBP1* was significantly negatively correlated in chicken adipose tissues (Fig. 6C), which further suggest that *SOCS3* was the target of HBP1 and that it might play a role in the proliferation of preadipocytes.

To confirm the above hypotheses, we used loss-of-function strategy to study the role of SOCS3 in the proliferation of preadipocytes. The results showed that knockdown of SOCS3 promoted the proliferation of preadipocytes (Fig. 7A-F), supporting SOCS3 is a negative regulator of proliferation [48]. However, our study found that knockdown of SOCS3 had no effect on the cell cycle (Fig. 7G-H). We speculated that knockdown of SOCS3 promoted chicken preadipocyte proliferation not by regulating the cell cycle but by regulating cell senescence or apoptosis. In addition, we found that knockdown of SOCS3 attenuated the inhibitory effect of knockout of HBP1 on preadipocytes proliferation (Fig. 8), indicating that SOCS3 mediated the regulation of HBP1 on the proliferation of preadipocytes.

5. Conclusion

In summary, we demonstrated that HBP1 directly binds to and inhibits *SOCS3* promoter and that HBP1 promotes chicken preadipocyte proliferation through downregulation of SOCS3 expression. Our findings expand the transcriptional regulatory network of preadipocyte proliferation, and they will be helpful in formulating a molecular breeding scheme to control excessive abdominal fat deposition and to improve meat quality in chickens.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2023.128414.

Funding

This work was supported by the National Key Research and Development Program of China (No. 2022YFF1000201), the Academic Backbone Project of Northeast Agricultural University (No. 21XG33), the Heilongjiang Province Natural Science Young Innovative Talents Project (No. 145209218), the Earmarked Fund for CARS-41 (No. CARS-41) and the Joint Guidance Project of Heilongjiang Natural Science Foundation (No. LH2021C037).

Ethical statement

Animal studies were conducted following the Guidelines for the Care and Use of Experimental Animals established by the Ministry of Science and Technology of the People's Republic of China (approval#: 2006-398) and were approved by the Laboratory Animal Management Committee and the Institutional Biosafety Committee of Northeast Agricultural University (Harbin, China).

CRediT authorship contribution statement

Hongyan Chen: Investigation, Writing – original draft, Funding acquisition. **Sitong Zhou:** Investigation, Visualization. **Youdong Wang:** Investigation, Visualization. **Qi Zhang:** Investigation, Visualization. **Li Leng:** Data curation, Funding acquisition. **Zhiping Cao:** Data curation. **Peng Luan:** Visualization. **Yumao Li:** Visualization. **Shouzhi Wang:** Software. **Hui Li:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Bohan Cheng:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

The authors appreciate the help from the Poultry Breeding Group at the College of Animal Science and Technology, Northeast Agricultural University. We thank International Science Editing (http://www.internationalscienceediting.com) for editing this manuscript.

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