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p300-Dependent Acetylation of Activating Transcription Factor 5 Enhances C/EBPβ Transactivation of C/EBPα during 3T3-L1 Differentiation

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Adipogenesis is a multistep process by which 3T3-L1 preadipocytes differentiate into mature adipocytes through mitotic clonal expansion (MCE) and terminal differentiation. The CCAAT/enhancer-binding protein β (C/EBPβ) is an important transcription factor that takes part in both of these processes. C/EBPβ not only transactivates C/EBPα and the peroxisome proliferator-activated receptor γ (PPARγ), which cause 3T3-L1 preadipocytes to enter terminal adipocyte differentiation, but also is required to activate cell cycle genes necessary for MCE. The identification of potential cofactors of C/EBPβ will help to explain how C/EBPβ undertakes these specialized roles during the different stages of adipogenesis. In this study, we found that activating transcription factor 5 (ATF5) can bind to the promoter of C/EBPα via its direct interaction with C/EBPβ (which is mediated via the p300-dependent acetylation of ATF5), leading to enhanced C/EBPβ transactivation of C/EBPα. We also show that p300 is important for the interaction of ATF5 with C/EBPβ as well as for the binding activity of this complex on the C/EBPα promoter. Consistent with these findings, overexpression of ATF5 and an acetylated ATF5 mimic both promoted 3T3-L1 adipocyte differentiation, whereas short interfering RNA-mediated ATF5 downregulation inhibited this process. Furthermore, we show that the elevated expression of ATF5 is correlated with an obese phenotype in both mice and humans. In summary, we have identified ATF5 as a new cofactor of C/EBPβ and examined how C/EBPβ and ATF5 (acetylated by a p300-dependent mechanism) regulate the transcription of C/EBPα.

Obesity is a significant risk factor for a number of health problems, such as heart disease, stroke, high blood pressure, and diabetes. The hyperplasia of adipocytes seen in obesity is mimicked during the adipogenic differentiation program of 3T3-L1 preadipocytes (1). The sequential expression of genes leading to terminal adipocyte differentiation is initiated during and after the period of mitotic clonal expansion (MCE). Previous studies have shown that the expression of CCAAT/enhancer-binding protein β (C/EBPβ) (2–4) is required for the activation of peroxisome proliferator-activated receptor γ (PPARγ) and C/EBPα (5), and that PPARγ and C/EBPα sequentially induce the expression of genes encoding the protein constituents of adipocytes, including 422/aP2, SCD1, and Glut4 (6–8). To more clearly understand obesity and the hyperplasia of adipocytes, it is crucial to understand the mechanisms underlying these transcriptional signaling cascades.

C/EBPβ plays important roles in both MCE and terminal adipocyte differentiation. C/EBPβ is induced early in adipocyte differentiation and binds to the promoter regions of C/EBPα and PPARγ, activating their expression and leading to the adipocyte phenotype (3). Previous studies have shown that phosphorylation of C/EBPβ is essential in order for it to bind to the C/EBPα promoter (9). Studies from our laboratory have also shown that C/EBPβ promotes MCE by controlling cell cycle gene expression (10). In order to explain how C/EBPβ transactivates different genes during MCE and terminal adipocyte differentiation, we examined its potential cofactors using a yeast two-hybrid system (see Fig. S1 in the supplemental material), and one of the candidate cofactors identified was ATF5 on the basis of the maximum number of clones identified (see Table S1 in the supplemental material).

ATF5 is a member of the cyclic AMP (cAMP) response element-binding protein (CREB)/ATF family of basic leucine zipper (bZIP) transcription factors and plays important roles in the regulation of a variety of cellular functions, including cell proliferation, survival, and the stress response (11). The expression of ATF5 in neural progenitors and in PC12 cells maintains these cells in a proliferative state and blocks their differentiation, whereas the loss of ATF5 function leads to premature differentiation of these cells (12–14). Previous studies have shown that ATF5 also regulates osteogenic differentiation in adult and tissue-specific stem cells (15). ATF5 is downregulated during osteoblast differentiation of adipose-derived stromal cells and may play a negative role in osteogenesis in these cells. Since adipogenesis and osteogenesis have been shown to be reciprocally regulated by a number of genes in adipose-derived stem cells (ADSCs) (i.e., proteins that promote adipogenesis often inhibit osteogenesis), we hypothesize that ATF5 regulates adipogenesis. In this study, we show that ATF5 is a novel cofactor of C/EBPβ and that it can activate C/EBPα during adipocyte differentiation.

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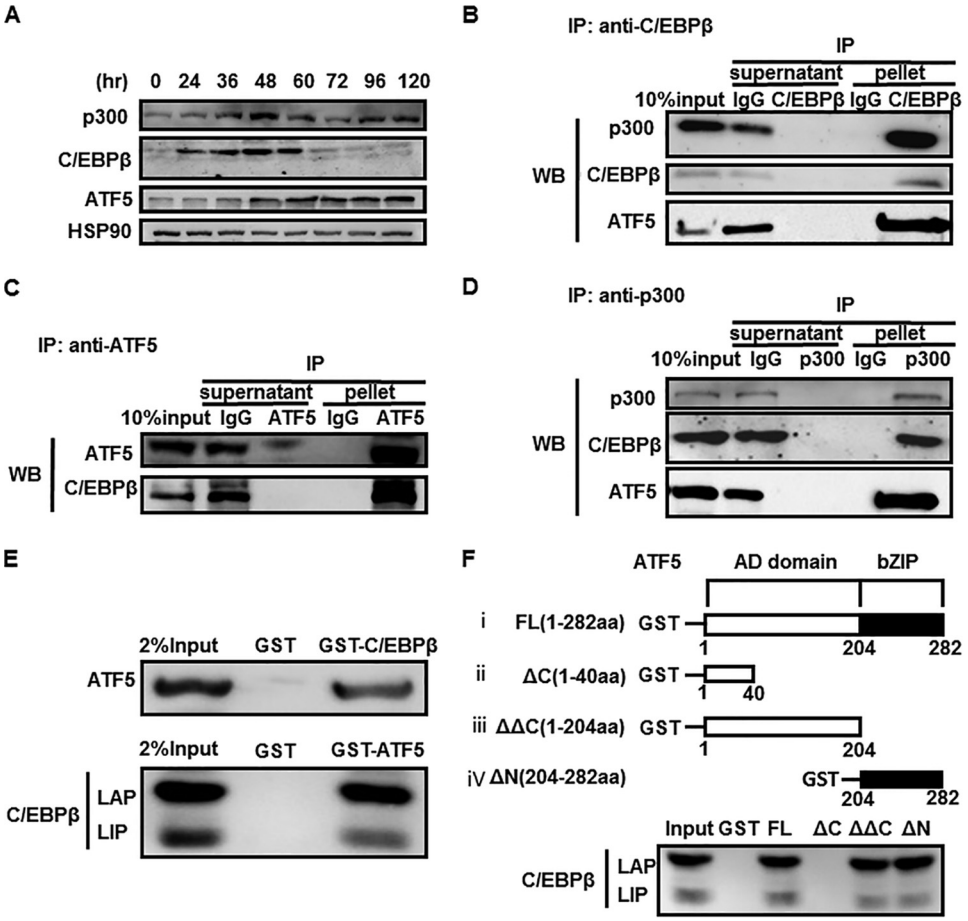

FIG 1 ATF5 interacts with C/EBPβ and p300 in 3T3-L1 cells. (A) p300, C/EBPβ, and ATF5 expression profiling in 3T3-L1 cells after induction. HSP90 served as a loading control. (B, C, and D) Interaction between ATF5, C/EBPβ, and p300. At 48 h postinduction, cell lysates were harvested and immunoprecipitated (IP) with antibodies against C/EBPβ (B), ATF5 (C), or p300 (D). The immunoprecipitated complexes were detected by Western blotting (WB) with the indicated antibodies. The band detected by the C/EBPβ antibody is liver-enriched activator protein (LAP) (34 kDa). (E) GST pulldown assays were performed to examine ATF5-C/EBPβ interaction. 3T3-L1 cell lysates were incubated with GST-fused C/EBPβ, GST-fused ATF5, and GST alone as a control. Western blotting was used to detect the complex precipitated with glutathione beads with the indicated antibodies. LIP, liver inhibitory protein. (F) Mapping of the ATF5 interaction domain within C/EBPβ using a GST pulldown assay. C/EBPβ was incubated with either GST or GST-fused ATF5, and a pulldown assay was performed to examine specific interactions. FL, full length.

MATERIALS AND METHODS

Human adipose tissue samples. Subcutaneous adipose tissue was obtained from patients ($n = 17$) undergoing plastic surgery (not related to metabolic disease) in the Shanghai Jiaotong University Affiliated Ninth People's Hospital. This study was approved by the ethics committees of the Fudan University Shanghai Medical College and is in accordance with the principles of Helsinki Declaration II. Written informed consent was obtained from each participant.

Mice. C57BL/6J mice were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. (SLAC). Mice were maintained under 12-h light/12-h dark cycles with unlimited access to food and water under specific-pathogen-free conditions. Mice were fed either a standard chow diet or a high-fat diet (51% kcal from fat beginning at 6 weeks of age). All studies were approved by the Animal Care and Use Committee of the Fudan University Shanghai Medical College and followed the NIH guidelines on the care and use of animals.

Cell culture and induction of differentiation. 3T3-L1 preadipocytes were propagated and maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% (vol/vol) calf serum (Gibco). Cells at two days postconfluence (designated day 0) were induced to differentiate with DMEM containing 10% (vol/vol) fetal bovine serum (FBS; Gibco), 1 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine until day 2. Cells were then fed with DMEM supplemented with 10% FBS and 1 μg/ml insulin for 2 days, after which they were fed every other day with DMEM containing 10% FBS. Cells were partially induced to differentiate with DMEM containing 10% (vol/vol) FBS (Gibco), 1 μg/ml insulin, and 0.5 mM 3-isobutyl-1-methyl-xanthine until day 2. Expression of adipocyte genes and acquisition of the adipocyte phenotype began on day 3 and reached a maximum on day 8. 293T cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Gibco).

ORO staining. Cells were washed three times with phosphate-buffered saline (PBS) and then fixed for 20 min with 3.7% formaldehyde. Oil Red O (ORO; 0.5% in isopropanol) was diluted with water (3:2) and incubated with the fixed cells for 2 h at room temperature. Cells were washed with water, and the stained fat droplets in the cells were visualized by light microscopy and photographed.

Plasmid constructs and siRNAs. The ATF5 gene coding sequence was amplified using the primers 5′-CCGCTCGAGATGTACCCATACGACG TCCCAGACTACGCTTCACTCCTGGCGACCCT-3′ (forward) and 5′-CCGGAATTCCTAGGTGCTGCGGGTCCTCTGGCTTCG-3′ (reverse) and then cloned into the XhoI (5′ end) and EcoRI (3′ end) restriction sites into the pcDNA3.1(−) vector. C/EBPβ was cloned into pcDNA3.1(−)

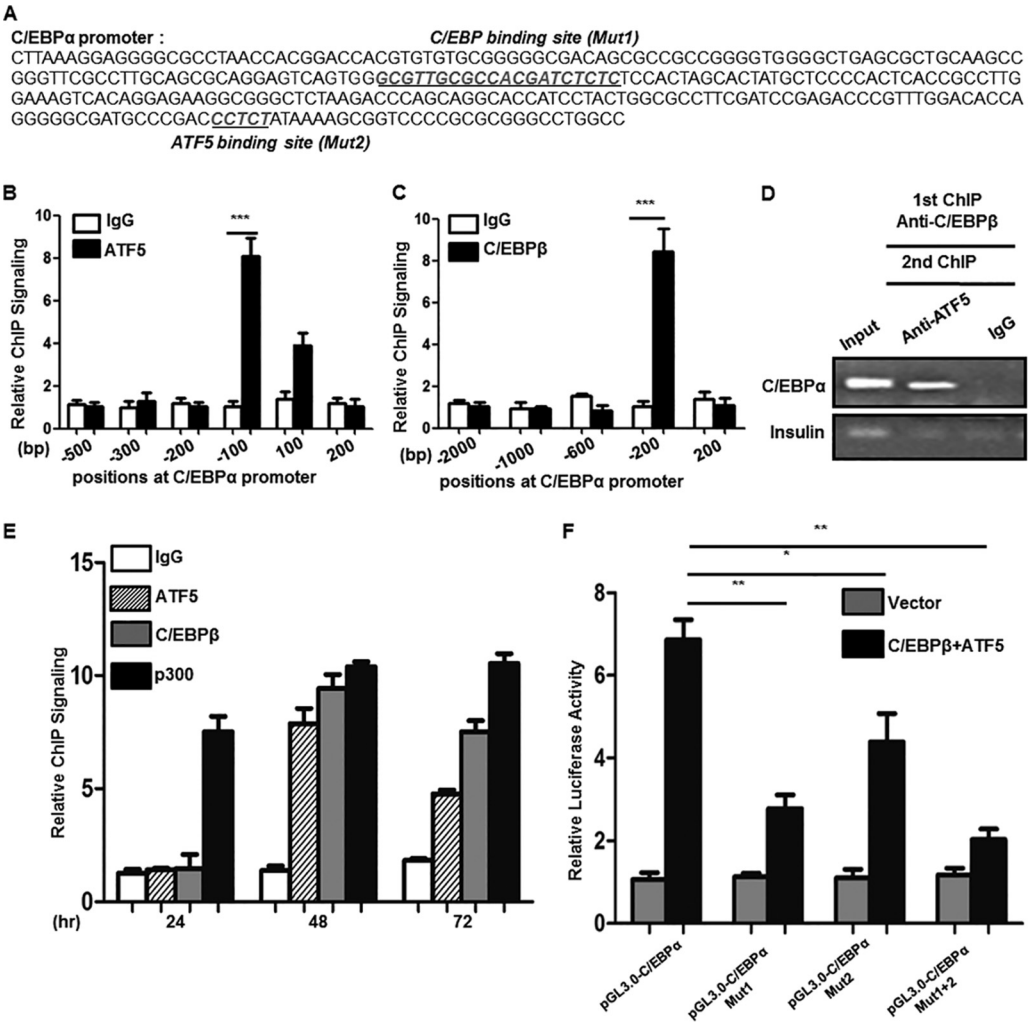

FIG 2 ATF5 and C/EBPβ bind to the C/EBPα promoter. (A) Potential C/EBPβ and ATF5 binding sites in the promoter of C/EBPα. (B) ChIP was performed to identify the binding site for ATF5 on the C/EBPα promoter. (C) ChIP was performed to identify the binding site of C/EBPβ on the C/EBPα promoter. (D) At 48 h postinduction, soluble chromatin was prepared for ChIPs and re-ChIPs with the indicated antibodies. Recovery of the C/EBPα promoter was analyzed by PCR. (E) At the different time points indicated after induction of 3T3-L1 cells, ChIP was performed to examine the binding activities of p300, ATF5, and C/EBPβ on the C/EBPα promoter. Data are normalized to the IgG controls at each time point. The insulin gene served as a negative control. (F) 293T cells were transfected with C/EBPα reporter constructs with or without the C/EBPβ (Mut1) or ATF5 (Mut2) binding site mutated and with or without the C/EBPβ and ATF5 expression vector. After 48 h, luciferase activity was analyzed and plotted. Data are presented as means ± standard deviations (SD) from three independent experiments.*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

vector for transient transfection, and C/EBPα was cloned into murine stem cell virus (MSCV) vector for generation of stably transfected cell lines. The K29 site of ATF5 was mutated to K29Q and K29R using a KOD plus mutagenesis kit (Toyobo, Japan) and the following primers: 5′-AAA CTCCCCCTGGCCCCTGCCCCCC-3′, 5′-CAGCTCCCCCTGGCCCC TGCCCCCC-3′, and 5′-CGGCTCCCCCTGGCCCCTGCCCCCC-3′. The expression vector for FLAG-p300 was kindly given by Qun-Ying Lei. For the protein pulldown assay, C/EBPβ, ATF5, and truncated domains of ATF5 proteins were inserted in frame with glutathione *S*-transferase (GST) in pGEX-6P1 (Amersham Pharmacia Biotech). When the 3T3-L1 cells reached 90 to 95% confluence, transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Small interfering RNAs (siRNAs) against p300 were purchased from Invitrogen Stealth RNAi. 3T3-L1 cells were transfected at about 50% confluence with RNA interference (RNAi) oligonucleotides using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

Immunoprecipitation. Cells were washed with PBS, scraped off plates, and collected by centrifugation. Cells were then suspended in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, and 0.5% sodium deoxycholate) for 1 h at 4°C. After centrifugation (4°C, 300 × *g*, 3 min), the supernatants were incubated with the following antibodies at 4°C overnight: anti-p300 (sc-585x), anti-ATF5 (sc-99205), anti-C/EBPβ (sc-150x), and antihemagglutinin (anti-HA; MBL561-5). The next day, protein A-agarose beads were added. After 2 h of incubation, the beads were washed with TBS-T (TBS plus 0.05% Tween 20). The immunoprecipitates were separated by SDS-PAGE and subjected to Western blotting.

Protein pulldown assay. GST fusion proteins expressed from *Escherichia coli* strain BL21 and purified on glutathione-Sepharose 4B beads. For protein pulldown assays, cells were collected and lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with protease inhibitors (Roche). Glutathione-Sepharose 4B beads were incubated with the cell lysates overnight at 4°C.

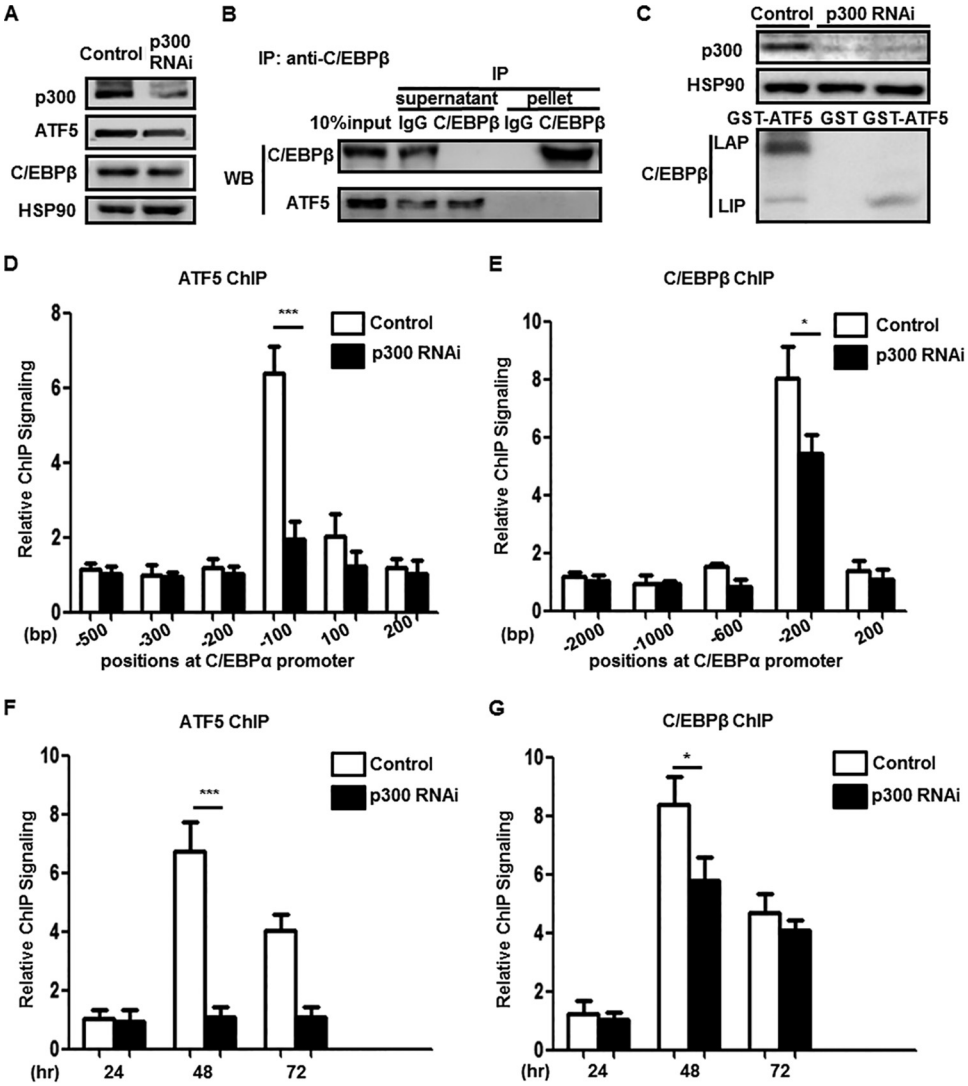

FIG 3 p300 is essential for the interaction and binding activities of ATF5 and C/EBPβ. (A) p300 siRNA was confirmed by Western blotting at 48 h postinduction. (B) The effect of p300 knockdown on the interaction between C/EBPβ and ATF5 was analyzed by immunoprecipitation. (C) GST pulldown assays were performed to examine ATF5-C/EBPβ interaction when p300 was knocked down. 3T3-L1 cell lysates were incubated with GST-fused ATF5 and GST alone as a control. Western blotting was used to detect the complex precipitated with glutathione beads with the indicated antibodies. (D and E) At 48 h postinduction in 3T3-L1 cells with or without p300 siRNA treatment, cell lysates were prepared and the enrichment levels of ATF5 (D) and C/EBPβ (E) on the C/EBPα promoter were detected via ChIP. (F and G) At the indicated times in 3T3-L1 cells treated with p300 siRNA or left untreated, ChIP was performed to examine the binding of ATF5 (F) and C/EBPβ (G) to the C/EBPα promoter. Data are presented as means ± SD from three independent experiments. *, $P < 0.05$; ***, $P < 0.001$.

The beads were washed with TTBS (TBS plus 0.05% Tween 20) 4 times. The immunoprecipitates were separated by SDS-PAGE for Western blotting.

Real-time reverse transcription-PCR (RT-PCR). Total RNA was extracted using the TRIzol method (Invitrogen) according to the manufacturer's protocol. To determine the expression level of ATF5, mRNA was reverse transcribed using a RevertAid first-strand cDNA synthesis kit (Fermentas) with random primers, followed by real-time PCR with SYBR green chemistry (Applied Biosystems). Primer sequences were the following: human ATF5, 5′-TGGCTTCTCTGACTGGATGA-3′ (forward) and 5′-TCCATCTGTTCCAGCTCCTT-3′ (reverse); 18S (GenBank accession number NM_003278), 5′-CGCCGCTAGAGGTGAAATTCT-3′ (forward) and 5′-CATTCTTGGCAAATGCTTTCG-3′ (reverse).

Luciferase reporter assay. The promoter region of the mouse C/EBPα gene (approximately bp −2044 to bp +100 from the transcription start site) was amplified from the genomic DNA of 3T3-L1 cells via PCR and cloned into the PGL3-basic vector (Promega, Madison, WI). Cells were transfected by Lipofectamine 2000 (Invitrogen), and luciferase activity was measured using the dual luciferase reporter assay (Promega), normalizing firefly luciferase to renilla activity.

ChIP analysis and re-ChIP. Samples underwent immunoprecipitation using antibodies against p300, C/EBPβ, ATF5, or a nonspecific IgG control (Santa Cruz Biotechnologies, Santa Cruz, CA). Immunoprecipitates were eluted and reverse cross-linked by incubation overnight at 65°C. Genomic DNA was extracted using a PCR purification kit (Qiagen, Valencia, CA). Chromatin immunoprecipitation (ChIP) data were normalized to control IgG or expressed as a percentage of the input. Re-ChIPs were done essentially the same as primary ChIPs. Beads from the first ChIPs were incubated with 10 mM dithiothreitol (DTT) at 37°C for 30 min, and the eluted immune complexes were diluted 1:50 in dilution buffer, followed by reimmunoprecipitation with the second antibody.

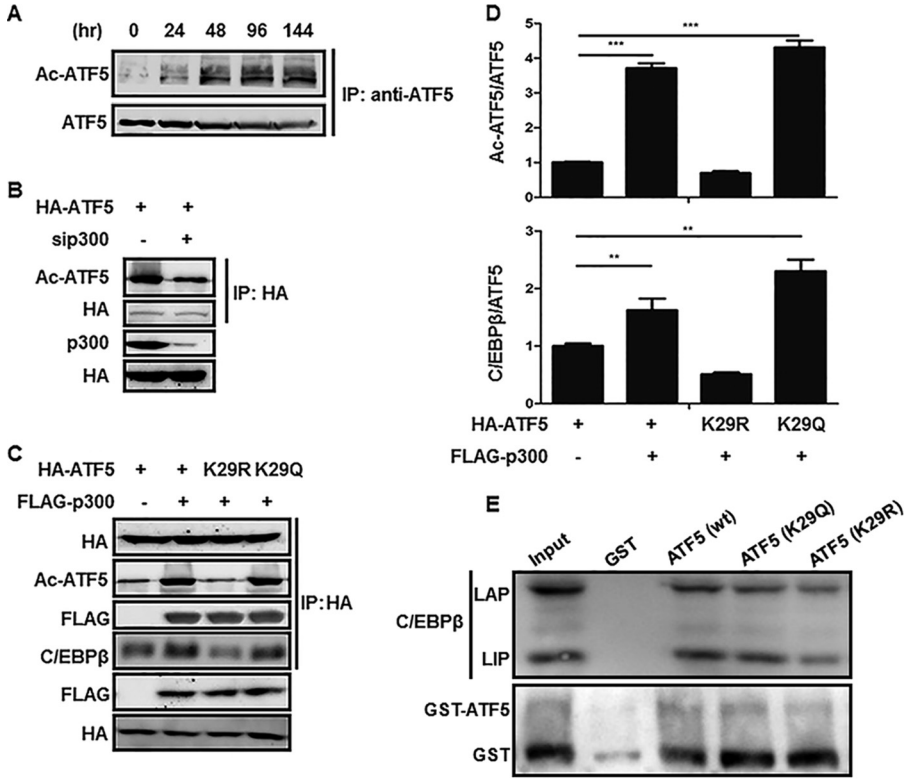

FIG 4 p300 acetylation of ATF5 enhances its interaction with C/EBPβ in 3T3-L1 cells. (A) Relative expression levels of acetylated ATF5 with equivalent amounts of ATF5 at the indicated times in 3T3-L1 cells after induction. (B) Anti-HA immunoprecipitates from 3T3-L1 cells transiently transfected with the indicated constructs and with or without p300 treatment were immunoblotted with antibodies against Ac-K or HA. The lower panel (input) shows expression of ATF5 and p300 in transfected cells. (C) 3T3-L1 cells were transiently transfected with the indicated constructs expressing ATF5 mutants. (D) Acetylated ATF5 and ATF5 shown in panel C were quantified by ImageJ, and the data were expressed as acetylated ATF5/ATF5 ratios (upper). The interaction activity of C/EBPβ with ATF5 was also quantified and expressed as C/EBPβ/ATF5 ratios (lower). (E) *In vitro* binding assays were performed to further examine the interaction between C/EBPβ and ATF5 mutants. 3T3-L1 cell lysates were incubated with equivalent amounts of GST-fused ATF5 (wt), ATF5 (K29Q), and ATF5 (K29R) or with GST alone as a control. Western blotting was used to detect the complex precipitated with glutathione beads with the indicated antibodies. Data are presented as means ± SD from three independent experiments. **, $P < 0.01$; ***, $P < 0.001$.

Western blotting. Cells were scraped into lysis buffer containing 2% SDS and 50 mM Tris-HCl (pH 6.8). Lysates were quantitated, and equal amounts of protein were subjected to SDS-PAGE and then immunoblotted with antibodies against C/EBPβ, ATF5, HSP90, C/EBPα, PPARγ, 422/aP2, p300, FLAG, hemagglutinin (HA), or acetyl (Ac). Antibodies against C/EBPβ and C/EBPα were obtained from the Department of Biological Chemistry at the Johns Hopkins University School of Medicine. Antibodies against ATF5 (ab69936) were obtained from Abcam (Cambridge, MA). Antibodies against p300 (sc-585x) and HSP90 (sc-7947) were obtained from Santa Cruz Biotechnology. Antibodies against FLAG (F1804) were obtained from Sigma (St. Louis, MO), and antibodies against HA (561-5) were obtained from MBL (Nagoya, Japan).

RESULTS

ATF5 interacts with C/EBPβ and p300 in 3T3-L1 cells. Previous studies have shown that C/EBPβ plays important roles during 3T3-L1 preadipocyte terminal differentiation and during MCE (3, 4, 16). To better understand the mechanisms of adipogenesis, we screened for potential cofactors of C/EBPβ using a yeast two-hybrid system (see Fig. S1 in the supplemental material). The most reliable protein interaction in this system was identified to be ATF5 (see Table S1 in the supplemental material). The expression of ATF5 gradually increased and reached a peak at 48 h postinduction during 3T3-L1 adipocyte differentiation (Fig. 1A), while the expression of C/EBPβ increased 4 h after induction and remained at a high level until 60 h after induction. In 3T3-L1 cells, coimmunoprecipitation studies showed that ATF5 could interact with C/EBPβ at 48 h postinduction (Fig. 1B and C). In order to further determine whether ATF5 interacted with C/EBPβ, we performed GST pulldown assays. GST-fused C/EBPβ rather than GST alone pulled down ATF5; likewise, GST-fused ATF5 pulled down C/EBPβ (Fig. 1E). We also identify that the interaction between ATF5 (amino acids 1 to 282) and C/EBPβ did not require the N-terminal (amino acids 1 to 40) domain of ATF5 (Fig. 1F).

Previous reports show that C/EBPβ recruits p300/CBP via interaction with its E1A-binding domain (17), and p300 is a transcriptional coactivator interacting with ATF5 (18). Therefore, we used coimmunoprecipitation experiments to examine whether p300 interacted with ATF5 and C/EBPβ to form a complex. We found that an anti-p300 antibody efficiently immunoprecipitated C/EBPβ and ATF5 (Fig. 1D), and that an anti-C/EBPβ antibody immunoprecipitated ATF5 and p300 (Fig. 1B), indicating that both ATF5 and p300 bind to C/EBPβ to form a complex. Collectively, our results indicate that ATF5 plays a role during adipocyte differentiation through its interaction with C/EBPβ and p300.

ATF5 and C/EBPβ bind to the C/EBPα promoter in 3T3-L1 cells. As ATF5 interacts with C/EBPβ, we hypothesize that ATF5

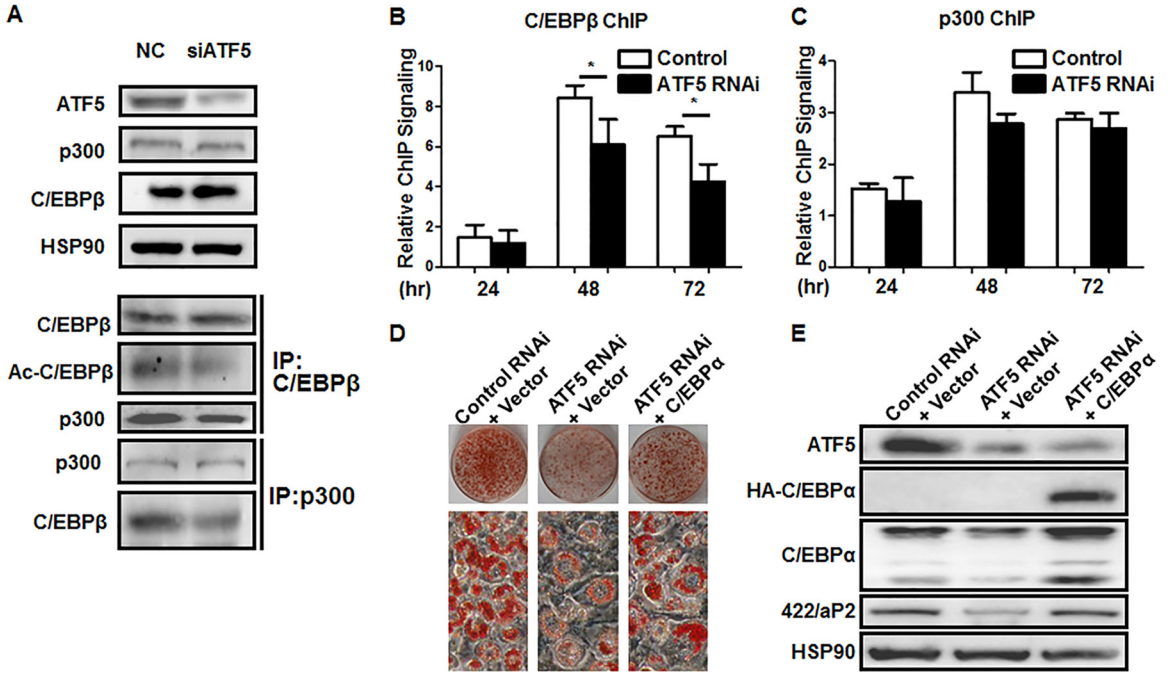

FIG 5 Downregulation of ATF5 inhibits adipogenesis through C/EBPα by impairing the interaction with p300-C/EBPβ. (A) 3T3-L1 preadipocytes were treated with ATF5 siRNA (siATF5) or negative-control siRNA (NC), and the expression levels of ATF5, p300, and C/EBPβ were analyzed by Western blotting. Anti-C/EBPβ or anti-p300 immunoprecipitates from 3T3-L1 cells treated with ATF5 siRNA or negative-control siRNA were immunoblotted with antibodies against C/EBPβ, Ac-K, or p300. (B and C) At the indicated times in 3T3-L1 cells with or without ATF5 siRNA treatment, ChIP was performed to examine the binding of C/EBPβ and p300 to the C/EBPα promoter. (D and E) 3T3-L1 preadipocytes were infected with empty MSCV vector or MSCV-C/EBPα with HA tag. Cells then were transfected with control RNAi or ATF5 RNAi. (D) Six days after induction, cells were ORO stained and photographed. (E) The expression levels of ATF5, HA-C/EBPα, C/EBPα, 422/aP2, and HSP90 were analyzed by Western blotting. Data are presented as means ± SD from three independent experiments. *, $P < 0.05$.

regulates the same target genes as C/EBPβ during adipogenesis. We searched the PubMed database to identify genes with potential ATF5 binding sites within ±2,000 bp of their transcription start site (19) and found that C/EBPα, but not PPARγ, has potential ATF5 binding sites (Fig. 2A). Interestingly, a potential ATF5 binding site on the C/EBPα promoter was identified very close to the C/EBPβ binding site. Using chromatin immunoprecipitation combined with quantitative PCR (ChIP-qPCR), we found that C/EBPβ and ATF5 bind to the C/EBPα promoter at bp −200 and −100, respectively (Fig. 2B and C). Furthermore, re-ChIP analysis showed that C/EBPβ and ATF5 both bind to C/EBPα promoter at 48 h postinduction (Fig. 2D). Similar ChIP assays were performed to detect the enrichment levels of ATF5, C/EBPβ, and p300 on the C/EBPα promoter at different time points during 3T3-L1 differentiation. As shown in Fig. 2E, the enrichment levels of ATF5 and C/EBPβ reached their highest levels at 48 h postinduction, while p300 remained high at all time points. The level of ATF5 decreased dramatically after 48 h, although the binding activity of C/EBPβ remained high. These data indicate that C/EBPα is a downstream target of both C/EBPβ and ATF5. Furthermore, we confirmed the binding sites of ATF5 and C/EBPβ on the CEBPα promoter by luciferase reporter assay. As indicated in Fig. 2F, C/EBPβ and ATF5 transactivate C/EBPα, and both C/EBPβ and ATF5 binding site mutations significantly reduce transactivation.

p300 is necessary for the interaction of ATF5 with C/EBPβ and for the DNA-binding activity of ATF5. As p300, ATF5, and C/EBPβ form a complex, in order to address whether p300 is required for the interaction between ATF5 and C/EBPβ, we knocked down p300 and found that ATF5 could no longer be immunoprecipitated with C/EBPβ (Fig. 3A and B). These data clearly indicate that p300 is required for the association of ATF5 with C/EBPβ. To further clarify the significance of p300, we have used siRNA to knock down p300 with 3T3-L1 cell lysates and found that ATF5 could no longer be pulled down with C/EBPβ-LAP, which is the transcriptional activity isoform (Fig. 3C), by GST pulldown assay.

We also detected whether p300 could regulate the DNA binding activities of ATF5 and C/EBPβ by ChIP assays. When p300 was knocked down using siRNA, at 48 h postinduction the enrichment levels of ATF5 and C/EBPβ on the C/EBPα promoter were reduced 3-fold (anti-ATF5) and 1.5-fold (anti-C/EBPβ) (Fig. 3D to G). These results indicate that p300 is more essential for the binding of ATF5 to the C/EBPα promoter than it is for the binding of C/EBPβ.

Acetylation of ATF5 influences its interaction with C/EBPβ in 3T3-L1 cells. p300 is an acetyltransferase, and previous studies have shown that p300-acetylated ATF5 plays a central role in ATF5-dependent gene activation (20). We examined the acetylation of ATF5 during 3T3-L1 differentiation and found that its level of acetylation was significantly increased at 48 h postinduction (Fig. 4A). To investigate whether ATF5 was acetylated by p300, downregulation of p300 significantly decreased ATF5 acetylation levels (Fig. 4B), while upregulation of p300 increased ATF5 acetylation (Fig. 4C). As p300 acetylates protein substrates at a Gly-Lys (GK) consensus motif (at K29 in ATF5) (20), we mutated ATF5 K29 to arginine (K29R) to mimic an ATF5 protein which cannot

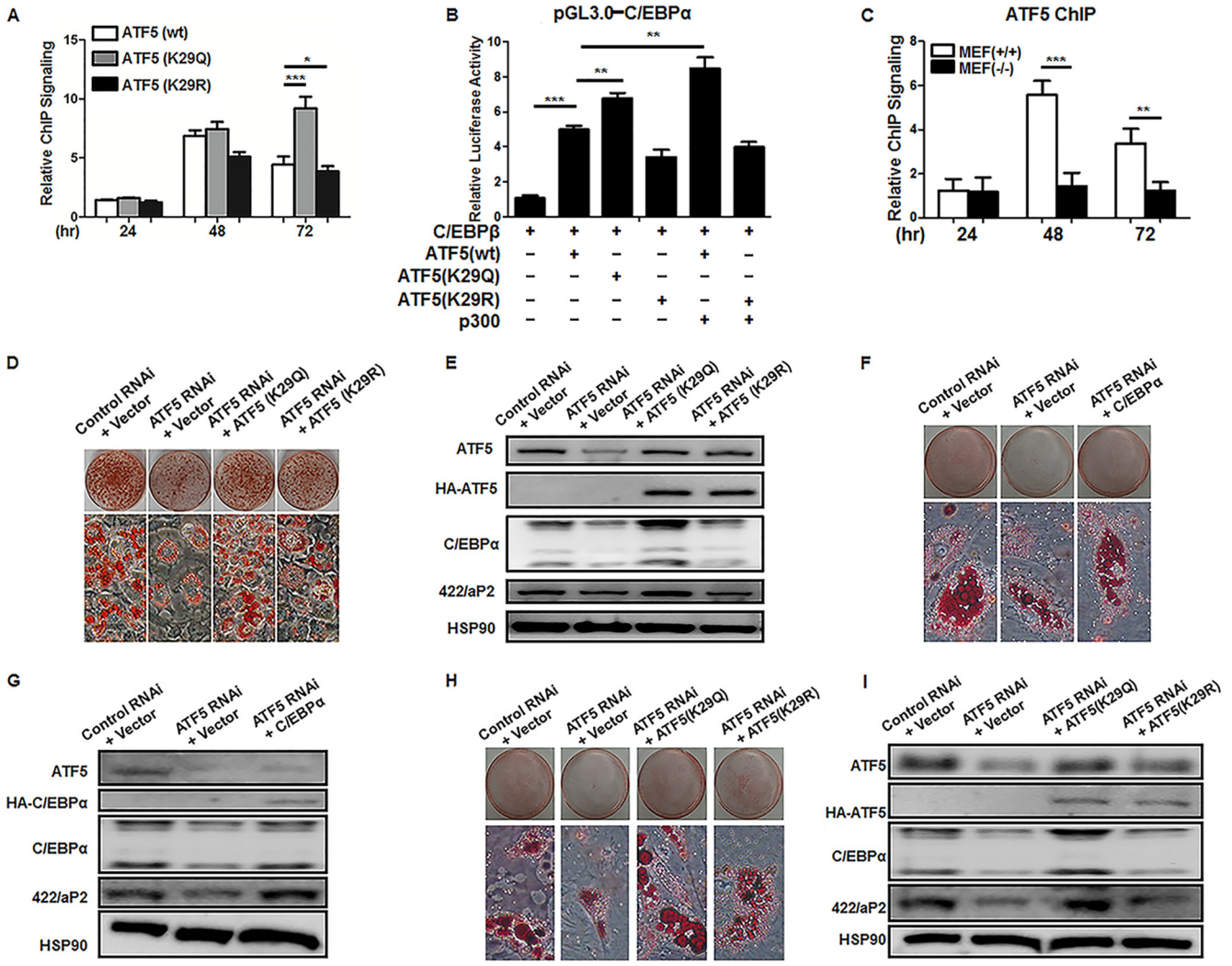

FIG 6 p300-dependent acetylated ATF5 promotes transactivation of C/EBPα by C/EBPβ, resulting in adipogenesis. (A) At the different time points indicated after induction of 3T3-L1 cells, ChIP was performed to examine the binding activities of ATF5 (wt), ATF5 (K29Q), and ATF5 (K29R) on the C/EBPα promoter using HA antibody. Data are normalized to the IgG controls at each time point. The insulin gene served as a negative control. (B) Luciferase assays were performed with 293T cells cotransfected with the indicated constructs. Cell extracts were prepared, and luciferase activity was measured and normalized to *Renilla* activity 48 h later. (C) At the indicated times in MEFs with C/EBPβ [MEF(+/+)] or without C/EBPβ [MEF(−/−)], ChIP was performed to examine the binding activities of ATF5 to the C/EBPα promoter. (D and E) HA-tagged ATF5 (K29Q) and HA-tagged ATF5 (K29R) were ectopically expressed in 3T3-L1 preadipocytes with ATF5 siRNA. (D) Six days after partial induction, cells were stained with Oil Red O (ORO). (E) The expression levels of ATF5, HA-ATF5, C/EBPα, 422/aP2, and HSP90 were analyzed by Western blotting. (F and G) hADSCs were infected with empty MSCV vector or MSCV-C/EBPα with HA tag. Cells then were transfected with control RNAi or ATF5 RNAi. (F) Ten days after induction, cells were ORO stained and photographed. (G) The expression levels of ATF5, HA-C/EBPα, C/EBPα, 422/aP2, and HSP90 were analyzed by Western blotting. (H and I) HA-tagged ATF5 (K29Q) and HA-tagged ATF5 (K29R) were ectopically expressed in hADSCs with ATF5 siRNA. (H) Ten days after partial induction, cells were stained with ORO. (I) The expression levels of ATF5, HA-ATF5, C/EBPα, 422/aP2, and HSP90 were analyzed by Western blotting. Data are presented as means ± SD from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

be acetylated by p300 (Fig. 4C and D, upper) and mutated to glutamine (K29Q) to mimic acetylated ATF5. Using immunoprecipitation experiments to determine whether acetylation at K29 influences the interaction between ATF5 and C/EBPβ, we found that p300 enhanced the interaction between ATF5 and C/EBPβ and the K29Q mutant showed an increase but K29R mutant a slight decrease in its association with C/EBPβ (Fig. 4C and D, lower). *In vitro* GST pulldown also showed that GST-fused ATF5 (K29R) decreased its interaction with C/EBPβ compared to that with ATF5 (wild type [wt]) and ATF5 (K29Q) (Fig. 4E). These results indicate that the acetylation of ATF5 influences its interaction with C/EBPβ during adipocyte differentiation.

Downregulation of ATF5 inhibits adipogenesis through C/EBPα by impairing the interaction with p300-C/EBPβ. Further investigations were performed to explore the role of ATF5 in adipogenesis. When ATF5 was downregulated with no change of p300 and C/EBPβ, the acetylation level of C/EBPβ was slightly decreased and the interaction between C/EBPβ and p300 was slightly impaired (Fig. 5A). Meanwhile, the enrichment level of C/EBPβ was decreased, but p300 was not impaired in ATF5

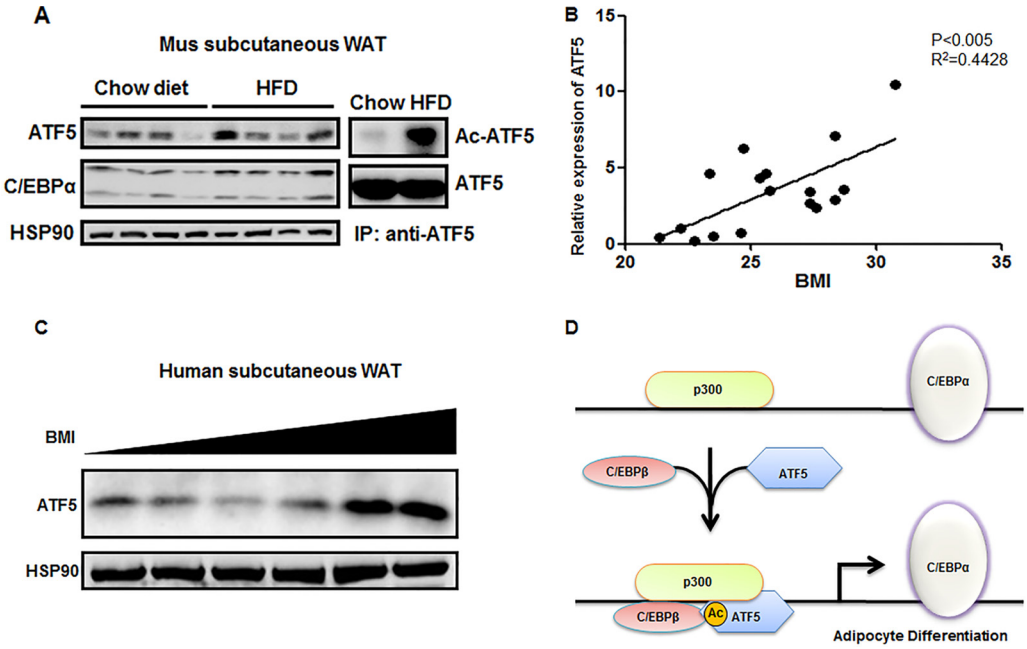

FIG 7 Elevated expression of ATF5 correlates with the obese phenotype. (A) Western blotting of ATF5, acetylated ATF5, and C/EBPα expression levels in subcutaneous white adipose tissue (WAT) from chow diet-fed ($n = 4$) and high-fat diet-fed mice ($n = 4$). (B) Linear regression analysis of body mass index (BMI) and ATF5 mRNA levels in subcutaneous ($n = 17$) adipose tissue from healthy individuals undergoing plastic surgery irrelevant to metabolic disease. (C) Western blotting of ATF5 expression in human subcutaneous WAT. (D) Scheme of the regulation of adipogenesis by ATF5. C/EBPβ/ATF5/p300 complex binds to the C/EBPα promoter to further regulate adipogenesis, in which C/EBPβ transactivates C/EBPα through ATF5 acetylation by p300.

knockdown cells (Fig. 5B and C). Downregulation of ATF5 by siRNA (Fig. 5E) inhibited C/EBPα expression and adipogenesis (Fig. 5E), as indicated by decreased ORO staining (Fig. 5D). To clarify whether ATF5 influences adipogenesis through C/EBPα, we overexpressed C/EBPα in ATF5-deficient cells. We found that forced expression of C/EBPα could rescue adipocyte differentiation impaired by ATF5 knockdown (Fig. 5D and E). These data reinforced that C/EBPα mediates the effect of ATF5 on the cells.

p300-acetylated ATF5 increases the transactivation of C/EBPβ and promotes 3T3-L1 adipocyte differentiation. ChIP assays with ATF5 (wt), ATF5 (K29Q), and ATF5 (K29R) were performed to determine whether the acetylation of ATF5 influences its binding activity to the C/EBPα promoter. As shown in Fig. 6A, the binding activity of wt ATF5 reached a peak at 48 h and then decreased at 72 h, but hyperacetylation of ATF5 remained high until 72 h; on the other hand, the hypoacetylation of ATF5 reduced this activity. These data indicate that hyperacetylation enhances the binding of ATF5. Furthermore, we used luciferase assays to analyze the effects of ATF5 and acetylated ATF5 on the transactivation properties of C/EBPβ. When cells were cotransfected with ATF5 (wt) and C/EBPβ, the transactivation of C/EBPα via C/EBPβ was increased (Fig. 6B) but the transactivation of PPARγ via C/EBPβ was not (data not shown), indicating that C/EBPα is the target gene of the ATF5-C/EBPβ complex. Compared to wt ATF5, the acetylated ATF5 mimic (K29Q) increased luciferase activity, while the nonacetylated ATF5 mimic (K29R) caused a modest decrease. Additionally, luciferase activity was increased 1.6-fold by overexpression of p300 and ATF5 (Fig. 6B), indicating that p300-acetylated ATF5 further increases the ability of C/EBPβ to transactivate C/EBPα. Actually, C/EBPβ is necessary for the effect of ATF5 on C/EBPα expression in mouse embryo fibroblasts (MEFs) (Fig. 6C; also see Fig. S2 in the supplemental material).

To examine the roles of acetylated ATF5 in adipogenesis, we forced the expression of ATF5 (K29Q) and ATF5 (K29R) in ATF5-deficient cells. We found that ATF5 (K29Q) overexpression intensively promoted adipogenesis, whereas ATF5 (K29R) overexpression only slightly partially recovered the adipogenesis (Fig. 6D and E). These data demonstrate that acetylated ATF5 mutants play stimulatory roles during 3T3-L1 adipocyte differentiation. Additionally, we found a similar phenotype in human ADSCs (hADSCs) (Fig. 6F to I), which further supports our claim that acetylation of ATF5 enhances adipogenesis.

DISCUSSION

C/EBPβ is a member of the transcription factor family of functionally and structurally related bZIP DNA-binding proteins (17). C/EBPβ is expressed in numerous tissues, including the liver, adipose tissue, ovary, lung, kidney, mammary gland, and hematopoietic tissues, and it regulates many genes involved in a number of cell processes, including metabolism, hematopoiesis, adipogenesis, immune responses, and morphogenesis (18, 21). Therefore, C/EBPβ plays various roles during different processes across a number of cell types. Previous studies have shown that forming different complexes with different cofactors enables C/EBPβ to function differently. For example, C/EBPβ interacts with the serum response factor (SRF) and antagonizes its effect, acting as a master regulator of the growth and differentiation of cardiomyocytes (22). In addition, calmodulin (CaM) directly interacts with C/EBPβ, inhibiting its ability to promote liver proliferation during the acute-phase response (APR) (23).

In 3T3-L1 cell differentiation, C/EBPβ acts as a multifunc-

tional protein, taking part in both MCE and terminal differentiation (3). In MCE, C/EBPβ and Kdm4b form a complex and transactivate cell cycle genes to promote the process of hyperplasia (10). When the cells exit MCE, C/EBPβ is dually phosphorylated and occupies the promoter of C/EBPα, leading to terminal differentiation of the cells. In this study, we have demonstrated that ATF5 forms a complex with C/EBPβ and p300, and that the p300-dependent acetylation of ATF5 enhances the transactivation of C/EBPα by C/EBPβ, leading to adipocyte differentiation.

C/EBPα and PPARγ are master regulators of the process of adipocyte differentiation. It is likely that C/EBPα stimulates adipogenesis and also induces the expression of genes that control adipocyte differentiation, so that there is homeostasis of the adipose tissue *in vivo* (24). In addition to acting as a critical adipogenic transcription factor, C/EBPα is required to confer insulin sensitivity on adipocytes (25), while PPARγ regulates fatty acid storage and glucose metabolism (26). Our data indicate that ATF5 binds to the promoter of C/EBPα but not the promoter of PPARγ, suggesting that ATF5 regulates adipogenesis as well as insulin sensitivity through C/EBPα. Since C/EBPα appears to be involved in a cross-regulatory loop with PPARγ (27), it is possible that ATF5 indirectly influences PPARγ through C/EBPα. At the same time, we found that the expression of C/EBPβ is downregulated when 3T3-L1 preadipocytes enter terminal differentiation, while the levels of C/EBPα and ATF5 remain high. Since C/EBPβ and C/EBPα belong to the same family of transcription factors and both have the leucine zipper motif, the possibility of the interaction between ATF5 and C/EBPα requires further investigation.

In conclusion, we have explored the novel transcription factor ATF5 in 3T3-L1 preadipocytes and revealed a novel mechanism for the regulation of C/EBPβ via acetylated ATF5 and p300, which contributes to the process of adipogenesis (Fig. 7D). Additionally, we found that the level of expression of ATF5 in the subcutaneous tissue of mice and humans was directly correlated with adiposity (Fig. 7A, B, and C), and the acetylated ATF5 levels in mice under a high-fat diet was higher than that under a chow diet (Fig. 7A). Although adipogenesis is not necessarily related to obesity, many reports showed that more severe obesity or obesity arising in childhood typically also involves an increased number of fat cells (hyperplastic obesity) (28). We tend to think that ATF5 contributes to obesity through promoting adipogenesis. Meanwhile, the reason why the expression of ATF5 obviously increased in obese subjects requires further investigation.

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