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Protein Inhibitor of Activated STAT 1 (PIAS1) Is Identified as the SUMO E3 Ligase of CCAAT/Enhancer-Binding Protein β (C/EBPβ) during Adipogenesis

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It is well recognized that PIAS1, a SUMO (small ubiquitin-like modifier) E3 ligase, modulates such cellular processes as cell proliferation, DNA damage responses, and inflammation responses. Recent studies have shown that PIAS1 also plays a part in cell differentiation. However, the role of PIAS1 in adipocyte differentiation remains unknown. CCAAT/enhancer-binding protein β (C/EBPβ), a major regulator of adipogenesis, is a target of SUMOylation, but the E3 ligase responsible for the SUMOylation of C/EBPβ has not been identified. The present study showed that PIAS1 functions as a SUMO E3 ligase of C/EBPβ to regulate adipogenesis. PIAS1 expression was significantly and transiently induced on day 4 of 3T3-L1 adipocyte differentiation, when C/EBPβ began to decline. PIAS1 was found to interact with C/EBPβ through the SAP (scaffold attachment factor A/B/acinus/PIAS) domain and SUMOylate it, leading to increased ubiquitination and degradation of C/EBPβ. C/EBPβ became more stable when PIAS1 was silenced by RNA interference (RNAi). Moreover, adipogenesis was inhibited by overexpression of wild-type PIAS1 and promoted by knockdown of PIAS1. The mutational study indicated that the catalytic activity of SUMO E3 ligase was required for PIAS1 to restrain adipogenesis. Importantly, the inhibitory effect of PIAS1 overexpression on adipogenesis was rescued by overexpressed C/EBPβ. Thus, PIAS1 could play a dynamic role in adipogenesis by promoting the SUMOylation of C/EBPβ.

In obesity, a major risk factor for type 2 diabetes, hypertension, hyperlipidemia, and arteriosclerosis (1), enlarged adipose tissue mass is due to the increase in both the number (hyperplasia) and size (hypertrophy) of adipocytes (2, 3). The 3T3-L1 preadipocyte line is widely used to investigate adipocyte hyperplasia during preadipocyte differentiation (4). The adipogenic differentiation program of 3T3-L1 cells has been well characterized. The hormone induction of growth-arrested 3T3-L1 preadipocytes triggers a cascade in which CCAAT/enhancer-binding protein β (C/EBPβ) is rapidly expressed, followed by induction of C/EBPα and peroxisome proliferator-activated receptor γ (PPARγ), which turn on the series of adipocyte genes that give rise to the adipocyte phenotype (5–8).

C/EBPβ, a basic leucine zipper (bZIP) transcriptional factor, is involved in many differentiation processes (9–12). C/EBPβ plays a role as a major regulator of mesenchymal stem cell fate by acting as an activator of adipogenesis (9, 10) and a repressor of osteoblastogenesis (11) and myogenesis (12). Our previous investigations have shown that C/EBPβ is rapidly expressed in the 3T3-L1 preadipocyte differentiation program and is maintained at a high level during the early stage of differentiation (6) and that upon sequential phosphorylation by mitogen-activated protein kinase (MAPK), cyclin-dependent kinase 2 (CDK2), and glycogen synthase kinase 3β (GSK3β) (13, 14), C/EBPβ acquires DNA-binding activity, activating the expression of C/EBPα and PPARγ as well as cell cycle genes essential for mitotic clonal expansion (MCE), a necessary step for terminal adipocyte differentiation (15–17). Other modifications, such as GlcNAcylation, can also regulate DNA-binding activity of C/EBPβ by preventing phosphorylation (18). It has also been reported that C/EBPβ, associated with a PR domain-containing protein 16 (PRDM16), initiates brown-fat formation from myoblastic precursors (19). These findings indicate that C/EBPβ plays an important role in adipogenesis.

C/EBPβ can be modified by the small ubiquitin-like modifier (SUMO) on Lys133 (20). SUMO regulates a number of cellular processes, including transcription, DNA repair, cell cycle progression, and signal transduction, in organisms from yeasts to humans (21–23). SUMOylation is catalyzed by Aos1/Uba2 (an E1-activating enzyme), Ubc9 (an E2-conjugating enzyme), and E3 ligases and can be reversed by SUMO-specific proteases (SENPs) (24). E3 ligases contribute to SUMOylation substrate specificity and efficiency. Three main subtypes of SUMO E3 ligases have been identified: PIAS proteins, RanBP2, and Pc2 (24, 25). PIAS (protein inhibitor of activated STAT) proteins were initially named for their ability to interact with and inhibit STAT factors (26, 27). It is now evident that PIAS family members influence the function of many transcription factors by acting as SUMO E3 ligases (28). The PIAS family contains potential regulators of cell proliferation (29), DNA damage responses (30), and inflammation responses (31), indicating that the PIAS family is essential for many cellular processes. PIAS1 has recently been shown to regulate cell differ-

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entiation, such as myogenic differentiation, but the role of PIAS1 in adipogenesis need clarification.

As C/EBPβ is a master gene during adipogenesis, it is important to identify the cofactors that regulate C/EBPβ. We used a yeast two-hybrid screen and found that PIAS1 is a candidate protein interacting with C/EBPβ. It has been shown that deSUMOylation of C/EBPβ dramatically inhibits its ubiquitination and subsequent degradation (32); however, C/EBPβ-specific SUMO E3 ligase has not been identified. PIAS1 was found to be the SUMO E3 ligase for C/EBPβ in the present study. Our results showed that PIAS1 was induced at the late stage during 3T3-L1 adipogenic differentiation, when the C/EBPβ protein level began to decline, and that PIAS1 interacted with C/EBPβ before SUMOylating it. Furthermore, overexpression of the wild-type PIAS1 but not the mutant PIAS1 defective in SUMO ligase activity led to the ubiquitination and subsequent degradation of C/EBPβ, thereby suppressing adipogenesis. Also, ectopic expression of C/EBPβ significantly abolished PIAS1-mediated inhibition of adipogenesis. These results demonstrated an essential role of PIAS1 in controlling adipogenesis.

MATERIALS AND METHODS

Cell culture and induction of differentiation. The 3T3-L1 preadipocytes were propagated and maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% calf serum. Two days after reaching confluence (designated day 0), 3T3-L1 preadipocytes were induced to differentiate into adipocytes with DMEM containing 10% fetal bovine serum (FBS), 0.5 mM 3-isobutyl-1-methylxanthine (M), 1 μM dexamethasone (D), and 1 μg/ml insulin (I) until day 2. The cells then received DMEM supplemented with 10% FBS and 1 μg/ml insulin for 2 days, after which the DMEM containing 10% FBS was changed every other day. Adipocyte gene expression and acquisition of the adipocyte phenotype began on day 3 and were maximal by day 8.

Plasmids and antibodies. Flag-PIAS1 and His-C/EBPβ were cloned into the vector pcDNA3.1 for transient transfection or the vector MSCV for generation of stably transfected cell lines. PIAS1 and C/EBPβ mutant forms were generated by site-directed mutagenesis. The expression vector for V5-SUMO1 was kindly given by Yong-Jun Dang. The vector for hemagglutinin-ubiquitin (HA-Ub) was a kind gift from Kun-Liang Guan. For the protein pulldown assay, C/EBPβ, PIAS1 and truncated domains of PIAS proteins were inserted in frame with glutathione *S*-transferase (GST) in pGEX-6P1 (Amersham Pharmacia Biotech). 422/aP2 antibody was a kind gift from M. Daniel Lane. Antibodies against C/EBPβ, C/EBPα, PPARγ, Ub, and Hsp90 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); PIAS1 antibody was purchased from Epitomics; antibodies against V5 and His were purchased from Aogma; Flag antibody was purchased from Sigma; HA antibody was purchased from MBL.

RNA isolation and real-time quantitative PCR. Total RNA was isolated using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized using the PrimeScript reverse transcriptase and random primers (TaKaRa Bio, Otsu, Japan). Quantitative PCR (qPCR) involved Power SYBR green PCR master mix (Applied Biosystems, Carlsbad, CA) and a Prism 7300 instrument (Applied Biosystems), with 18S rRNA as an endogenous control. Analysis was done in triplicate and repeated at least 3 times. Results are presented as means and standard deviations (SD) from three independent experiments. Forward and reverse primers (5′ to 3′) used in qPCR were as follows: PIAS1, GCGGACAGTGCGGAACTAAA and ATGCAGGGCTTTTGTAAGAAGT; PIAS2, TTCAGCTTGGATGGTAGCTCA and TGTAATTGAAGTCGAAGGCAACG; PIAS3, TTCGCTGGCAGGAACAAGAG and GGGCGCAGCTAGACTTGAG; PIAS4, TGAGCTTCCGAGTATCAGACC and GGGCTACAGTCGAACTGCAC; C/EBPβ, ACGACTTCCTCTCCGACCTCT and CGAGGCTCACGTAACCGTAGT; C/EBPα, CAAGAACAGCAACGAGTACCG and GTCACTCGTCAACTCCAGCAC; PPARγ, GTGCCAGTTTCGATCCGTAGA and GGCCAGCATCGTGTAGATGA; 422/aP2, CCTTTGTGGGAACCTGGAA and CTGTCGTCTGCGGTGATT; adiponectin, TGTTCCTCTTAATCCTGCCCA and CCAACCTGCACAAGTTCCCTT; 18S rRNA, CGGCTACCACATCCAAGGAA and GCTGGAATTACCGCGGCT.

RNA interference. Synthetic small interfering RNA (siRNA) oligonucleotides specific for PIAS1 (5′ to 3′, UUAAACUGUAUCUCGUCACAGUCUG) and Stealth siRNA negative-control duplexes were designed and synthesized by Invitrogen. 3T3-L1 preadipocytes were transfected with the siRNA oligonucleotide by using Lipofectamine RNAiMAX (Invitrogen) at 50% confluence. Two days after confluence was reached (about the 4th day after transfection), 3T3-L1 preadipocytes received adipogenic induction (designated day 0 postinduction). 3T3-L1 cells on days 0, 1, 2, 4, 6 after adipogenic differentiation were subjected to analyze the knockdown of PIAS1. The silencing effects and the specificity of RNAi oligonucleotides were tested through analysis of the expression of PIAS1 and other family members by reverse transcription-qPCR (RT-qPCR) and Western blotting. Stealth RNAi-negative duplexes with a similar GC content were used as negative controls.

Western blotting. The cells were lysed with a buffer containing 2% sodium dodecyl sulfate (SDS), 10 mM dithiothreitol, 50 mM Tris-HCl (pH 6.8), 10% glycerol, 0.002% bromphenol blue, and protease inhibitor mixture (Roche). Equal amounts of protein were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA), immunoblotted with antibodies, and visualized with horseradish peroxidase-coupled secondary antibodies.

Protein pulldown assay. Glutathione *S*-transferase (GST) fusion proteins were expressed from *Escherichia coli* strain BL21 and purified on glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech, Inc.). GST fusion proteins were examined on SDS-PAGE gels subsequently stained with Coomassie blue. For protein pulldown assays, cells were collected and lysed in radioimmunoprecipitation assay (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). After incubating with the equal amount GST fusion proteins, glutathione-Sepharose 4B beads were washed and then incubated overnight with the cell lysates at 4°C. The beads were washed with TBS-T (Tris-buffered saline plus 0.05% Tween 20) 4 times. The immunoprecipitates were separated by SDS-PAGE for Western blotting.

Immunoprecipitation assay. The 3T3-L1 cells were collected at the indicated times, washed with phosphate-buffered saline (PBS), scraped off, and collected by centrifugation, and then lysed in RIPA buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) in the presence of protease inhibitors (Roche) at 4°C. After centrifugation, the lysates were incubated with the indicated antibodies overnight at 4°C and with protein A-Sepharose beads (Invitrogen) for the next 2 h. The beads were washed 4 times with TBS-T (TBS + 0.05% Tween 20), and the immunoprecipitates were separated by SDS-PAGE for Western blotting.

***In vivo* SUMOylation assay.** The cells transfected with His-tagged C/EBPβ, V5-tagged SUMO1, and Flag-tagged PIAS proteins were collected and lysed in buffer (250 mM NaCl, 50 mM Na_2HPO_4-NaH_2PO_4 [pH 8.0], 0.01% Tween 20, 1% Triton X-100, 10 mM imidazole) with protease inhibitors (Roche) and *N*-ethylmaleimide (Sigma). Lysates were mixed with 50 μl Dynabeads for His-tag isolation and pulldown (Invitrogen) and incubated at 4°C for 1 h. The beads were washed in washing buffer (250 mM NaCl, 50 mM Na_2HPO_4-NaH_2PO_4 [pH 8.0], 0.01% Tween 20, 10 mM imidazole) with protease inhibitors (Roche) and *N*-ethylmaleimide (Sigma). Washed beads were incubated with 40 μl elution buffer (200 mM imidazole, 50 mM Na_2HPO_4-NaH_2PO_4 [pH 8.0], 30% glycerol, 1% SDS, 0.72 M β-mercaptoethanol) at room temperature. The input and the eluates were analyzed by Western blotting.

***In vivo* ubiquitination assay.** The 3T3-L1 cells were collected after induction and lysed in 1% SDS buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM dithiothreitol [DTT], 1% NP-40, 1% SDS) in the presence of protease inhibitors (Roche) and boiled for 10 min. For the pull-

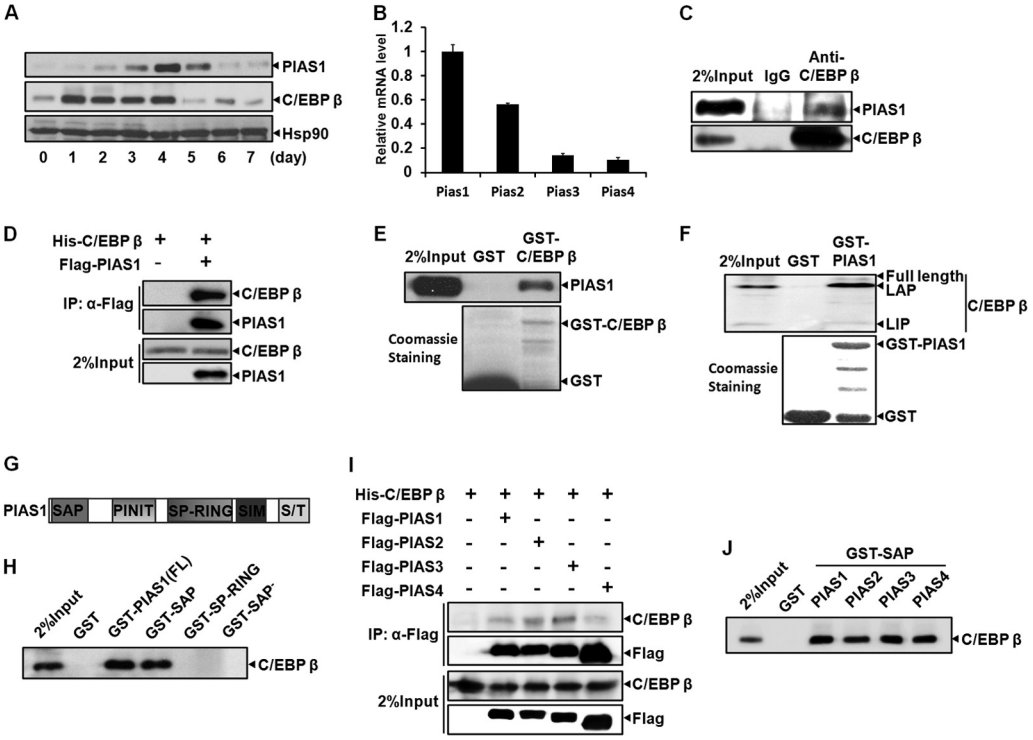

FIG 1 PIAS1 interacts with C/EBPβ. (A) PIAS1 and C/EBPβ proteins were detected by Western blotting at the indicated time points after MDI treatment. (B) PIAS family member mRNA levels were monitored via qPCR on day 4 postinduction. Data are normalized to the expression level of PIAS1 (C) Whole-cell extracts on day 4 postinduction were immunoprecipitated with anti-C/EBPβ antibody. Immunoprecipitated complexes were detected by Western blotting with anti-PIAS1 or anti-C/EBPβ antibodies. (D) The 3T3-L1 cells transfected with His-tagged C/EBPβ or Flag-tagged PIAS1 were collected on day 4 postinduction and immunoprecipitated with anti-Flag antibody, followed by Western blotting with anti-Flag or anti-His antibodies. (E and F) GST pulldown assays were performed to examine PIAS1-C/EBPβ interaction. 3T3-L1 cell lysates were incubated with GST-fused C/EBPβ (E), GST-fused PIAS1 (F), and GST alone as a control (E and F), and the resulting complexes were precipitated with glutathione beads. Western blotting was used to detect the complex with the indicated antibodies. GST-fused C/EBPβ, GST-fused PIAS1, and GST alone were stained with Coomassie brilliant blue. (G) Schematic representation of PIAS1 functional domains. (H) GST-fused full-length PIAS1, SAP motif, and SP-RING domain and the SAP motif deletion mutants (SAP$^-$) were used in GST pulldown assays. After incubation with 3T3-L1 cell lysates, Western blotting was used to detect the complex with anti-C/EBPβ antibody. GST alone was used as a negative control. (I) Coimmunoprecipitation assays using 293T cells. Flag-tagged PIAS1, PIAS2, PIAS3, and PIAS4 were transfected separately with His-tagged C/EBPβ in 293T cells. At the 36th hour after transfection, coimmunoprecipitation assays were performed with anti-Flag antibody, followed by Western blotting with the indicated antibodies. (J) SAP domains from PIAS1, PIAS2, PIAS3, and PIAS4 were fused to GST and purified for incubation with 3T3-L1 cell lysates. Western blotting was used to detect the complex with anti-C/EBPβ antibody. GST alone was used as a negative control.

down assay (His purification or immunoprecipitation assay), the lysates were diluted 10-fold in Tris-HCl buffer. After His purification or immunoprecipitation assay, Western blotting was used to detect the ubiquitination of C/EBPβ.

Luciferase reporter assays. The promoters of C/EBPα and PPARγ which contain the C/EBPβ regulatory element were cloned into the pGL3-basic luciferase vector (Promega Corp., Madison, WI). The promoters were sequenced, and their authenticity was verified. The report assays used 293T cells. Wild-type C/EBPβ [C/EBPβ(WT)], C/EBPβ(K133R), and PIAS1 were transfected separately into 293T cells with the transfection kit Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. At the 36th hour, luciferase activity was measured using the dual-luciferase reporter assay (Promega), normalizing the firefly luciferase activity against *Renilla* luciferase activity. The data are presented as means and SD from three independent experiments.

Oil Red O staining. After MDI induction, the cells were washed three times with PBS and fixed for 10 min with 3.7% formaldehyde. Oil Red O (0.5% in isopropanol) was diluted with water (3:2), filtered through a 0.45-μm filter, and incubated with the fixed cells for 3 to 4 h at room temperature. The cells were washed with water, and stained fat droplets in the adipocytes were examined by light microscopy and photographed.

Statistical analysis. Results were expressed as means ± SD from at least 3 independent experiments. *P* values were determined by Student's *t* test, with *P* values of <0.05 being taken as significant.

RESULTS

PIAS1 expression is transiently induced during adipocyte differentiation. To identify the proteins that interact with C/EBPβ, we used a Gal4-based yeast two-hybrid screen in which C/EBPβ fused to the Gal4 DNA binding domain was used as bait and a 3T3-L1 preadipocyte line library fused to Gal4 activation domain was used as prey, which revealed that PIAS1 interacted with C/EBPβ (data not shown). To investigate the role of PIAS1 in 3T3-L1 adipogenesis, we analyzed the expression of PIAS1 via Western blotting during 3T3-L1 differentiation (Fig. 1A), with the result that PIAS1 protein reached a peak on day 4 after adipogenic induction, whereas C/EBPβ protein began to decrease at that time, which demonstrated that the expressions of PIAS1 and C/EBPβ were inversely correlated during adipogenesis (Fig. 1A).

Since mammalian PIAS family members share a highly conserved sequence, we monitored the expression of other PIAS pro-

teins during adipogenesis. The expression of PIAS2, PIAS3, and PIAS4 was significantly lower than that of PIAS1 during adipogenesis (Fig. 1B). In addition, unlike with PIAS1, no obvious change in the protein level of PIAS2 was detected during adipogenesis (data not shown). Together, these data suggest that PIAS1 was more likely to be the functional molecule of the PIAS family members during adipogenesis.

PIAS1 physically interacts with C/EBPβ through its SAP (scaffold attachment factor A/B/acinus/PIAS) motif. Endogenous coimmunoprecipitation was carried out with 3T3-L1 whole-cell extracts (WCE) collected on day 4 of differentiation to test the interaction between C/EBPβ and PIAS1. Immunoprecipitation with C/EBPβ antibody pulled down PIAS1 (Fig. 1C), which is consistent with the finding in the yeast two-hybrid screen. Coimmunoprecipitation analysis using cells transfected with His-tagged C/EBPβ and Flag-tagged PIAS1 was also carried out; Flag-tagged PIAS1 clearly interacted with C/EBPβ (Fig. 1D).

In vitro binding assays were performed to further verify the interaction between C/EBPβ and PIAS1. GST-fused C/EBPβ, GST-fused PIAS1, and GST alone were expressed and purified. 3T3-L1 cell lysates were incubated with GST-fused C/EBPβ or GST-fused PIAS1. GST-fused C/EBPβ rather than GST alone pulled down PIAS1 (Fig. 1E), and likewise, GST-fused PIAS1 pulled down C/EBPβ, especially the LAP isoform (Fig. 1F). GST pulldown assays were then employed to identify which PIAS1 domain functionally recognized C/EBPβ; therefore, 3T3-L1 cell lysates were incubated with equivalent amounts of GST fusions of the full-length PIAS1, the SAP motif, the SP-RING domain, and the SAP motif deletion mutant (termed SAP$^-$), as well as with GST alone. the PIAS1 SAP motif proved to be the region that effectively recognized C/EBPβ, whereas the SP-RING domain, the SAP$^-$ mutant, and GST alone did not interact with C/EBPβ (Fig. 1G and H). These results together indicate that PIAS1 physically interacts with C/EBPβ through the SAP motif.

Since all PIAS proteins contain the SAP domain, we doubted whether other family members interacted with C/EBPβ. By the coimmunoprecipitation assay conducted in 293T cells, it was found that other PIAS proteins, in addition to PIAS1, interacted with C/EBPβ in similar abundances (Fig. 1I). GST pulldown assays were performed to examine whether the SAP domains from PIAS2, PIAS3, and PIAS4 could interact with C/EBPβ. As shown in Fig. 1J, all GST-fused SAP domains from different PIAS proteins could pull down C/EBPβ (Fig. 1J).

SUMOylation of C/EBPβ is significantly enhanced by PIAS1. To determine the functional consequences of the PIAS1-C/EBPβ interaction, we examined whether PIAS1 enhanced C/EBPβ SUMOylation by transfecting 3T3-L1 cells with His-tagged C/EBPβ, V5-tagged SUMO1, Flag-tagged PIAS1(WT), or the catalytically inactive mutant form of PIAS1, termed PIAS1(DN) (Fig. 2A). On day 4 postinduction, the cell lysates were assayed by histidine purification, followed by immunoblotting with anti-V5 antibody. Consequently, V5-tagged SUMO1-C/EBPβ conjugates were detected when SUMO1 was overexpressed and accumulated significantly upon PIAS1(WT) and SUMO1 coexpression (Fig. 2A). In contrast, PIAS1(DN) impaired the SUMOylation of C/EBPβ (Fig. 2A). To rule out the possibility that PIAS1(DN) failed to SUMOylate C/EBPβ because it did not interact with C/EBPβ, a coimmunoprecipitation assay was performed, confirming that both PIAS1(WT) and PIAS1(DN) associated with C/EBPβ (Fig. 2B), suggesting that PIAS1(DN) failed to SUMOylate C/EBPβ primarily due to the deficiency of its catalytic activity.

Lys133 of C/EBPβ was identified as the SUMO acceptor site (20); to test whether PIAS1 SUMOylates C/EBPβ on this site, a mutant form of C/EBPβ was generated by replacing Lys133 with Arg [termed C/EBPβ(K133R)]. His-C/EBPβ or His-C/EBPβ(K133R) was expressed in 3T3-L1 cells with or without V5-SUMO1 and Flag-PIAS1; consequently, ectopic expression of PIAS1 led to a dramatic increase in SUMOylated C/EBPβ (Fig. 2C), as in the case of the results above (Fig. 2A). In contrast, the bands of SUMO1-C/EBPβ(K133R) conjugates were totally absent when C/EBPβ(K133R) was expressed, regardless of whether PIAS1 was coexpressed (Fig. 2C). The coimmunoprecipitation assay showed that PIAS1 interacted with both C/EBPβ(WT) and C/EBPβ(K133R) in similar abundances (Fig. 2D).

Since C/EBPβ also interacted with other PIAS proteins, SUMOylation assays were performed in 293T cells to test the SUMOylation of C/EBPβ by PIAS2/3/4. All PIAS proteins were found to increase SUMOylated C/EBPβ to different degrees, though PIAS1 produced much more than others did (Fig. 2E), suggesting that PIAS1, but not other PIAS proteins, might play a major role in SUMOylating C/EBPβ during adipogenesis.

As SUMOylation regulates transcriptional activity of transcription factor, C/EBPβ transcriptional activity was monitored by measuring the transactivation of C/EBPα and PPARγ promoters by C/EBPβ. The promoters of C/EBPα and PPARγ which contain the C/EBPβ regulatory element were cloned into pGL3-basic luciferase vectors to be transfected into 293T cells; the results indicated that both C/EBPβ(WT) and C/EBPβ(K133R) overexpression transactivated the promoters of C/EBPα and PPARγ (Fig. 2F and G), as indicated by the increased luciferase activity, and that the ectopic expression of PIAS1 decreased the luciferase activity triggered by C/EBPβ(WT), whereas it had no effect on the transcriptional activity of C/EBPβ(K133R) (Fig. 2F and G).

PIAS1 leads to the ubiquitination and subsequent degradation of C/EBPβ. Since de-SUMOylation of C/EBPβ was reported to inhibit its ubiquitination and subsequent degradation (32), we hypothesized that PIAS1 would decrease the stability of endogenous C/EBPβ during adipogenesis. 3T3-L1 preadipocytes that stably overexpressed PIAS1(WT) or PIAS1(DN) were generated. Western blotting showed the overexpression of PIAS1 (Fig. 3A). On day 4 of differentiation, the stable cells were incubated with cycloheximide, and C/EBPβ protein was measured at various time points after treatment (Fig. 3B), showing that forced expression of PIAS1(WT) instead of PIAS1(DN) resulted in a marked decrease in the stability of C/EBPβ and that the overexpression of PIAS1(DN) had a C/EBPβ half-life similar to that of the control cells (Fig. 3B and C). This result indicated that PIAS1 decreased the stability of C/EBPβ, which was dependent on SUMO E3 ligase activity. The effect of PIAS1 on endogenous protein level of C/EBPβ was also explored during 3T3-L1 preadipocyte differentiation, which demonstrated that PIAS1(WT) overexpression led to a significant decrease in C/EBPβ, whereas PIAS1(DN) overexpression showed no obvious effect on the expression of C/EBPβ (Fig. 3D), which was consistent with the results of C/EBPβ half-life assays shown in Fig. 3B and C. Moreover, both PIAS1(WT) and PIAS1(DN) overexpression displayed little effect on the transcription levels of C/EBPβ (Fig. 3E).

The results above indicated that PIAS1 downregulated C/EBPβ protein level through E3 ligase activity. Based on previ-

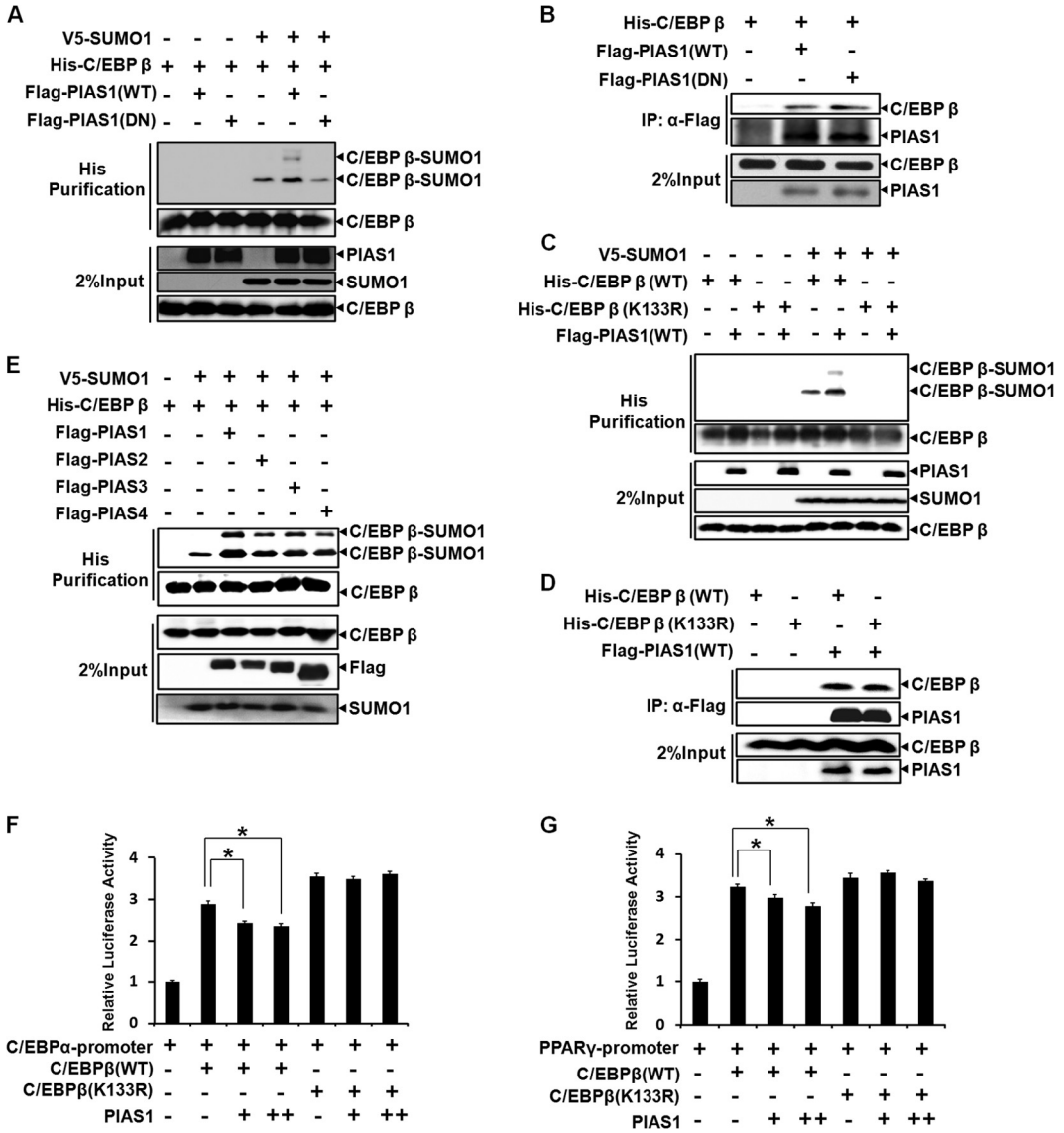

FIG 2 PIAS1 promotes C/EBPβ SUMOylation directly through its E3 ligase activity. (A) 3T3-L1 preadipocytes were transfected with the vectors expressing His-tagged C/EBPβ, V5-tagged SUMO1, Flag-tagged PIAS1(WT), or PIAS1(DN), as indicated. On day 4 after induction, histidine purification assays were used to purify His-tagged C/EBPβ. After purification, the SUMO1-C/EBPβ conjugates were analyzed with anti-V5, anti-His, and anti-Flag antibodies by Western blotting. (B) 3T3-L1 cells transfected with His-C/EBPβ, Flag-PIAS1(WT), or PIAS1(DN) were used for coimmunoprecipitation assays using anti-Flag antibody, followed by Western blotting to detect the complex with anti-Flag or anti-His antibodies. (C) C/EBPβ-SUMOylation was assayed as described above using 3T3-L1 cells transfected with the indicated expression vectors for His-C/EBPβ(WT), His-C/EBPβ(K133R), V5-SUMO1, and Flag-PIAS1(WT), followed by Western blotting with the indicated antibodies. (D) His-tagged C/EBPβ(WT), His-tagged C/EBPβ(K133R), and Flag-tagged PIAS1(WT) were transfected into 3T3-L1, as indicated. Coimmunoprecipitation assays were performed with anti-Flag antibody. The complexes were monitored with anti-Flag and anti-His antibodies. (E) 293T cells were transfected with vectors expressing His-C/EBPβ, V5-SUMO1, Flag-PIAS1, Flag-PIAS2, Flag-PIAS3, or Flag-PIAS4. At the 36th hour after transfection, histidine-purification assays were employed to purify His-tagged C/EBPβ. After purification, the SUMO1-C/EBPβ conjugates were analyzed with anti-V5, anti-His, and anti-Flag antibodies by Western blotting. (F and G) The promoters of C/EBPα (0.05 μg) (F) and PPARγ (0.05 μg) (G) were used separately to transfect 293T cells with PIAS1 (0.2, 0.3 μg) and C/EBPβ(WT) (0.2 μg) or C/EBPβ(K133R) (0.2 μg). Luciferase activity was measured using the dual-luciferase reporter assay at the 36th hour, and firefly luciferase activity was normalized to *Renilla* luciferase activity. *, $P < 0.05$.

ous reports, it was hypothesized that the mechanism could be SUMOylation-directed ubiquitination that facilitates the proteasomal degradation of C/EBPβ (33). 3T3-L1 preadipocytes were cotransfected with HA-Ub, V5-SUMO1, His-C/EBPβ, Flag-PIAS1(WT), or Flag-PIAS1(DN) (Fig. 4A). From the histidine-purification assays and Western blotting conducted to measure ubiquitination of C/EBPβ on day 4 of adipogenic induction, it was found that SUMOylation of C/EBPβ led to its ubiquitination (Fig. 4A), which was consistent with previous reports (33). PIAS1(WT), in addition to generating SUMOylated C/EBPβ, significantly augmented the ubiquitination of C/EBPβ, whereas PIAS1(DN) markedly decreased C/EBPβ ubiquitination, probably due to competition with endogenous PIAS1 (Fig. 4A). To confirm this result, the ubiquitination of endogenous C/EBPβ was carried out by immunoprecipitation assay. A consistent increase in the level of endogenous ubiquitinated C/EBPβ occurred when

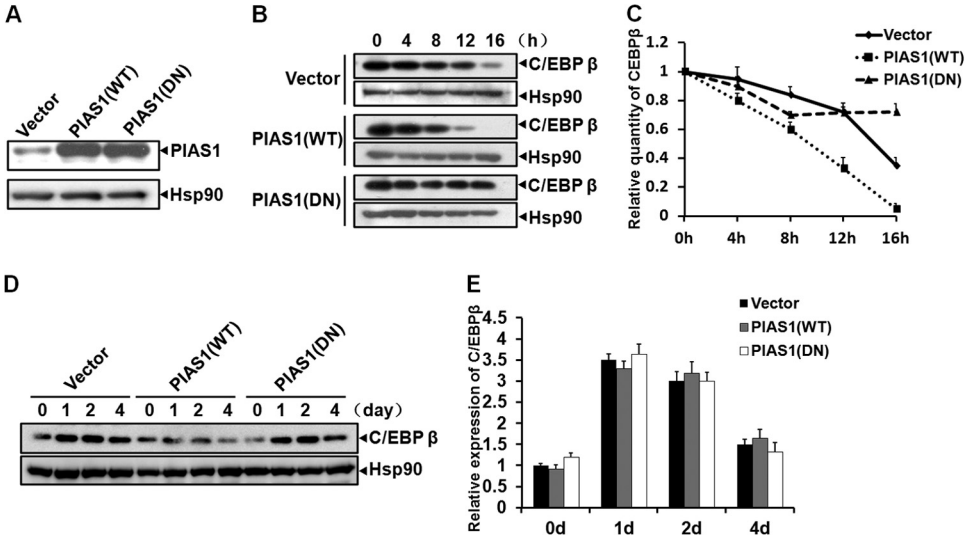

FIG 3 Overexpression of PIAS1 decreases the protein stability of C/EBPβ. (A) PIAS1(WT) and PIAS1(DN) were stably overexpressed in 3T3-L1 preadipocytes by using retroviruses harboring PIAS1 expression vectors. Control cells were generated by infection with a retrovirus harboring an empty vector. Four days after MDI treatment, Western blotting was used to test the overexpression of PIAS1 with anti-PIAS1 antibody. (B) On day 4 of induction, three types of cells were incubated with cycloheximide (5 μM) as shown. After treatment, cell lysates were Western blotted with the anti-C/EBPβ antibody. (C) The C/EBPβ protein level after cycloheximide treatment was quantified after normalization to 0 h and plotted as shown. The data are means and SD from three independent experiments. (D and E) 3T3-L1 preadipocytes stably overexpressing PIAS1(WT) or PIAS1(DN) were collected after MDI induction. Western blotting (D) and real-time quantitative PCR (E) were used to monitor the expression of C/EBPβ at the indicated time points.

PIAS1(WT) was overexpressed, whereas ubiquitinated C/EBPβ was relatively weak when PIAS1(DN) was overexpressed (Fig. 4B), suggesting that PIAS1 promoted C/EBPβ ubiquitination in a SUMOylation-dependent manner. In agreement with the notion that PIAS1 promoted C/EBPβ ubiquitination, the inhibition of proteasomal degradation with MG132 completely abolished PIAS1 inhibition of endogenous C/EBPβ protein (Fig. 4C). These results demonstrated that PIAS1 SUMOylated C/EBPβ to facilitate its ubiquitination and subsequent proteasomal degradation.

PIAS1 knockdown increases the protein stability of C/EBPβ. Further investigations were performed to explore the effect of PIAS1 knockdown on C/EBPβ by transfecting 3T3-L1 preadi-

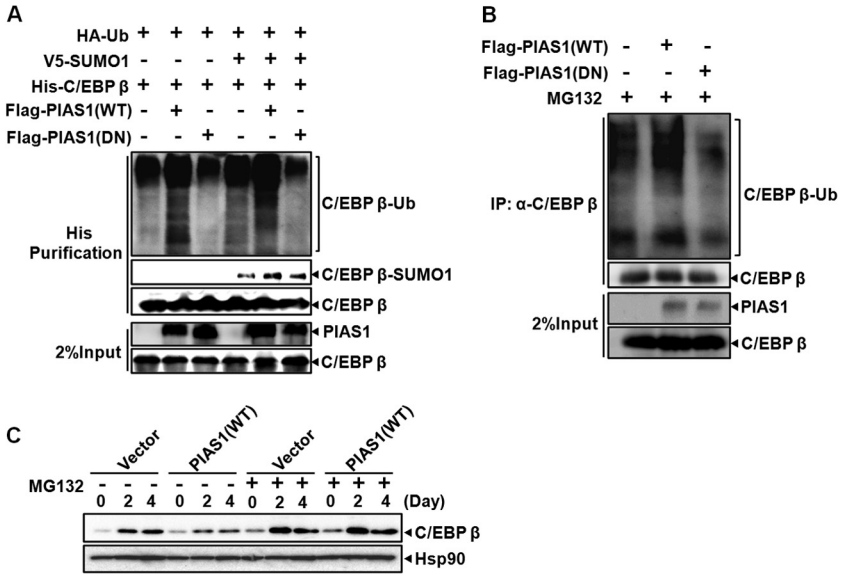

FIG 4 PIAS1 dramatically enhances C/EBPβ ubiquitination and subsequent degradation. (A) 3T3-L1 preadipocytes were cotransfected with His-tagged C/EBPβ, HA-tagged Ub, V5-tagged SUMO1, Flag-tagged PIAS1(WT), or PIAS1(DN), as indicated. On day 4 of adipogenic induction, cells were collected and subjected to histidine-purification assays. Western blotting was used to analyze the conjugates with anti-HA or anti-V5 antibodies. (B) 3T3-L1 preadipocytes were transfected with Flag-tagged PIAS1(WT), or PIAS1(DN). After MDI induction for 4 days, cells were treated with MG132 (10 μM) for 5 h. These cells were assayed by immunoprecipitation with anti-C/EBPβ antibody and followed by Western blotting with anti-Ub, anti-C/EBPβ or anti-Flag antibodies. (C) 3T3-L1 preadipocytes stably overexpressing PIAS1(WT) and control cells were treated with MDI for the indicated times before being incubated with or without MG132. The expression of C/EBPβ was detected with anti-C/EBPβ antibody.

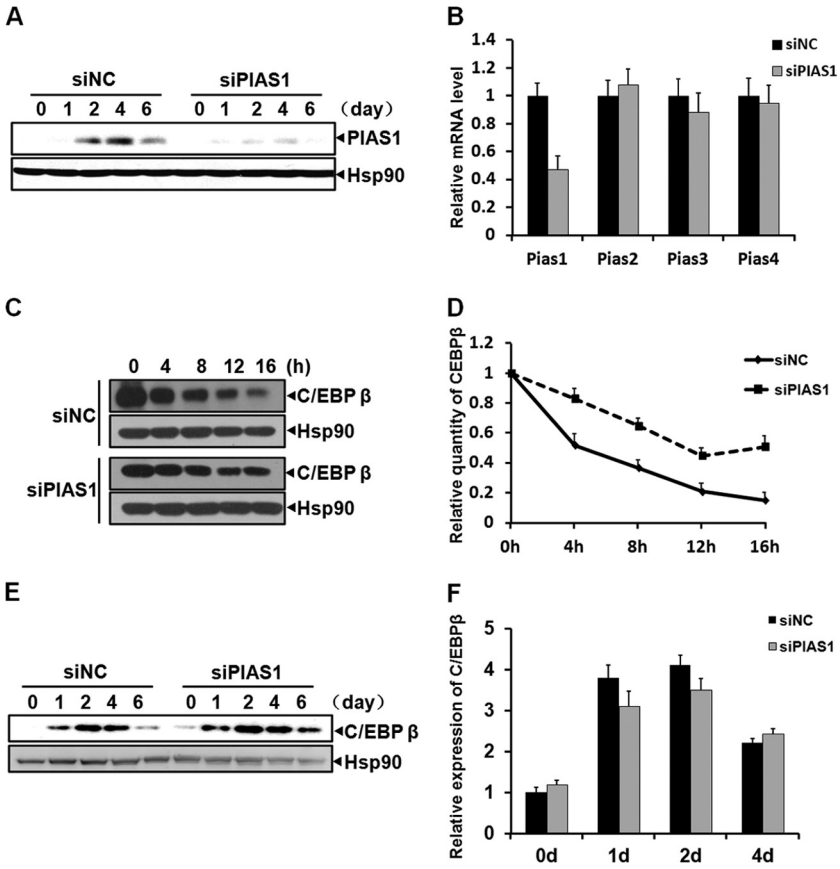

FIG 5 PIAS1 knockdown augments the protein stability of C/EBPβ. (A) 3T3-L1 preadipocytes were transfected with the siPIAS1 or siNC oligonucleotide at 50% confluence. Transfected cells received the adipogenic induction and were used to monitor PIAS1 protein on days 0, 1, 2, 4, and 6 postinduction by Western blotting. (B) Transfected 3T3-L1 cells were collected on day 4 after MDI treatment and analyzed by qPCR for the expression of PIAS1, PIAS2, PIAS3, and PIAS4. Data are normalized to the expression level in the control cells. (C) 3T3-L1 preadipocytes transfected with siPIAS1 or siNC were treated with MDI and incubated with cycloheximide (5 μM) for the indicated times. Cell lysates were checked by Western blotting with anti-C/EBPβ antibody. (D) The C/EBPβ protein level after cycloheximide treatment was quantified, normalized to 0 h, and plotted as shown. The data are means and SD from three independent experiments. (E and F) 3T3-L1 preadipocytes transfected with siPIAS1 or siNC were collected at the indicated time points after MDI induction. Western blotting (E) and real-time quantitative PCR (F) were used to monitor the expression of C/EBPβ. qPCR data are normalized to the expression of control siNC-transfected cells on day 0.

pocytes with a PIAS1-specific siRNA (siPIAS1) or a nonspecific control siRNA (siNC) at confluence by 50%. The transfected cells were subjected to analysis of the silencing effect of PIAS1 on days 0, 1, 2, 4, and 6 postinduction. PIAS1 knockdown by siPIAS1 led to a significant reduction in the protein (Fig. 5A) and mRNA (Fig. 5B) levels of PIAS1 during adipogenesis, without any effect on the PIAS2, PIAS3, or PIAS4 mRNA level (Fig. 5B). Similar experiments (Fig. 3) to determine the half-life and endogenous protein level of C/EBPβ showed that the transfection of siPIAS1 significantly increased the half-life of C/EBPβ (Fig. 5C and D) and that the C/EBPβ protein level was partially increased by PIAS1 knockdown at the indicated time points (Fig. 5E), whereas the silencing of PIAS1 exhibited little effect on the mRNA level of C/EBPβ (Fig. 5F). The data clearly indicated that PIAS1 knockdown enhanced the stability of C/EBPβ, which supported our results above.

PIAS1 modulates 3T3-L1 adipocyte differentiation by inhibiting C/EBPβ. PIAS1 SUMOylated C/EBPβ and contributed to the ubiquitin-mediated degradation of C/EBPβ, implying an important role for PIAS1 in adipogenesis. To test this possibility, 3T3-L1 preadipocytes stably overexpressing PIAS1(WT) or PIAS1 (DN) were produced by retrovirus infection. The effect of PIAS1 on adipogenesis was assessed by means of the time course studies of lipid droplet formation and the expression of adipogenic genes. Overexpression of PIAS1(WT) impaired lipid droplet formation during adipogenesis (Fig. 6A). Similarly, the expression of adipocyte-specific genes, such as those encoding C/EBPα, PPARγ, and 422/aP2, was weakened by PIAS1(WT) at the mRNA (Fig. 6B and C) and protein (Fig. 6D) levels. The mutant PIAS1, however, failed to repress adipogenesis, suggesting that the SUMO ligase activity was required for PIAS1 to suppress adipocyte differentiation.

The effect of PIAS1 knockdown on adipogenesis was also investigated, with the results that PIAS1 knockdown promoted the earlier appearance of lipid droplets (day 2) (Fig. 6E), that lipid droplets in those silenced cells for PIAS1 were larger than those in the controls (Fig. 6E), and that the expression of adipocyte-specific genes was induced earlier and to a higher level upon PIAS1 knockdown (Fig. 6F to H).

To evaluate whether C/EBPβ suppression is critical for PIAS1 to inhibit adipogenesis, 3T3-L1 preadipocytes stably overexpressing C/EBPβ(WT) or C/EBPβ(K133R) were produced through retrovirus infection. It was confirmed that C/EBPβ protein re-

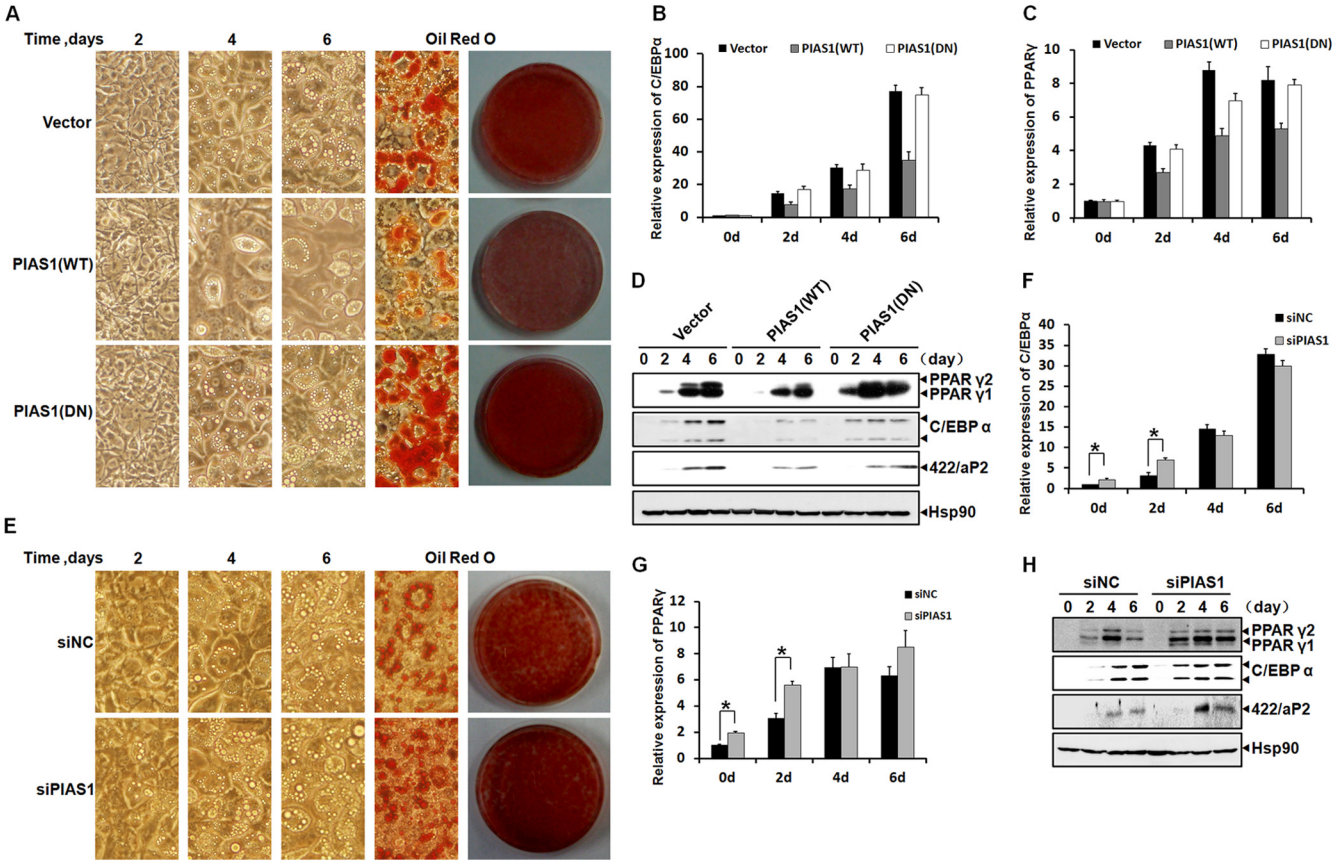

FIG 6 PIAS1 modulates adipogenesis through SUMO E3 ligase activity. (A to D) PIAS1 overexpression restrains 3T3-L1 preadipocytes adipogenesis in an E3 ligase-dependent manner. (A) 3T3-L1 preadipocytes were infected with retrovirus harboring PIAS1(WT) or PIAS1(DN) expression vectors or an empty vector. Lipid droplet formation in stable cells was observed on days 2, 4, and 6 postinduction, and cells were stained with Oil Red O on day 6. (B and C) Stable cells were collected and assayed by qPCR to monitor mRNA level of C/EBPα (B) and PPARγ (C) on days 0, 2, 4, and 6 postinduction. Data are normalized to the expression in control cells on day 0. (D) Western blotting was performed with the indicated antibodies to detect the protein level of C/EBPα, PPARγ, and 422/aP2 in the stable cells. (E to H) PIAS1 knockdown promotes adipogenesis. (E) 3T3-L1 preadipocytes were transfected with the siPIAS1 or siNC oligonucleotide at 50% confluence. Cells were subjected to the standard differentiation protocol as described above. Lipid droplet formation and Oil Red O staining of the transfected 3T3-L1 cells were observed on days 2, 4, and 6 after adipogenic differentiation. The mRNA level (F and G) and protein level (H) of adipogenic genes were measured by qPCR and Western blotting during adipogenesis. Data are normalized to the expression in control siNC-transfected cells on day 0. *, $P < 0.05$.

mained overexpressed, even after PIAS1 overexpression. The effect of C/EBPβ(WT) and C/EBPβ(K133R) on adipogenesis was investigated when PIAS1 was overexpressed, as indicated by Oil Red O staining (Fig. 7A) and expression of adipogenic genes, such as those encoding adiponectin, C/EBPα, PPARγ, and 422/aP2 (Fig. 7B to D). C/EBPβ(K133R) overexpression completely rescued impaired adipogenesis by PIAS1 overexpression, whereas C/EBPβ(WT) only partially restored adipogenesis (Fig. 7A, B, and D). Compared with C/EBPβ(WT), C/EBPβ(K133R) protected itself from SUMOylation by PIAS1 (Fig. 2C); therefore, it might be more stable and maintained at a higher protein level even upon PIAS1 overexpression (Fig. 7C). Thus, C/EBPβ(K133R) played a more powerful role in adipogenesis rescue than C/EBPβ(WT) did, suggesting that PIAS1 modulates adipogenesis by SUMOylating C/EBPβ.

DISCUSSION

The present study identified PIAS1 as a SUMO E3 ligase of C/EBPβ which regulates adipogenesis. Based on this, we propose a model in which PIAS1 plays a role in the control of 3T3-L1 adipogenic differentiation (Fig. 8). During the early stage of adipogenic differentiation, C/EBPβ is rapidly activated and sequentially phosphorylated to obtain DNA-binding activity. C/EBPβ protein is maintained at a high level, leading to the induction of downstream effectors, including C/EBPα and PPARγ. In contrast, PIAS1 is kept silent during the same period to ensure the low level of SUMOylated C/EBPβ (Fig. 8A). The PIAS1 protein level reaches a peak on day 4 of adipogenesis, when C/EBPβ has accomplished its mission. Subsequently, PIAS1 acts as a C/EBPβ SUMO E3 ligase, which results in SUMOylation of C/EBPβ. This leads to elevated ubiquitination and degradation of C/EBPβ, thereby regulating adipogenesis (Fig. 8B).

Adipocyte differentiation is a complicated process involving many transcription factors and coregulators interacting to establish the gene expression pattern of mature adipocytes (34). In the case of 3T3-L1 preadipocyte differentiation, C/EBPβ is an indispensable member among these transcription factors. On the one hand, C/EBPβ activates the expression of C/EBPα and PPARγ, which turn on a series of adipocyte genes, giving rise to the adipocyte phenotype (5, 6). On the other, C/EBPβ, associated with other cofactors, such as Kdm4b, induces the expression of cell

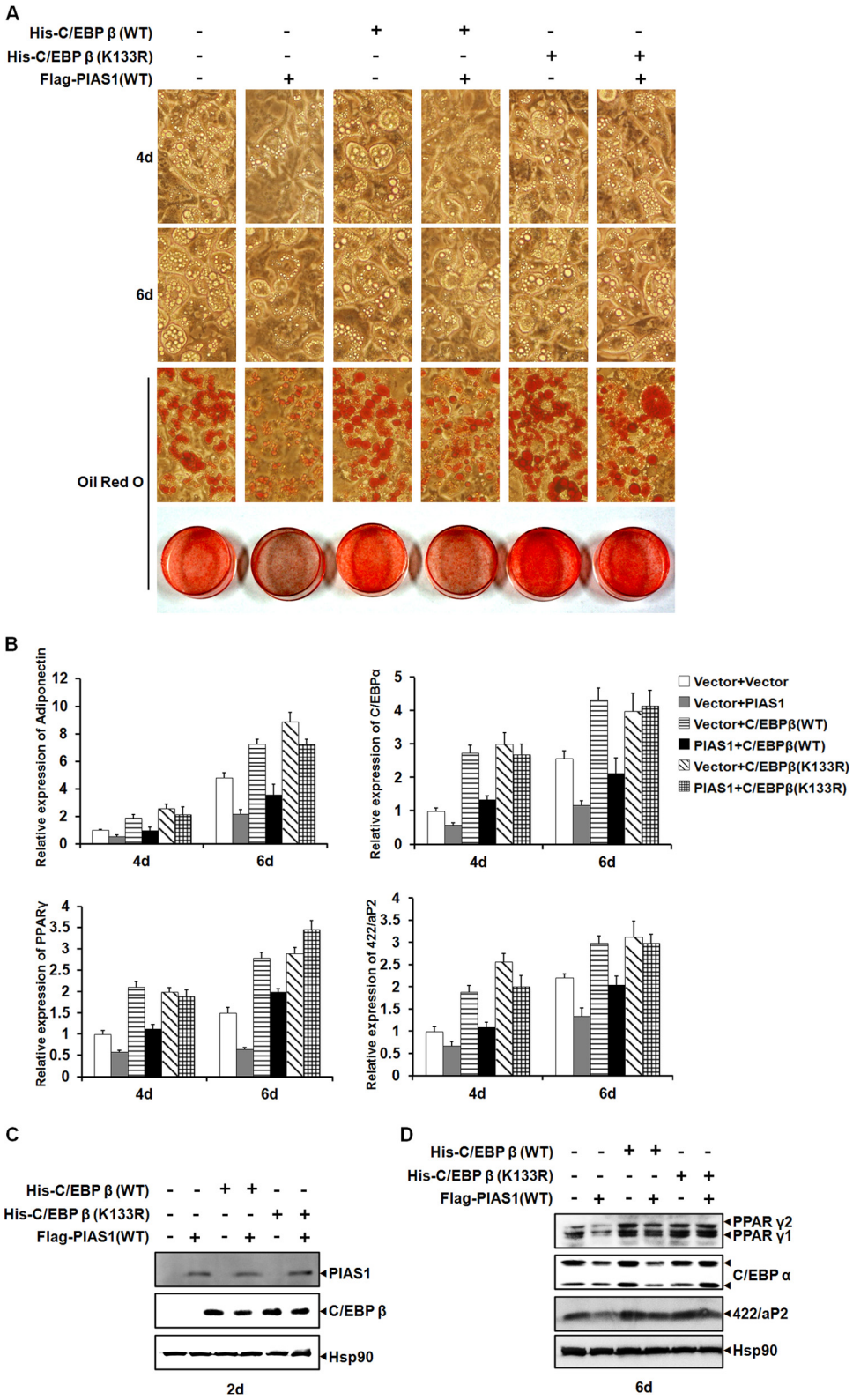

FIG 7 Forced expression of C/EBPβ reverses PIAS1-mediated inhibition of adipogenesis. (A) His-tagged C/EBPβ(WT) and C/EBPβ(K133R) were ectopically expressed in stable cells that overexpress Flag-PIAS1(WT), as well as the control cells. The stable cells were subjected to analysis for lipid droplet formation on days 4 and 6 postinduction and Oil Red O staining on day 6. (B) On days 4 and 6 postinduction, adiponectin, C/EBPα, PPARγ, and 422/aP2 mRNA levels were assayed by qPCR. Data are normalized to the expression in control cells on day 4. (C) Western blotting was used to test the overexpression of PIAS1 and C/EBPβ(K133R) on day 2 with anti-His or anti-Flag antibodies. (D) C/EBPα, PPARγ, and 422/aP2 proteins were monitored by Western blotting on day 6 postinduction in the indicated cells.

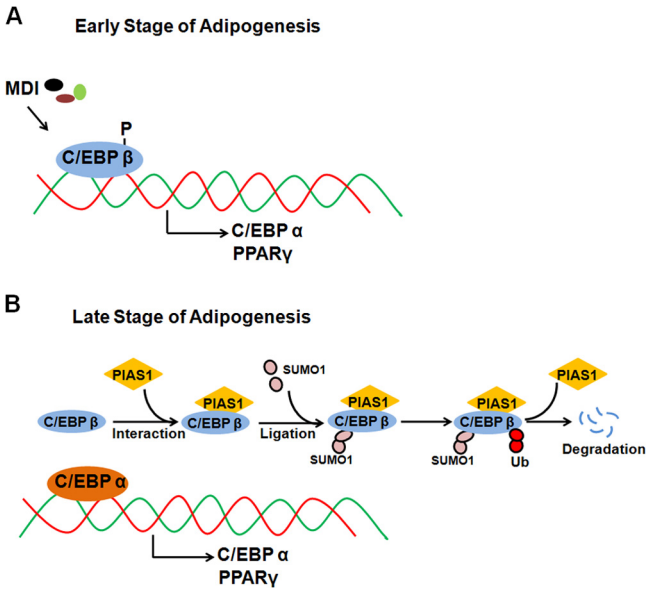

FIG 8 Model of the role of PIAS1 in regulating C/EBPβ and adipogenesis. (A) During the early stage of adipogenic differentiation, C/EBPβ was rapidly activated and sequentially phosphorylated to obtain DNA-binding activity, leading to the induction of downstream effectors, including C/EBPα and PPARγ. In contrast, PIAS1 was kept silent during the same period to ensure a low level of SUMOylated C/EBPβ. (B) PIAS1 protein level reached a peak on day 4 of adipogenesis, when C/EBPβ had accomplished its mission. Subsequently, PIAS1 interacted with C/EBPβ and then catalyzed its SUMOylation, causing elevated ubiquitination and degradation of C/EBPβ and thereby regulating adipogenesis.

cycle genes essential for mitotic clonal expansion (MCE) (15). C/EBPβ also associates with glucocorticoid receptor, retinoid X receptor, and STAT5 during the early stages of adipogenesis, forming a powerful transcription network to establish and maintain the adipocyte phenotype (35, 36). In view of the critical role of C/EBPβ, some negative regulators of C/EBPβ, such as CHOP-10 (37) and TRB3 (38), were suppressed during the initial days of adipogenic differentiation; therefore, the upregulation of these factors inhibited the adipogenesis (37, 38). In the present study, PIAS1, a novel negative regulator of C/EBPβ, was shown to SUMOylate C/EBPβ, thereby promoting its ubiquitination and subsequent degradation during the late stage of adipogenesis. Upregulation of PIAS1 caused a marked decrease in C/EBPβ protein level and inhibition of adipogenesis. It was reasonable that the PIAS1 protein level was low during the early stage to keep C/EBPβ stable; therefore, the expression of PIAS1 must have been tightly regulated, and this needs to be further investigated.

C/EBPβ was modified by SUMO on Lys133 (20). SUMOylation led to impaired transcriptional activity of C/EBPβ in addition to its ubiquitination, and de-SUMOylation of C/EBPβ by SENP2 promotes adipogenesis (32). C/EBPα and PPARγ could also be modified by SUMO, thereby inhibiting their function, for example, by impairing transcriptional activity (39, 40). As a SUMO E3 ligase (41), PIAS1 regulates many other transcription factors, including STAT1 (27), NF-κB (42), and liver X receptors (LXRs) (43). Although PIAS1 SUMOylated C/EBPβ through direct interaction during adipogenesis, we cannot rule out the possibility that PIAS1 regulates other adipogenic factors. In the present study, however, expression of C/EBPβ significantly rescued impaired adipogenesis by PIAS1 overexpression, indicating that PIAS1 played an essential role in adipogenesis mainly by regulating C/EBPβ stability. In addition, it has been reported that C/EBPβ played an important role in regulating brown-adipocyte gene expression (19). We found that PIAS1 was expressed in brown adipose tissue, and the change in its expression was detected during brown-adipocyte development (data not shown), indicating that PIAS1 might play a role in brown-adipocyte differentiation. Whether the regulation model of PIAS1-C/EBPβ exists in brown adipocytes needs to be investigated.

Originally, PIAS proteins were recognized for their ability to interact with and inhibit STAT factors, but it was recently shown that their interactions and functions are not restricted to STAT proteins. Mammalian PIAS proteins share a highly conserved sequence, especially at their N terminus and the central region. The serine/threonine-rich C-terminal region of PIAS proteins is the least conserved part. Four structural motifs have been identified in PIAS proteins: an N-terminal SAP motif, a PINIT motif, a putative RING-type zinc-binding structure, and a SIM (SUMO-interacting motif) motif (44). Containing numerous positively charged amino acids that might make contacts with the backbone of the DNA, the SAP motif is a putative DNA-binding motif involved in chromosomal organization. As such, the SAP motif has been found in many chromatin-associated proteins (45). In addition to functioning as SUMO E3 ligases, PIAS family members act as repressors of some transcription factors through direct binding to gene promoters. SAP domain in PIAS proteins is required for this process. Moreover, it is known that the PIAS SAP motif is an amphipathic, four-helix bundle conformation which is consistent with known protein-protein interaction interfaces. The SAP motif contributes to protein-protein interaction and the assembly of nuclear receptor-coregulator complexes. As shown in the present study, the SAP domain mediated the association between PIAS1 and C/EBPβ. Since the SAP motif exists in all PIAS family members and displays a highly conserved sequence, PIAS1, PIAS2, PIAS3, and PIAS4 recognized C/EBPβ through the SAP domain, as confirmed in our study. However, PIAS1 might be the major member that modulated adipocyte differentiation through C/EBPβ, on account of the findings that PIAS1 protein level was induced by adipogenic treatment and inversely correlated with C/EBPβ during adipogenesis (Fig. 1A), that mRNA level of PIAS1 was apparently higher than that of PIAS2, PIAS3, and PIAS4 (Fig. 1B), and that PIAS2 protein level exhibited no obvious changes during adipogenesis (data not shown).

PIAS1 has been identified as an important regulator in cell differentiation, including T cell differentiation (46), plasma cell differentiation (47), and muscle cell differentiation (48–50). For instance, PIAS1 promoted smooth muscle cell differentiation by its inhibitory effect on the JAK/STAT pathway (48, 49). In contrast, it was reported that PIAS1 repressed myogenin gene expression and skeletal muscle differentiation by associating with SnoN and Msx1 (50). Despite many investigations on myogenesis, the relationship between PIAS1 and adipogenesis was unexplored until the present study, which clarified the critical role of PIAS1 in adipocyte differentiation. Our findings could provide a potential therapeutic target for treating obesity and its associated disorders.

It has been reported that PIAS1 regulates lipogenesis in the liver, where its expression is inversely associated with the genes involved in lipogenesis, and suppresses LXR ligand-dependent transcriptional activation of lipogenic genes, including those en-

coding Srebp1c and Fas in hepatocytes, which in turn inhibits liver lipogenesis (43). The effect of PIAS1 on liver lipogenesis and its role in adipocyte differentiation suggest that PIAS1 could be a common regulator of lipogenesis in both adipocytes and hepatocytes. However, the function of C/EBPβ in liver lipogenesis is not clear; therefore, it would be meaningful to explore PIAS1-C/EBPβ regulation in liver lipogenesis.

In the present study, we demonstrated that PIAS1 can promote SUMOylation of C/EBPβ, facilitating its ubiquitination and subsequent degradation, and that, intriguingly, forced expression of C/EBPβ rescued PIAS1 inhibition on adipogenesis. In conclusion, PIAS1 modulated adipogenesis by functioning as SUMO E3 ligase of C/EBPβ. This model offers new insight into the role of PIAS1 in adipogenesis and helps us better understand the regulation of C/EBPβ.

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No potential conflicts of interest relevant to this article were reported.

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