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Ajuba functions as a co-activator of C/EBPβ to induce expression of PPARγ and C/EBPα during adipogenesis

Han Yan [a,b], Qi Li [a], Mengying Li [a], Xiuqun Zou [a], Ningning Bai [c], Zichao Yu [d], Jie Zhang [a], Dan Zhang [a], Qun Zhang [a], Jiamin Wang [a], Hao Jia [a,*], Yingjie Wu [d,e,**], Zhaoyuan Hou [a,b,***]

[a] Hongqiao International Institute of Medicine, Tongren Hospital/Faculty of Basic Medicine, Shanghai Jiaotong University School of Medicine, Shanghai, 200025, China
[b] Shanghai Key Laboratory for Tumor Microenvironment and Inflammation, Department of Biochemistry & Molecular Cellular Biology, Shanghai Jiaotong University School of Medicine, Shanghai, 200025, China
[c] Shanghai Key Laboratory of Diabetes, Shanghai Institute for Diabetes, Shanghai Clinical Medical Centre of Diabetes, Shanghai Key Clinical Centre of Metabolic Diseases, Department of Endocrinology and Metabolism, The Sixth People's Hospital, Shanghai Jiaotong University, Shanghai, 200233, China
[d] Shandong Provincial Hospital, Shandong Laboratory Animal Center, Science and Technology Innovation Center, Shandong First Medical University & Shandong Academy of Medical Sciences, Jinan, Shandong, 250021, China
[e] Department of Molecular Pathobiology, New York University College of Dentistry, New York, 10010, USA

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ABSTRACT

Adipogenesis is regulated by a complicated network of transcription factors among which PPARγ and C/EBP family members are the major regulators. During adipogenesis, C/EBPβ is induced early and then transactivates PPARγ and C/EBPα, which cooperatively induce genes whose expressions give rise to the mature adipocyte phenotype. Identifying the factors that influence the expression and activity of C/EBPβ should provide additional insight into the mechanisms regulating adipogenesis. Here, we demonstrate that depletion of Ajuba in 3T3-L1 cells significantly decreases mRNA and protein levels of PPARγ and C/EBPα and impairs adipocyte differentiation, while overexpression increases expression of these genes and promotes adipocyte differentiation. Moreover, restoration of C/EBPα or PPARγ expression in Ajuba-deficient 3T3-L1 cells improves the impaired lipid accumulation. Mechanistically, Ajuba interacts with C/EBPβ and recruits CBP to facilitate the binding of C/EBPβ to the promoter of PPARγ and C/EBPα, resulting in increased H3 histone acetylation and target gene expression. Collectively, these data indicate that Ajuba functions as a co-activator of C/EBPβ, and may be an important therapeutic target for combating obesity-related diseases.

1. Introduction

Obesity is a public health problem, correlating closely with various diseases such as cardiovascular disease, type 2 diabetes and serval types of cancer (Haslam. and James., 2005). The expansion of adipose tissue size can be driven by hyperplasia (differentiation of resident pre-adipocytes to adipocytes) and/or hypertrophy (amplification of existing adipocytes)(Wang et al., 2013). Adipogenesis is a two-step process: the commitment of mesenchymal stem cells (MSCs) to adipocyte lineage without any morphological changes and the differentiation of pre-adipocytes into triglyceride-filled mature adipocytes (Tontonoz and Spiegelman, 2008; Tang and Lane, 2012; Rosen and MacDougald, 2006; Poulos et al., 2016; Ghaben. and Scherer., 2019; Farmer, 2006). BMP and Wnt pathways regulate the commitment of stem cell to adipocyte lineage (Huang et al., 2009; Bowers et al., 2006; Yuan et al., 2016; Ross et al., 2000). The regulatory mechanisms governing differentiation of preadipocytes into mature adipocytes have been investigated in vitro using 3T3-L1 preadipocytes (Ruiz-Ojeda et al., 2016). Induction of differentiation is triggered in the presence of a cocktail consisting of insulin, dexamethasone and isobutyl-methylxanthine in fetal calf serum-containing medium (Farmer, 2006). Adipocyte differentiation is a highly orchestrated process governed by a transcription factor cascade

* Corresponding author.
** Corresponding author. Shandong Provincial Hospital, Shandong Laboratory Animal Center, Science and Technology Innovation Center, Shandong First Medical University & Shandong Academy of Medical Sciences, Jinan, Shandong 250021, China.
*** Corresponding author. Shanghai Key Laboratory for Tumor Microenvironment and Inflammation, Department of Biochemistry & Molecular Cellular Biology, Shanghai Jiaotong University School of Medicine, Shanghai, 200025, China.
E-mail addresses: fonney@sjtu.edu.cn (H. Jia), yingjiewu@dmu.edu.cn (Y. Wu), houzy@sjtu.edu.cn (Z. Hou).

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among which PPARγ (peroxisome proliferator-activated receptor) and C/EBP (the CCAAT/enhancer-binding protein) family members are the major regulators (Ghaben. and Scherer., 2019). In response to hormonal stimulation, C/EBPβ is induced early and undergoes phosphorylation to acquire DNA-binding activity (Tang and Lane, 2012). "Activated" C/EBPβ then transactivates PPARγ and C/EBPα, which cooperatively induce adipogenic genes to produce the mature adipocytes phenotype (Tontonoz and Spiegelman, 2008). Consequently, identifying the factors that influence the expression and activity of C/EBPβ is extremely important in providing additional insight into the mechanisms regulating adipogenesis.

C/EBPβ (the CCAAT/enhancer-binding protein β) belongs to C/EBP transcription factor family which includes six members named as C/EBPα, β, γ, δ, ε, and ζ, characterized by a leucine zipper domain for dimerization and a DNA-binding domain in the C-terminal region (Ramji and Foka, 2002; Lane et al., 1999). Accumulating evidence suggests that C/EBPβ participates in a variety of cell programs such as cell differentiation and proliferation, cell death and survival, metabolism as well as immune responses (Pulido-Salgado et al., 2015). Double disruption of C/EBPβ and C/EBPδ (which is considered to be redundancy function of C/EBPβ) genes in mice remarkably reduce adipose tissue mass, suggesting that C/EBPβ is indispensable for adipocyte tissue formation (Tanaka et al., 1997). During the adipogenesis in 3T3-L1 cells, C/EBPβ up-regulates the expression of mitosis-associated genes to facilitate the mitotic clonal expansion at the early stage of differentiation and transactivates PPARγ and C/EBPα at the late stage of differentiation (Guo et al., 2015). C/EBPβ transcriptional activities are affected by many factors including posttranslational modifications and association with co-factors (Guo et al., 2015). Co-factors include co-activators, such as CBP/p300 and P/CAF, and co-repressors, such as NCoR/SMRT and HDACs/SIRTs, modulate C/EBPβ transcriptional activity via chromatin remodeling and direct interactions with basal transcription complexes (Pulido-Salgado et al., 2015).

Ajuba is a member of the Ajuba/Zyxin protein family and is characterized by three LIM domains at their C-terminus and a proline-rich amino-terminal preLIM region (Schimizzi and Longmore, 2015). The LIM domain consists of two tandem zinc fingers that primarily mediate protein-protein interactions (Schimizzi and Longmore, 2015). Ajuba protein contains a nuclear export sequence (NES) in the preLIM region and a nuclear localization sequence in the LIM region, which confers its ability to shuttle between the nucleus and cytoplasm (Kanungo et al., 2000). In the nucleus, Ajuba plays an important role as transcriptional co-regulators (Jia et al., 2020). Ajuba was identified as a corepressor of Snail/Slug mediating epithelial-mesenchymal transition by coupling Snail1 with PRMT5 (protein arginine methyltransferase 5) to form a transcriptional repressor complex (Hou et al., 2008). Ajuba restricts the transcriptional activity of Isl1 and repress the SHF (second heart field) progenitor cell expansion, migration, and differentiation (Witzel et al., 2012). Ajuba interacts with RARs (retinoic acid receptors) and RXRs (rexinoid receptor) and negatively regulates retinoic acid signaling in P19 cells (Hou et al., 2010). Ajuba enhances the ERα transcriptional activity via recruiting DBC1 and CBP/p300 to modify the acetylation of ERα in the breast cancer cells (Xu et al., 2019).

We have previously shown that Ajuba promotes adipogenesis via functioning as a co-activator of PPARγ(Li et al., 2016). However, we observed that depletion of Ajuba in 3T3-L1 cells decreased the PPARγ expression at the early stage of differentiation, leading us to hypothesizing that Ajuba may act upstream of PPARγ to regulate adipogenesis. Here, we report that Ajuba promotes adipogenesis by enhancing the transactivity of C/EBPβ to increase the expression of PPARγ and C/EBPα.

2. Materials and methods

2.1. Plasmids

The promoter region of mouse PPARγ (−650 to + 32 bp) was amplified via PCR from the genomic DNA of 3T3-L1 cells and subcloned into pGL3-basic-vector. The PPARγ and C/EBPα gene coding sequences were amplified from the cDNA of 3T3-L1 cells and subcloned into PCDH-CMV-GFP-vector. PLKO.1-shAjuba, pbabe-myc-Ajuba, pMEX-6myc-Ajuba, and its truncation mutants were constructed as previously described (Hou et al., 2010). PGL3-C/EBPα was gifted by professor Qiqun Tang. PLKO.1-shC/EBPβ were purchased (Thermal). The target sequences for the shRNA in lentivirus vectors were as follows: siAjuba: 5′-CACCTGTATCAAGTGCAACAA-3′, siC/EBPβ: 5′-CAAGGCCAAGATGCGCAACCT-3'.

2.2. Cell culture and 3T3-L1 cell differentiation

3T3-L1 cells and 293 T cells were cultured in DMEM (MA0212, Meilunbio) supplemented with 10% FBS and 1% Penicillin/Streptomycin at 37 °C, 5% CO_2. Two days' post confluence, 3T3-L1 cells were induced in the presence of a cocktail containing 0.5 mM IBMX (I7018, Sigma-Aldrich), 1uM dexamethasone (D4902, Sigma-Aldrich), 1ug/ml insulin (I5500, Sigma-Aldrich) and 1uM rosiglitazone (R2408, Sigma-Aldrich) in FBS-containing medium for two days. Cells were then maintained in 10%FBS/DMEM supplemented with 1ug/ml insulin for 2 days, then cells were cultured in 10%FBS/DMEM for another four days.

2.3. Lentiviral packages and infection

Plasmids were transfected into 293 T cells according to the manufacturer's instructions. The supernatants containing lentivirus were collected at 48 h and 72 h after transfection and used to infect target cells with polybrene (10 μg/ml). After 72 h post-infection, 3T3-L1 cells were subjected to 4 μg/mL puromycin selection for three days.

2.4. Oil red o (ORO) staining

ORO working buffer was prepared by mixing six parts of ORO stock solution (0.5% ORO dissolved in isopropanol) with four parts of distilled water, and was filtered through a 45-μm filter to remove precipitates. 3T3-L1 cells were fixed in 4% Paraformaldehyde at room temperature for 15mins and then incubated in fresh ORO working solution for 15mins at room temperature. Samples were rinsed four times with H_2O to remove precipitates and bright-field images were captured with a light microscope.

2.5. RNA isolation, RT-PCR, and qRT-PCR

Total RNAs were isolated using TRIzol reagent (15596026, Ambion) following the manufacturer's instruction. 3ug of total RNAs was used for cDNA synthesis in a reaction mixture with Superscript II reverse transcriptase (18064022, Invitrogen). Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green Master Mix reagents (11201ES03, Yeasen) and LightCycler 480II (Roche). 36B4 was used as an internal control. Primer sequences for qRT-PCR were as follows: 36B4 (F:5′-GCTTCATTGTGGGAGCAGAC-3′, R:5′-ATGGTGTTCTTGCCCATCAG-3′); mAjuba (F:5′-TGTGTCTGCGGCCACTTGATTCTA-3′, R:5′-CGTGGCAGAGCAAATGTCCATCAA-3′), mC/EBPβ (F:5′-ACGACTTCCTCTCCGACCTCT-3′, R:5′-CGAGGCTCACGTAACCGTAGT-3′), mPPARγ (F:5′-CCGTAGAAGCCGTGCAAGAG-3′, R:5′-GGAGGCCAGCATCGTGTAGA-3′), mC/EBPα (F:5′-CAAGAACAGCAACGAGTACCG-3′, R:5′-GTCACTCGTCAACTCCAGCAC-3′).

2.6. Western blot

Cell lysates were prepared in RIPA lysis buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Sodium deoxycholate, 1% NP-40, 0.1% SDS, 1 mM EDTA) containing 1 x protease inhibitor (Roche) and 1 x phosphostop (Roche), and were quantified using the BCA Protein Assay kit (23227, Pierce). Lysates were separated using 10% SDS-PAGE gels and

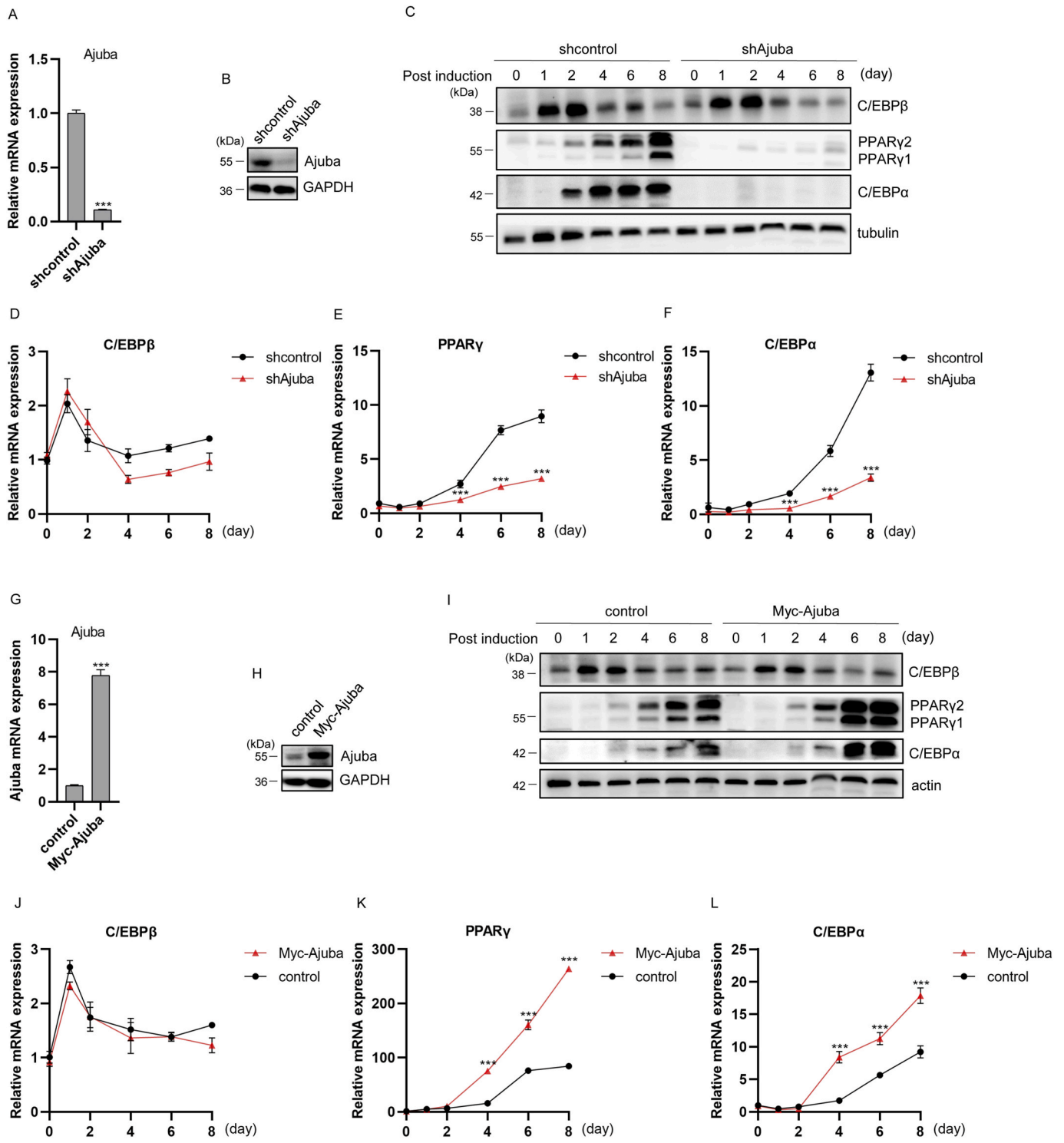

Fig. 1. Ajuba promotes the expression of PPARγ and C/EBPα in the process of differentiation. A–B: mRNA and protein expression of Ajuba in control and Ajuba knockdown 3T3-L1 cells. C: C/EBPβ, PPARγ, and C/EBPα protein levels in control and Ajuba knockdown 3T3-L1 cells during differentiation. D–F: C/EBPβ, PPARγ, and C/EBPα mRNA levels in control and Ajuba knockdown 3T3-L1 cells during differentiation. G–H: mRNA and protein expression of Ajuba in control and Ajuba overexpression 3T3-L1 cells. I: C/EBPβ, PPARγ, and C/EBPα protein levels in control and Ajuba overexpression 3T3-L1 cells during differentiation. J–L: C/EBPβ, PPARγ and C/EBPα mRNA levels in control and Ajuba overexpression 3T3-L1 cells during differentiation. Data were presented as mean ± SEM from three independent experiments. Statistical significance was determined by Student's *t*-test. $^{**}p < 0.01$, $^{***}p < 0.001$.

then transferred onto PVDF membranes (IPVH00010, Millipore). The process of Western blot assays were performed as previously described (Hou et al., 2008). An Imaging System (LAS 4000mini, ImageQuant) was used for signal detection. GAPDH, Tubulin or Actin were used as internal controls. The following antibodies were used: Anti-flag (F3165, Sigma-Aldrich), Anti-Myc (13–2500, Invitrogen), Anti-HA (901501, BioLegend), Anti-C/EBPα (2295, CST), Anti-PPARγ (sc7273, Santa Cruz), Anti-C/EBPβ (sc-7962X, Santa Cruz), Anti-Ajuba (4897, CST), Normal rabbit IgG (2729, CST), Anti-GAPDH (60004-1-Ig, Proteintech), Anti-actin (9601, AOGMA), Anti-Tubulin (66031-Ig, Proteintech),

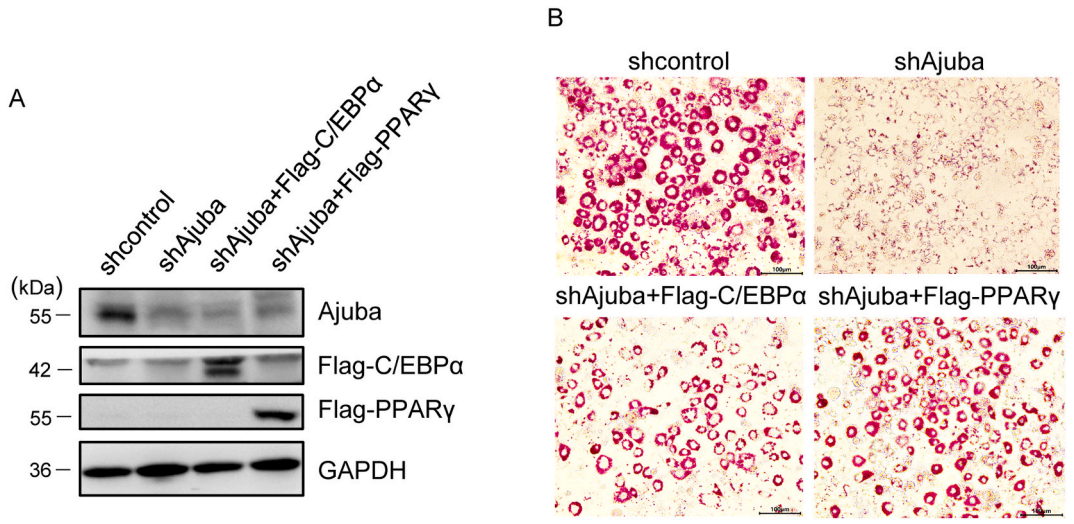

Fig. 2. Overexpression of C/EBPα or PPARγ in Ajuba-deficient 3T3-L1 cells improves the impaired lipid accumulation. A: Ajuba knockdown 3T3-L1 cells were infected with a retrovirus vector or retroviruses carrying the genes expressing Flag-C/EBPα or Flag-PPARγ. Western blot was performed to exam the protein expression of Ajuba, Flag- C/EBPα, Flag-PPARγ. B: Oil Red O staining in differentiated 3T3-L1 cells on day 8.

Anti-Rabbit IgG peroxidase-conjugated (98385, Jackson), Anti-Mouse IgG peroxidase-conjugated (97910, Jackson).

2.7. Immunoprecipitation

3T3-L1 cells were homogenized with IP lysis buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA) containing 1 x protease inhibitor (Roche) and 1 x phosphostop (Roche). Cell lysates were subjected to Immunoprecipitation with indicated antibodies overnight at 4 °C. Then, immune complexes were washed four times in cold IP lysis buffer. The input and IP samples were detected by Western blot.

2.8. Immunofluorescence

3T3-L1 cells were fixed in 4% Paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, and blocked with 2% BSA. Cells were incubated with a cocktail of primary antibodies, including rabbit anti-Ajuba (1:400) and mouse anti-C/EBPβ (1:400) diluted in blocking solution overnight at 4 °C. After three times washing, cells were then incubated with the secondary antibody mixture including 1:400 dilutions of Alexa Fluor 488 Donkey anti-Rabbit IgG (A21206, Invitrogen) and Alexa Fluor 568 rabbit anti-mouse IgG (A11061, Invitrogen). Images were captured on a fluorescence microscope (Intensilight C-HGFI, Nikon).

2.9. Luciferase reporter assay

Luciferase reporter assay was performed after 24 h transfection of indicated plasmids. Cells were suspended with luciferase lysis buffer. Firefly luciferase activity (MA0519, Meilunbio) and β-galactosidase activity (631712, Takara) were detected using the Commercial reagents in accordance with the manufacturer's instruction. Firefly luciferase activity was normalized to β-galactosidase activity.

2.10. Chromatin immunoprecipitation(ChIP)

Cells were cross-linked in 1% Paraformaldehyde and neutralized with 2 M glycine. Samples were lysed with ChIP lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.5% NP-40, 1% Triton X-100, 0.5% SDS) to release the chromatin. Cell lysates were sonicated, and underwent immunoprecipitation using antibodies against CBP (sc-7300, Santa Cruz), C/EBPβ, Ajuba (sc-374610, Santa Cruz), Ac-H3 (06–559, Millipore) and nonspecific IgG control (sc-2025, Santa Cruz). Protein-DNA crosslinks were reversed by incubation overnight at 65 °C. Genomic DNA was extracted using a Cycle-Pure kit (D6492-02, Omega) and target fragments were detected by qRT-PCR. ChIP data were normalized to control IgG. Primer sequences for ChIP qRT-PCR were as follows: C/EBPα (F:5′-TCCCTAGTGTTGGCTGGAAG-3′, R:5′-CAGTAGGATGGTGCCTGCTG-3′), PPARγ (F:5′-TTCAGATGTGTGATTAGGAG-3′, R:5′-AGACTTGGTACATTACAAGG-3′).

2.11. Quantification and statistical analysis

All experiments were repeated more than three times. Statistical analysis was performed using GraphPad Prism. Data were presented as mean ± SEM and analyzed using Student's *t*-tests. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Ajuba increases the expression of PPARγ and C/EBPα in the process of 3T3-L1 differentiation

To explore the impact of Ajuba on adipocyte differentiation, we examined expression levels of C/EBPβ, PPARγ, and C/EBPα in the course of differentiation. 3T3-L1 cells were infected with a lentiviruses vector or lentiviruses bearing a shRNA specifically targeting Ajuba. Knockdown of Ajuba was successfully achieved at both mRNA and protein levels (Fig. 1A and 1B). Depletion of Ajuba in 3T3-L1 cells remarkably suppressed the expression of PPARγ and C/EBPα at the early stage of adipogenesis (Fig. 1C, E, and 1F). Conversely, overexpression of Myc-Ajuba in 3T3-L1 cells enhanced both mRNA and protein expression levels of PPARγ and C/EBPα during adipogenesis (Fig. 1G, H, 1I, 1K and 1L). Notably, silencing or overexpression of Ajuba in 3T3-L1 cells did not significantly affect C/EBPβ expression at both protein and mRNA levels (Fig. 1C, D, 1I, and 1J).

To certify that Ajuba affects lipid accumulation by influence on the expression of PPARγ and C/EBPα, we overexpressed C/EBPα or PPARγ in Ajuba-deficient cells (Fig. 2A). Ajuba-deficient adipocytes accumulated less and smaller fat droplets, while forced expression of C/EBPα or PPARγ ameliorated the lipid formation impaired by Ajuba depletion (Fig. 2B). These data provide compelling evidence that knockdown of Ajuba inhibits adipogenesis via downregulation of PPARγ and C/EBPα.

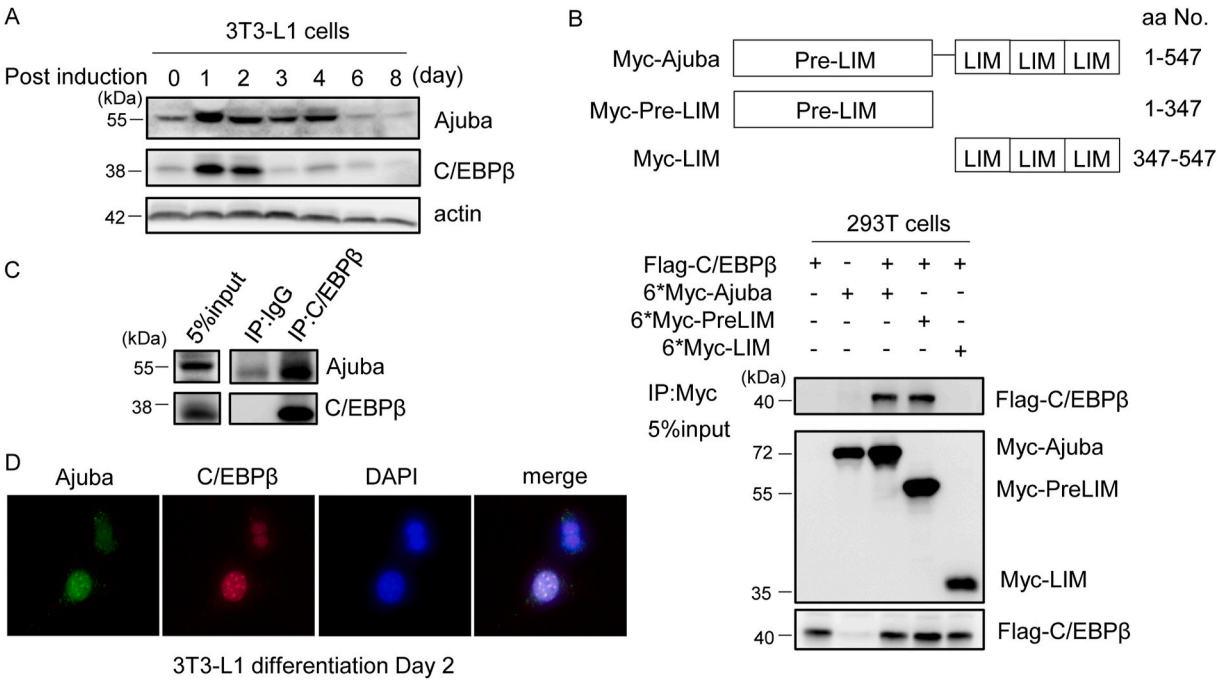

Fig. 3. Ajuba interacts with C/EBPβ via its preLIM region. A: The expression profile of Ajuba and C/EBPβ in 3T3-L1 cells in the course of differentiation. B: Myc-Ajuba, its mutants, and Flag-C/EBPβ were transiently expressed in 293 T cells. Immunoprecipitation was performed with Myc antibody. C: Endogenous co-immunoprecipitation experiments were conducted with C/EBPβ antibodies at 48 h postinduction. Mouse IgG was used as a control. D: Subcellular localization of endogenous Ajuba and C/EBPβ were demonstrated using immunofluorescence assays in 3T3-L1 cells after two-day differentiation.

3.2. Ajuba interacts with C/EBPβ via its preLIM region

To dissect the underlying mechanism by which Ajuba enhances the expression of PPARγ and C/EBPα, we examined Ajuba expression profile during the process of adipocyte differentiation, and found that Ajuba protein remained at high level from 1 d to 4 d postinduction and then declined at 6 d (Fig. 3A). This expression pattern was well correlated with that of C/EBPβ. Given the fact that PPARγ and C/EBPα are dominantly regulated by C/EBPβ during adipogenesis, we postulated that Ajuba may be functionally related to C/EBPβ.

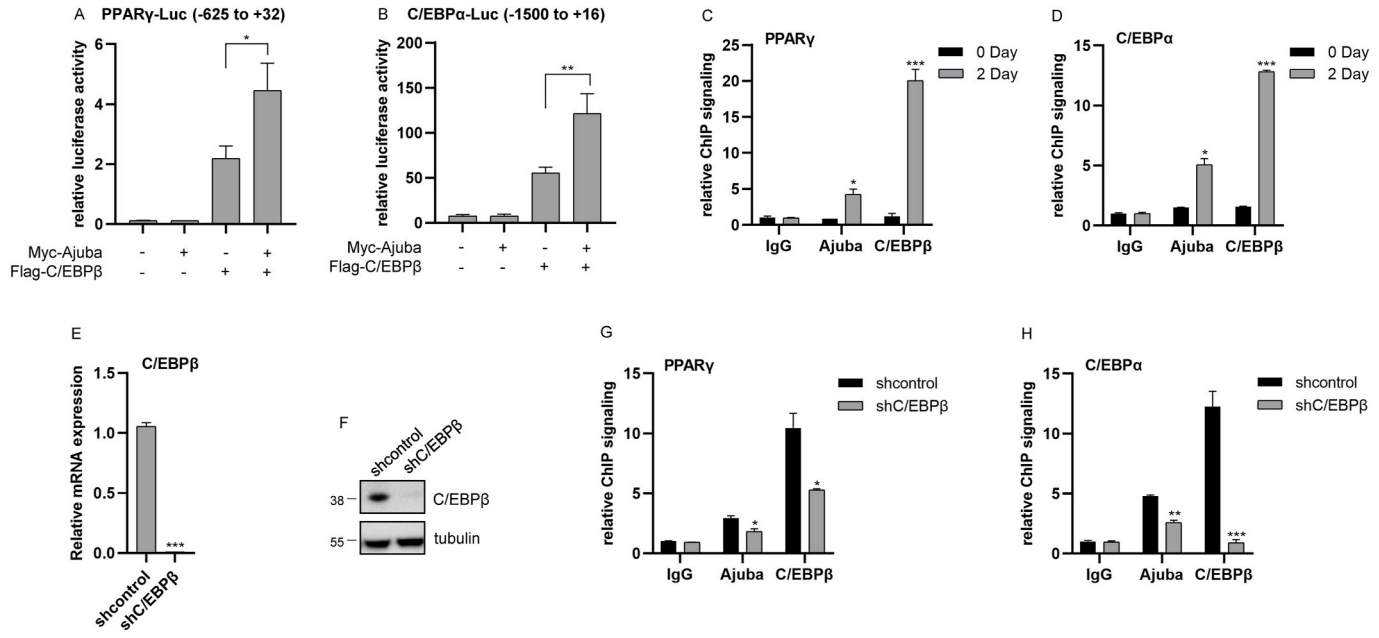

Fig. 4. Ajuba enhances the transactivity of C/EBPβ. A–B: Luciferase assays were performed in 293 T cells co-transfected with the indicated constructs and luciferase activity was normalized to β-galactosidase activity. C–D: ChIP assays were performed to examine the binding activity of Ajuba and C/EBPβ on the promoter of PPARγ and C/EBPα using the control IgG, Ajuba, and C/EBPβ antibodies at 0 d and 2 d postinduction. Data were normalized to the IgG controls at each time point. E–F: mRNA and protein expression of C/EBPβ in control and C/EBPβ knockdown 3T3-L1 cells. G–H: 3T3-L1 cells were infected with a lentiviruses vector or lentiviruses carrying siRNA targeting C/EBPβ. ChIP assays were performed to examine the binding activity of Ajuba and C/EBPβ on the promoter of PPARγ and C/EBPα using indicated antibody at 48 h postinduction. Data were presented as mean ± SEM from three independent experiments. Statistical significance was determined by Student's *t*-test. $^{**}p < 0.01$, $^{***}p < 0.001$.

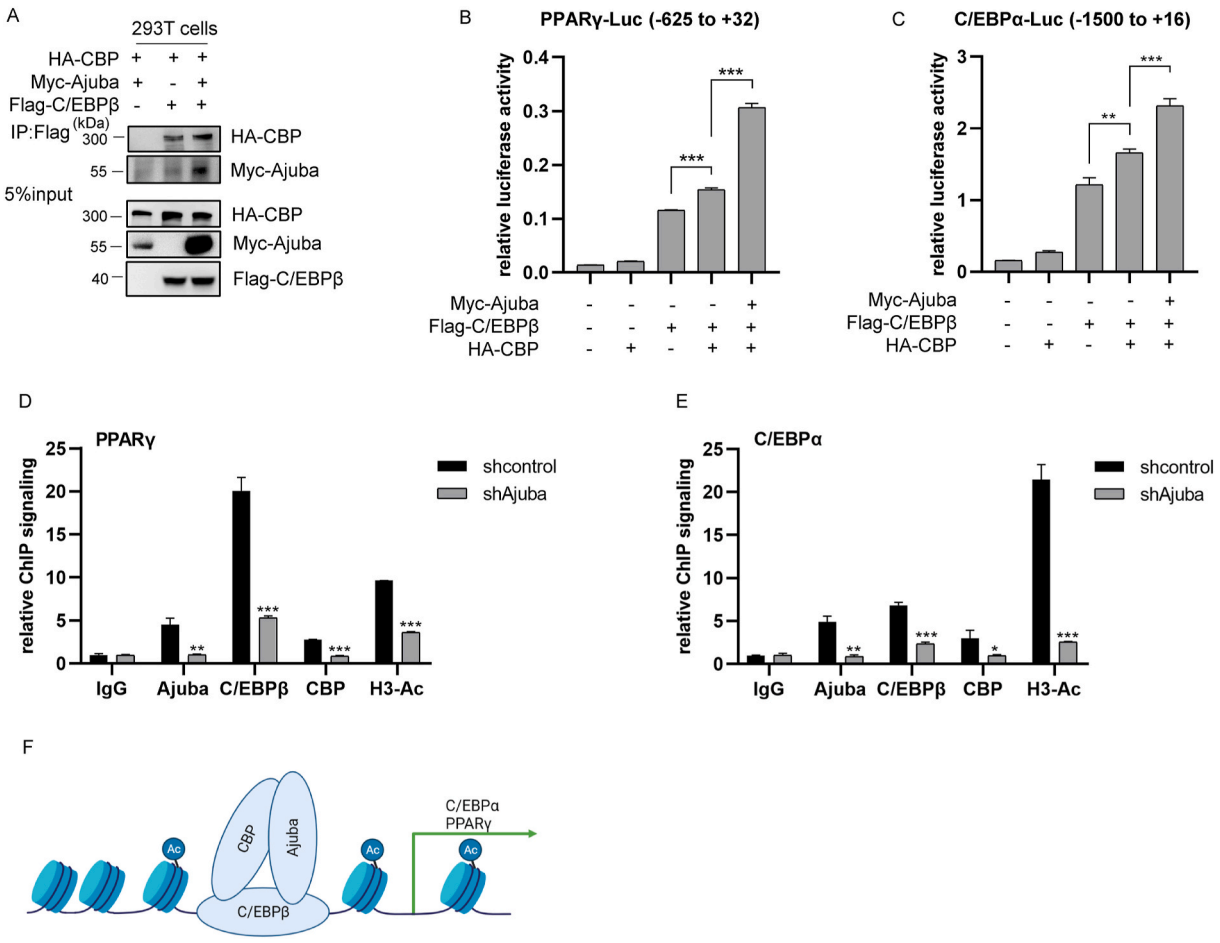

Fig. 5. Ajuba recruits CBP to facilitate the binding of C/EBPβ on the promoter of PPARγ and C/EBPα. A: Myc-Ajuba, Flag-C/EBPβ, and HA-CBP were transiently expressed in 293 T cells. Immunoprecipitation assays were performed with Flag antibody. B–C: Luciferase assays were performed in 293 T cells co-transfected with the indicated constructs and luciferase activity was normalized to β-galactosidase activity. D–E: 3T3-L1 cells were infected with a lentiviruses vector or lentiviruses carrying siRNA targeting Ajuba. ChIP assays were carried out to exam the enrichment of Ajuba, C/EBPβ, CBP, Ac-H3 on the promoter of PPARγ and C/EBPα using indicated antibody at 48 h postinduction. F: Working model. Ajuba recruits CBP to enhance the transcriptional activity of C/EBPβ to increase the transcription of PPARγ and C/EBPα. Data were presented as mean ± SEM from three independent experiments. Statistical significance was determined by Student's *t*-test. $^{**}p < 0.01$, $^{***}p < 0.001$.

To test this hypothesis, we transiently expressed Myc-Ajuba and Flag-C/EBPβ in 293 T cells and performed co-immunoprecipitation experiments to determine if Ajuba interacts with C/EBPβ. Indeed, anti-Myc antibody precipitated Flag-C/EBPβ (Fig. 3B). To identify the regions of Ajuba that mediated the interaction with C/EBPβ, we constructed truncation mutants of Myc-preLIM and Myc-LIM. Co-IP assays demonstrated that preLIM rather than LIM was required for their binding (Fig. 3B).

To verify the interaction between endogenous Ajuba and C/EBPβ proteins, we conducted co-IP experiments in cell lysates prepared from 3T3-L1 cells at 48 h postinduction with antibody against C/EBPβ. Consistently, C/EBPβ protein co-precipitated with Ajuba (Fig. 3C). Moreover, Immunofluorescence assay was carried out in 3T3-L1 cells at 48 h after differentiation. C/EBPβ was located in the nucleus in the form of a punctate pattern which was consistent with previous studies (Fig. 3D)(Tang et al., 2003). Ajuba was distributed in the nucleus and cytoplasm, while C/EBPβ and Ajuba were overlapped in the nucleus (Fig. 3D). Taken together, these data indicate that the Ajuba interacts with C/EBPβ.

3.3. Ajuba enhances the transactivity of C/EBPβ

To examine the effects of Ajuba on the transactivation properties of C/EBPβ, we performed luciferase reporter assays with PPARγ-luc or C/EBPα-luc reporters which contained C/EBPβ responsive elements, respectively. Notably, expression of Ajuba alone did not affect the reporter activities, while a combination with C/EBPβ markedly increased C/EBPβ-driven reporter activities (Fig. 4A and B).

To detect the DNA-binding activity of Ajuba on the promoter of C/EBPβ target genes, we performed ChIP assays in 0 h and 48 h differentiated 3T3-L1 cells. The DNA binding activity of C/EBPβ reached higher at 48 h postinduction in agreement with previous studies (Fig. 4C and D) (Zhao et al., 2014). Ajuba barely bound to the PPARγ and C/EBPα promoter before differentiation, but at 48 h postinduction, the binding level of Ajuba on the PPARγ and C/EBPα promoters increased to four folds. To test whether the binding of Ajuba on the promoter was dependent on CEBPβ, we used siRNA to knockdown CEBPβ in 3T3-L1 cells (Fig. 4E and F) and performed ChIP experiments two days after differentiation. Indeed, depletion of C/EBPβ decreased the enrichment of Ajuba on the promotor of PPARγ and C/EBPα (Fig. 4G and H), indicating that the binding of Ajuba to the promoter regions is dependent on C/EBPβ.

3.4. Ajuba recruits CBP to facilitate the binding of C/EBPβ on the promoter of PPARγ and C/EBPα

CBP (CREB binding protein) is endowed with histone acetyltransferase (HAT) activity and acts as a co-activator for many

transcription factors including C/EBPs and nuclear receptors (PPARs) (Yamauchi et al., 2002; Takahashi et al., 2002). Previous research has established that C/EBPβ interacts with the E1A-binding domain of CBP, and CBP binds to the LIM domain of Ajuba (Li et al., 2016; MINK. et al., 1997). Therefore, we performed immunoprecipitation assays to determine whether CBP, Ajuba and C/EBPβ could form a complex. We co-expressed HA-CBP, myc-Ajuba, and flag-C/EBPβ into 293 T cells and immunoprecipitation experiments were carried out with Flag antibody. Western blot results showed that Ajuba facilitated C/EBPβ and CBP interaction, as evidenced by the fact that flag-CEBPβ could precipitate more HA-CBP when co-transferred with myc-Ajuba (Fig. 5A).

To examine whether the ternary complex of C/EBPβ, Ajuba and CBP influenced the transactivation of PPARγ and C/EBPα, we conducted luciferase reporter assays. CBP alone only weakly enhanced the C/EBPβ transcriptional activity, while a combination of Ajuba, CBP, and C/EBPβ strongly increased the C/EBPβ-driven transactivation, indicating that CBP and Ajuba synergistically enhance the transactivation mediated by C/EBPβ (Fig. 5B and C). We next performed ChIP assays to examine whether Ajuba influenced the DNA-binding activity of C/EBPβ. shRNA-mediated depletion of Ajuba in 3T3-L1 cells was exposed to differentiation inducers for 48 h and ChIP assays were carried out. Depletion of Ajuba in 3T3-L1 cells reduced the binding affinities of C/EBPβ and CBP on the promoter of PPARγ and C/EBPα, and was concomitantly associated with decreased H3 histone acetylation at these regions (Fig. 5D and E). Therefore, we conclude that Ajuba recruits CBP to enhance the transcriptional activity of C/EBPβ to increase the transcription of PPARγ and C/EBPα (Fig. 5F).

4. Discussion

Ajuba regulates a variety of cellular phenotypes, such as proliferation, differentiation, apoptosis, migration and adhesion, but the impact of Ajuba on adipose differentiation is scarcely studied (Jia et al., 2020). Here, we demonstrated that knockdown of Ajuba in 3T3-L1 cells significantly decreased mRNA and protein levels of PPARγ and C/EBPα during adipocyte differentiation. Mechanistically, Ajuba acted as a co-activator by enhancing C/EBPβ and CBP interaction to facilitate their binding at the target promoter regions, leading to elevated histone acetylation and concomitant target gene expression. In addition, we have previously reported that Ajuba promotes adipogenesis by functioning as an obligate co-activator of PPARγ(Li et al., 2016). Ajuba binds to PPARγ in a ligand independent manner and recruits p300/CBP via its LIM domain to enhance PPARγ target gene expression (Li et al., 2016). These findings indicate that Ajuba regulates adipogenesis at the both early and late stages of differentiation. However, the definitive role of Ajuba in adipogenesis needs to be confirmed in genetic engineering mice.

Adipogenesis is regulated by a complicated network of transcription factors that act at different stages of differentiation (Rosen and MacDougald, 2006). In the course of differentiation, C/EBPβ is induced early, and bind to the promoters of multiple TFs such as C/EBPα and PPARγ(Farmer, 2006). However, overexpression of C/EBPβ alone has limited effect on adipocyte differentiation, suggesting that adipogenesis involves more complex regulatory events (Lee et al., 2019). Accumulating assays report that chromatin structure see dramatic changes during adipocyte differentiation (Zhang et al., 2012). It is widely believed that transcription factors-regulated adipogenesis involves co-factors, which modify chromatin directly or act as platforms for recruitment of chromatin modifiers (Siersbaek et al., 2011). Many co-factors have been identified to affect C/EBPβ activity in various tissues, only a few co-factors such as ATFs, HDAC1 and p300/CBP are interrogated during adipogenesis (Pulido-Salgado et al., 2015). How C/EBPβ and its associated proteins are interacted to control the expression of adipogenic genes is largely unknown. Here, we show that Ajuba functions as a coactivator of C/EBPβ and recruits CBP to enhance the expression of PPARγ and C/EBPα during adipogenesis. Our findings highlight the important role of Ajuba on the transactivation of PPARγ and C/EBPα mediated by C/EBPβ during adipocyte differentiation.

In conclusion, Ajuba is a promotor of adipogenesis by acting as co-activator of C/EBPβ and PPARγ, indicating that Ajuba may be an important therapeutic target for combating obesity-related diseases.

Disclosure of potential conflicts of interest

The authors declare no conflicts of interest.

CRediT authorship contribution statement

Han Yan: Conceptualization, Methodology, Investigation, Software, Formal analysis, Writing – original draft. **Qi Li:** Investigation. **Mengying Li:** Resources. **Xiuqun Zou:** Methodology. **Ningning Bai:** Resources. **Zichao Yu:** Resources. **Jie Zhang:** Formal analysis. **Dan Zhang:** Formal analysis. **Qun Zhang:** Methodology. **Jiamin Wang:** Resources. **Hao Jia:** Funding acquisition. **Yingjie Wu:** Funding acquisition. **Zhaoyuan Hou:** Supervision, Writing – review & editing.

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