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Control of Adipogenesis by the SUMO-Specific Protease SENP2▽

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Here, we demonstrate that SENP2, a desumoylating enzyme, plays a critical role in the control of adipogenesis. SENP2 expression was markedly increased upon the induction of adipocyte differentiation, and this increase was dependent on protein kinase A activation. Remarkably, knockdown of SENP2 led to a dramatic attenuation of adipogenesis with a marked decrease in PPARγ and C/EBPα mRNA levels. Knockdown of SENP2 also caused a marked reduction in the level of C/EBPβ protein but not in that of C/EBPβ mRNA. Interestingly, sumoylation of C/EBPβ dramatically increased its ubiquitination and destabilization, and this increase could be reversed by SENP2. In addition, overexpression of C/EBPβ could overcome the inhibitory effect of SENP2 knockdown on adipogenesis. Furthermore, SENP2 was absolutely required for adipogenesis of preadipocytes implanted into mice. These results establish a critical role for SENP2 in the regulation of adipogenesis by desumoylation and stabilization of C/EBPβ and in turn by promoting the expression of its downstream effectors, such as PPARγ and C/EBPα.

A large number of transcription factors are modified by the small ubiquitin (Ub)-related modifier (SUMO), and this covalent modification regulates their transcriptional activities (12, 16, 17). Unlike ubiquitination, SUMO modification is not a signal for protein degradation. SUMO modification regulates the target proteins through various mechanisms such as affecting cellular localization, protein-protein interaction, or stability of the target proteins. SUMO modification (sumoylation) is a reversible process that is catalyzed by SUMO-specific proteases (SENPs) (27). Six SENPs (SENP1, -2, -3, -5, -6, and -7) have been identified in humans, and they have different cellular localization and substrate specificities (42). Although the biochemical properties of SENPs have been well documented, their specific targets and physiological roles are known in a limited number of cases. SENP1 plays a key role in the hypoxic response by regulating HIF1α stability (6). SUSP4, a newly identified mouse SENP, inhibits cell growth by positively regulating p53 by promoting the self-ubiquitination of Mdm2 (21). In addition, overexpression of SENP2 is involved in the down-regulation of β-catenin, whereas the direct target of SENP2 in this process is unknown (19). It has also been reported that SENP5 regulates cell division and mitochondrial morphology; however, the targets of SENP5 have not been identified (9, 46).

The adipose tissues function as a reservoir of excessive energy. They also secrete adipokines that regulate physiological and pathological events involving energy metabolism, insulin sensitivity, atherogenesis, and inflammatory responses. Adipocyte differentiation from preadipocytes occurs by serial inductions of transcription factors, and this process is tightly regulated. Expression of C/EBPβ and C/EBPδ is induced immediately after stimulation, followed by the induction of PPARγ and C/EBPα (5, 43). PPARγ induces several adipocyte-specific genes, including aP2 and adiponectin, and C/EBPα acts in concert with PPARγ in this process (25, 26, 33, 34). CREB (cyclic AMP [cAMP] response element-binding protein) is also involved in the induction of C/EBPβ and PPARγ (11). In addition, several reports have shown that additional transcription factors, such as KLF5, KLF4 and Foxo-1, are involved in adipogenesis (4, 28, 31). While C/EBPβ and C/EBPδ are expressed immediately after treatment with differentiation inducers, expression of their downstream genes, the PPARγ and C/EBPα genes, does not begin until day 2 after treatment. It has been suggested that mitotic clonal expansion is a prerequisite for the induction of PPARγ and C/EBPα (38, 39). It has also been reported that C/EBPβ proteins are phosphorylated sequentially by mitogen-activated protein kinase (MAPK), CDK2, and GSK3β to acquire their C/EBPα and PPARγ promoter DNA-binding activities (22, 37), suggesting that C/EBPβ is a master regulator of adipocyte differentiation.

Several transcription factors regulating adipogenesis can be modified by SUMO. Sumoylation of C/EBPα and C/EBPβ negatively regulates their transcriptional activities in a transient transfection and reporter assay system (10, 36); however, a mechanism(s) by which sumoylation regulates their transcriptional activities has not been identified. PPARγ is also modified by SUMO, and a PPARγ mutant in which the major sumoylation site Lys107 is replaced with Arg stimulates adipogenesis more efficiently than does wild-type PPARγ (29, 41). In

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addition, sumoylation of KLF5 regulates lipid metabolism involving PPARδ (30) and SUMO-conjugating enzyme Ubc9 regulates glucose transporter 4 turnover in adipocytes (23). These results indicate the possibility that regulation of sumoylation plays a role in adipogenesis and lipid metabolism.

In the present study, we tested whether a SENP is involved in adipogenesis. We demonstrated that SENP2 was induced in a cAMP-dependent fashion in the early stage of adipogenesis. SENP2 desumoylated and stabilized C/EBPβ, whereas knockdown of SENP2 led to a decrease in the stability of the transcription factor and, in turn, inhibition of adipogenesis. In addition, overexpression of C/EBPβ could overcome the inhibitory effect of SENP2 knockdown on adipogenesis. Collectively, our findings indicate that SENP2 plays an essential role in the control of adipogenesis.

MATERIALS AND METHODS

Plasmids and antibodies. pFLAG-SUMO1, pMyc-SUMO1, pMyc-SENP1, pFLAG-SENP2, and pFLAG-SENP3 were prepared as described previously (8). Expression vectors for mouse C/EBPβ and CREB were obtained from J. B. Kim (Seoul National University, Seoul, South Korea). To generate the hemagglutinin (HA)-C/EBPβ expression vector, a cDNA fragment of mouse C/EBPβ (LAP) was inserted into pcDNA-HA. SENP2 mutant forms in which Cys548 was replaced with Ser (referred to as C548S) (19) and Arg576 and Lys577 were replaced with Leu and Met, respectively (RK576/577LM) (3), were generated by site-directed mutagenesis (Stratagene). To generate SENP2 reporter vectors, the promoter region of mouse SENP2 (bp −1980 to +93) was ligated to the luciferase (Luc) gene in the pGL2-basic vector (Promega). Serial deletions of the promoter with the sequences from −868 or −157 to +93 were also generated. In the deletion construct with the −157 to +93 sequence, ACG was replaced with GTT by site-directed mutagenesis (i.e., −103 to −96, TGACGTCA → TGGTTTCA). Small interfering RNAs (siRNAs) of SENP1 and SENP2 were purchased from Dharmacon. Antibodies against PPARγ, C/EBPα, CEBPβ, CEBPδ, aP2, SENP2, ubiquitin, SUMO1, GAPDH, CREB, and c-Myc (9E10) were purchased from Santa Cruz Biotechnology. Antibody against HA was obtained from Roche, and antibodies against FLAG and β-actin were purchased from Sigma-Aldrich.

Cell culture and adipocyte differentiation. 3T3-L1 preadipocytes were obtained from J. W. Kim (Yonsei University, Seoul, South Korea). They were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (Invitrogen). Differentiation of 3T3-L1 cells was induced by treatment with DMI [5 μg/ml insulin, 0.25 μM dexamethasone (Dex), 0.5 mM 3-isobutyl-α-methylxanthine (IBMX)] in DMEM supplemented with 10% fetal bovine serum (FBS). Two days after treatment, cells were maintained in DMEM supplemented with 10% FBS and 1 μg/ml insulin for up to 10 days with a medium change every other day. For knockdown of SENP2 or SENP1, 3T3-L1 cells were transfected with siRNA (50 nM) using Lipofectamine 2000 (Invitrogen) 1 day before the addition of DMI. Lipid droplets were stained by 5% Oil Red O in 60% isopropanol. COS-7 cells were maintained in DMEM supplemented with 10% FBS.

Generation of stable preadipocyte cell lines. cDNA for C/EBPβ (LAP) or C/EBPβ K133R was inserted into pBabe-Puro. Bosc cells were transfected with pBabe-Puro or pBabe-Puro-C/EBPβ. The resulting retrovirus was used to infect 3T3-L1 cells, and cells harboring pBabe-Puro, pBabe-Puro-C/EBPβ, or pBabe-Puro-C/EBPβ K133R were then selected by puromycin treatment. To generate stable preadipocytes (3T3-F442A) in which SENP2 expression is knocked down, the RNAi-Ready pSIREN-RetroQ (Clontech, Mountain View, CA)-mediated retroviral system was used. The DNA sequence used for SENP2 knockdown was AAAGTTATTGGAGCGACT.

Animals. Animals were handled in compliance with the Guide for Experimental Animal Research from the Laboratory for Experimental Animal Research, Clinical Research Institute, Seoul National University Hospital. C57BL/6J, *ob*/*ob*, and *db*/*db* mice (all 11 weeks old) were used in this study. The control group (9-week-old C57BL/6J male mice) was fed a 78% sucrose diet (Research Diet Inc.), and the high-fat diet group was fed a 60% fat diet for 4 weeks. The body weight and blood glucose level of the control group were 25.9 ± 0.9 g and 147.0 ± 13.9 mg/dl, and those of the high-fat diet group were 31.0 ± 1.5 g and 213.6 ± 11.2 mg/dl, respectively.

RNA preparation, Northern blot analysis, and real-time PCR. Aliquots (5 to 10 μg) of total RNAs prepared with Trizol (Invitrogen) were electrophoresed in 1% agarose-formaldehyde gels and transferred to Nytran membranes (Schleicher & Schuell). Membranes were hybridized with α-^{32}P-labeled probes in ExpressHyb solution (BD Stratagene) and then autoradiographed. Probes were prepared by PCR using primers 5′-ACTACGGTTACGTGAGCCTCGG-3′ and 5′-GCTGCTTGAACAAGTTCCGCAG-3′ for mouse C/EBPβ, 5′-TCGACTTCAGCGCCTACATTGAC-3′ and 5′-ACAACTCCACCAGCTTCTGCTG-3′ for mouse C/EBPδ, 5′-CAATGCCCCGCCTGGTCTTCTA-3′ and 5′-GCACTCCAGCATTAGAGAAGCTAGC-3′ for mouse SENP2, and 5′-GAGGATTCGGCAGCGGTCTTAGG-3′ and 5′-ACAGCTGGAGCCTGGGTCCTCTG-3′ for mouse RPS2. For real-time PCR, TaqMan Master Mix reagents and a TaqMan ABI PRISM 7000 sequence detector system (Applied Biosystems) were used. Primers and probes were purchased from Applied Biosystems. 18S rRNA was used as an endogenous control. Analysis was performed in triplicate and repeated at least three times.

Transient transfection of plasmids and Luc reporter assay. 3T3-L1 cells were plated in 12-well plates and transfected with 300 ng of Luc reporter vectors carrying serial deletions of the SENP2 promoter region and 200 ng of pCMV-βgal. Cells were treated with forskolin (10 μM) for 18 h and then harvested. The Luc reporter assay was performed in accordance with the manufacturer's instructions (Invitrogen). For the C/EBPβ transcriptional activity assay, COS-7 cells were transfected with a p21 promoter-Luc reporter vector (−1458 p21-Luc) (200 ng) (32) and expression vectors for C/EBPβ (20 ng), SENP2 (20, 50, 100 ng), or SENP2 C548S (100 ng) and pCMV-βgal (100 ng).

EMSA. To prepare nuclear extracts, cells were lysed in 10 mM HEPES (pH 7.9)–1.5 mM $MgCl_2$–10 mM KCl–0.5 mM dithiothreitol (DTT). After centrifugation at 3,300 × *g*, nuclei were suspended in 20 mM HEPES (pH 7.9)–1.5 mM $MgCl_2$–0.42 M NaCl–25% (vol/vol) glycerol–0.2 mM EDTA–0.5 mM phenylmethylsulfonyl fluoride (PMSF)–0.5 mM DTT. They were then dialyzed in 20 mM HEPES (pH 7.9)–100 mM KCl–20% glycerol–0.2 mM EDTA–0.5 mM PMSF–0.5 mM DTT. To determine CREB binding to the SENP2 promoter by electrophoretic mobility shift assay (EMSA), an α-^{32}P-labeled oligonucleotide probe with the bp −113 to −81 sequence (5′-CGGAAGAAGGTGACGTCAACGGCTTCTAGAGA-3′) of the promoter was incubated with nuclear extracts (2 μg) in 10 mM HEPES (pH 7.9)–50 mM KCl–0.1 mM EDTA–0.25 mM DTT–0.1 mg/ml poly(dI-dC)–0.01% Nonidet P-40–10% glycerol for 10 min. Competition assays were performed by supplementing increasing amounts of unlabeled wild-type or mutated probes. Supershift assays were carried out by incubation of nuclear extracts with anti-CREB antibody for 15 min before addition of the labeled probe. For the EMSA of C/EBPβ binding, the C/EBP binding site of the C/EBPα promoter, 5′-ttGCAGTTGCGCCACGATC-3′ (−191 to −172), was used as the probe (38). After incubation, the samples were subjected to electrophoresis on 5% gels under nondenaturing conditions, followed by autoradiography.

ChIP. For chromatin immunoprecipitation (ChIP), 3T3-L1 cells were incubated with DMI or IBMX for 8 h. After incubation, cells were treated with 1% formaldehyde for 10 min, harvested, and resuspended in 100 mM Tris-HCl (pH 9.4)–10 mM DTT–protease inhibitors. Nuclei were isolated by centrifugation and resuspended in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA–0.5 mM EGTA–1 mM DTT–protease inhibitors. After sonication, nuclear extracts were incubated with anti-CREB antibody, salmon sperm DNA, and protein G-Sepharose in radioimmunoprecipitation assay buffer overnight. Precipitates were resolved in 1% sodium dodecyl sulfate (SDS)–0.1 M $NaHCO_3$ and incubated with 200 mM NaCl. DNA was purified and subjected to PCR using primers 5′-CCTGTTGCTAGGCTTACAAGGAGC-3′ (bp −186) and 5′-CTTCAGCCGTAGCCAGGATCAG-3′ (bp +4) of the SENP2 promoter.

Immunoprecipitation and immunoblot analysis. COS-7 cells plated on 60-mm dishes were transfected with 0.25 μg of pHA-C/EBPβ, 1 μg of pFLAG-SUMO1, 1 μg of pMyc-ubiquitin, and 0.25, 0.5, or 1 μg of SENP expression vectors by using Lipofectamine plus (Invitrogen). After incubation for 24 h, cells were lysed in 20 mM HEPES (pH 7.4)–150 mM NaCl–1% Triton X-100–1% sodium deoxycholate–1% SDS–1 mM EDTA. Cell lysates (1 mg) were incubated with 2 μg of anti-HA antibody for 4 h at 4°C and then with protein A-Sepharose for the next 1 h. Precipitates were subjected to SDS-polyacrylamide gel electrophoresis, followed by immunoblot analysis. Lysates of 3T3-L1 cells were also subjected to immunoprecipitation with anti-C/EBPβ or anti-SUMO antibody.

Implantation of 3T3-F442A preadipocytes and examination of fat pads. 3T3-F442A preadipocytes were grown to 90% confluence and transfected with siNS or siSENP2 (50 nM) for 4 h. Cells were trypsinized and then suspended in DMEM with 10% calf serum. After centrifugation, cell pellets were resuspended in DMEM with 10% calf serum. Cells (3×10^7 cells) were injected into the subcutis of the abdomens of 9-week-old BALB/c nude mice (CAnN.Cg-Foxn1-

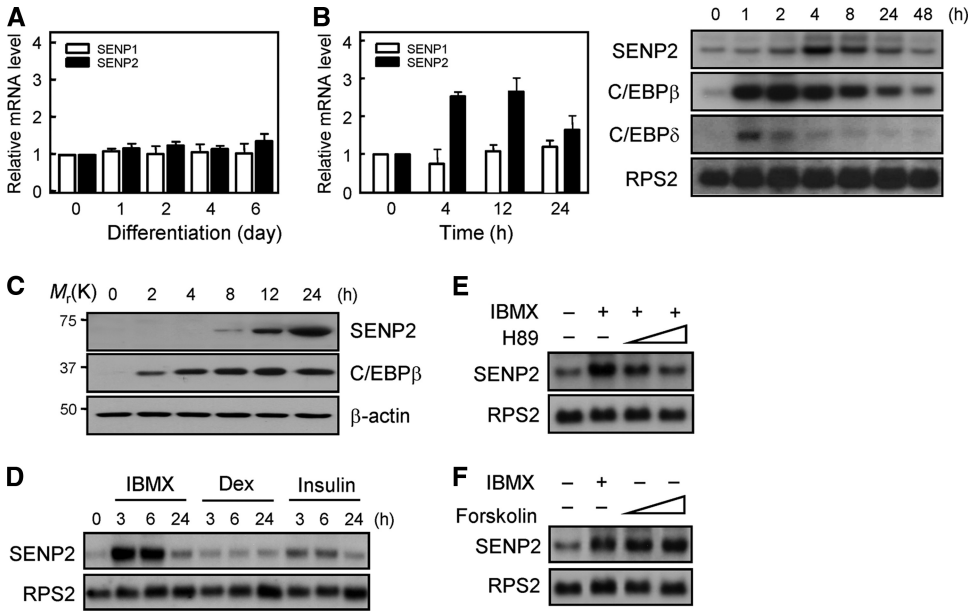

FIG. 1. Expression of SENP2 mRNA during adipogenesis. (A) 3T3-L1 cells were incubated with DMI for induction of adipogenesis. Total RNAs were prepared at the indicated times after induction and subjected to real-time PCR using primers specific for SENP1 (□) and SENP2 (■). The mRNA levels seen immediately after DMI treatment were set to 1.0, and the others are expressed as relative values. Data represent the means ± the standard errors of the means of three independent experiments. (B) 3T3-L1 preadipocytes were incubated with DMI for the indicated periods. After incubation, total RNAs were prepared and subjected to real-time PCR (left panel) or Northern blot analysis (right panel). A specific probe for ribosomal protein s2 (RPS2) was used as an internal control in Northern blot analysis. (C) After incubation with DMI for the indicated periods, cell lysates were prepared and subjected to immunoblot analysis. (D) Cells were incubated with IBMX, Dex, or insulin for the indicated periods. (E) Cells were incubated for 8 h with IBMX in the absence or presence of H89 (10 or 50 μM). RNAs were then prepared. (F) Cells were incubated with or without forskolin (10 or 50 μM) for 8 h.

nu/CrljBgi athymic mice; Orient Bio, Inc., Gyeonggi-Do, South Korea) (24). Fat pads generated from the implanted cells were removed, fixed in buffered formalin, and stained with hematoxylin and eosin (H&E) or subjected to immunohistochemistry with antiperilipin antibody (Research Diagnostics, Concord, MA) and detected with an LSM 710 confocal microscope (Carl Zeiss, Jena, Germany) 5 weeks after implantation.

Statistical analysis. Statistical analysis was performed using SPSS version 12.0 (SPSS Inc., Chicago, IL). Statistical significance was tested using the Mann-Whitney U test. A P value of <0.05 was considered statistically significant.

RESULTS

Expression of SENP2 mRNA is induced at the early stage of adipogenesis. To test whether SENP activity is involved in adipogenesis, the changes in SENP1 and SENP2 mRNA levels during adipogenesis of 3T3-L1 cells were monitored. Neither the SENP1 nor the SENP2 mRNA level was substantially changed at 24 or 48 h after treatment with the adipocyte differentiation inducers Dex, IBMX, and insulin (henceforth referred to as DMI) (Fig. 1A). When monitoring the SENP1 and SENP2 mRNA levels at the very early stage of differentiation, we found that the SENP2 mRNA level markedly increased within 4 h after the induction of adipocyte differentiation (Fig. 1B). The SENP1 mRNA level, unlike the SENP2 mRNA level, was not induced. In accord with a previous report (5), the transcript levels of C/EBPβ and C/EBPδ were also dramatically increased within 1 to 2 h after treatment with DMI and gradually declined thereafter, indicating that the increased expression of C/EBPβ and C/EBPδ precedes that of SENP2 mRNA (Fig. 1B, right panel). Consistent with the induction of the C/EBPβ and SENP2 mRNA levels, the C/EBPβ protein level was rapidly increased by DMI and the SENP2 protein level was gradually increased within 24 h (Fig. 1C). These results implicate SENP2 in adipogenesis.

SENP2 mRNA expression is induced by PKA activation. Since DMI consists of Dex, IBMX, and insulin, we examined whether an individual component by itself could affect the expression of SENP2 mRNA. IBMX dramatically increased the SENP2 mRNA level, unlike insulin and Dex, which had relatively little or no effect (Fig. 1D). Since IBMX is known to elevate the intracellular cAMP level, we tested whether the increase in the SENP2 mRNA level was mediated by cAMP-dependent activation of protein kinase A (PKA). H89, a PKA inhibitor, diminished the IBMX-mediated increase in the SENP2 mRNA level (Fig. 1E). On the other hand, forskolin, a PKA activator, alone could increase the SENP2 mRNA level (Fig. 1F). These results suggest that activation of PKA is responsible for the induction of SENP2 mRNA expression.

The SENP2 promoter has a functional CRE. Activated PKA phosphorylates CREB, which in turn binds to *cis*-acting cAMP response elements (CRE) in the promoters and stimulates the expression of the target genes (13). To identify a functional CRE, we generated a serial deletion of the SENP2 promoter region (Fig. 2A, upper panel). Transient transfection into 3T3-L1 preadipocytes and reporter assays reveal that deletion of the sequence from bp −1980 to bp −868 or −157 has little effect on the forskolin-mediated increase in Luc activity (Fig. 2A, lower panel), indicating that a CRE is located downstream of bp −157 of the promoter. Since a sequence similar to a consensus CRE is located at bp −103 to −96 from the tran-

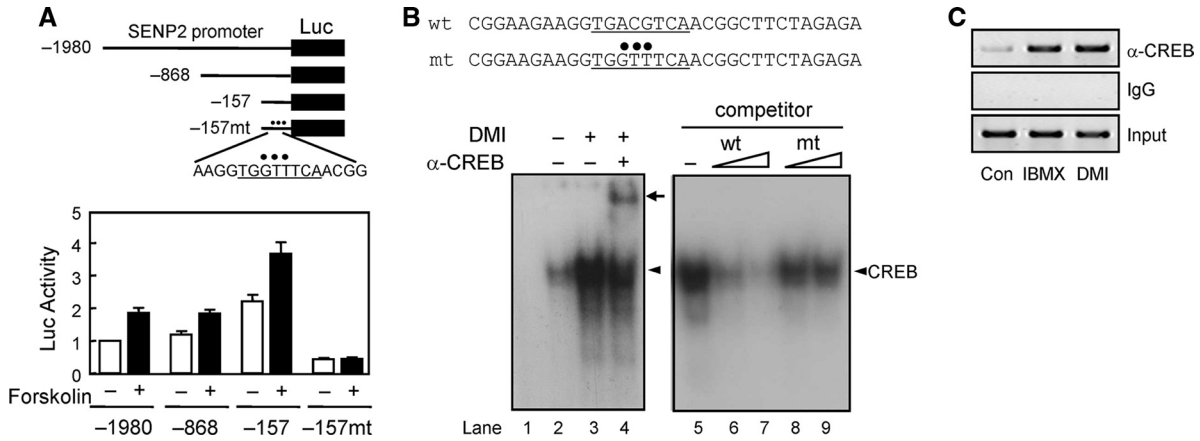

FIG. 2. Identification of a CRE in the SENP2 promoter. (A) Serial deletions of the SENP2 promoter region were fused to the Luc reporter gene, and the resulting constructs were transfected into 3T3-L1 preadipocytes with pCMV-βgal. Cells were incubated with or without forskolin for 18 h and then assayed for Luc and β-galactosidase. Luc activities were normalized to β-galactosidase activity. The normalized activity seen with the construct without deletion was set to 1.0, and the others are expressed as relative values. Data represent the means ± the standard errors of the means of six independent experiments. The dots indicate the mutations at bp −101 to −99 of the SENP2 promoter. A putative CRE sequence is underlined. (B) An oligonucleotide probe (wt) with the bp −113 to −81 sequence of the SENP2 promoter and a mutant form (mt) with GTT in place of ACG (indicated by dots) were synthesized. Nuclear extracts were prepared from 3T3-L1 cells that had been incubated for 8 h with or without DMI. For competition assays, the unlabeled probe (wt) or the mutant form (mt) was also included at 10- and 50-fold molar excesses over the radiolabeled probe. In lane 1, the radiolabeled probe only was loaded. (C) 3T3-L1 cells were incubated in the absence (control [Con]) or presence of IBMX or DMI for 8 h. They were then subjected to ChIP analysis by using anti-CREB antibody or control IgG as described in Materials and Methods.

scription start site, mutations were inserted into this region (i.e., replacement of ACG with GTT). This mutation abrogated the increase in Luc activity caused by forskolin and also led to a significant decrease in basal transcription activity (Fig. 2A, lower panel). These results indicate that the SENP2 promoter has a functional CRE. To confirm this finding, we performed an EMSA by using a radiolabeled oligonucleotide probe (wt) with the bp −113 to −81 sequence of the SENP2 promoter (Fig. 2B, upper panel). A slow-migrating band appeared upon incubation of the probe with the nuclear extracts prepared from 3T3-L1 preadipocytes that had not been treated with DMI (Fig. 2B, lane 2 in lower panel). Significantly, the binding activity was dramatically increased upon incubation with the extract prepared 8 h after inducer treatment (lane 3). Furthermore, supplementation with antibody against CREB led to the appearance of a supershifted band (lane 4), and unlabeled wild-type oligomers, but not mutated oligomers, competed for the binding of CREB to the radiolabeled probe in a dose-dependent fashion (lanes 5 to 9). These results indicate that CREB binds to the probe and PKA activation is involved in the binding of CREB to the SENP2 promoter. In addition, a ChIP analysis was performed with anti-CREB antibody and 3T3-L1 cells left untreated (control) or treated with IBMX or DMI. CREB binding to the endogenous SENP2 promoter region (−184/+4 bp) was dramatically increased after the treatment of cells with IBMX or DMI (Fig. 2C). Collectively, these results indicate that an elevated cAMP level induces SENP2 expression through CREB binding to a functional CRE in the SENP2 promoter.

SENP2 is required for adipocyte differentiation. Our finding that SENP2 mRNA expression dramatically increases at the early stage of adipocyte differentiation implies an important role for SENP2 in adipogenesis. To test this possibility, 3T3-L1 preadipocytes were transfected with a SENP2-specific siRNA (referred to as siSENP2) or a nonspecific control siRNA (siNS) 1 day before DMI treatment. Transfection of siSENP2 caused a dramatic reduction in the SENP2 mRNA level at the indicated times after DMI treatment (Fig. 3A). We then examined the effect of SENP2 mRNA knockdown on adipocyte differentiation by transfection of siSENP2. Oil-red O staining of lipid droplets in cells reveals that knockdown of SENP2 mRNA strongly attenuates adipocyte differentiation (Fig. 3B). To rule out the possibility of an off-target effect of the siRNA treatment, we tested four different types of siSENP2, and all of the siSENP2 types tested significantly reduced SENP2 mRNA levels and inhibited adipocyte differentiation (data not shown). To determine whether this effect is specific to SENP2, we compared the effect of siSENP1 to that of siSENP2. Of the SENP family members, SENP1 is evolutionarily most closely related to SENP2 and both SENPs are, at least in part, localized in the nucleus (1, 27, 44). The SENP1 mRNA level was significantly reduced by the transfection of siSENP1 (Fig. 3C). In contrast to siSENP2, siSENP1 had little or no effect on adipocyte differentiation (Fig. 3D). In addition, adenovirus-mediated overexpression of SENP2 in 3T3-L1 or 3T3-F442A cells increased the adipogenesis of both cell lines (data not shown). These results suggest that SENP2 specifically plays a critical role in the control of adipocyte differentiation.

SENP2 is required for the expression of C/EBPα and PPARγ. To explore the mechanism by which SENP2 controls adipogenesis, we examined the effect of SENP2 mRNA knockdown on the expression of mRNAs of adipogenic transcription factors. Transfection of siSENP2 had little or no effect on the transcript levels of C/EBPβ and C/EBPδ (Fig. 3E, upper panels). In contrast, expression of PPARγ and C/EBPα mRNAs was strongly attenuated by knockdown of SENP2 mRNA

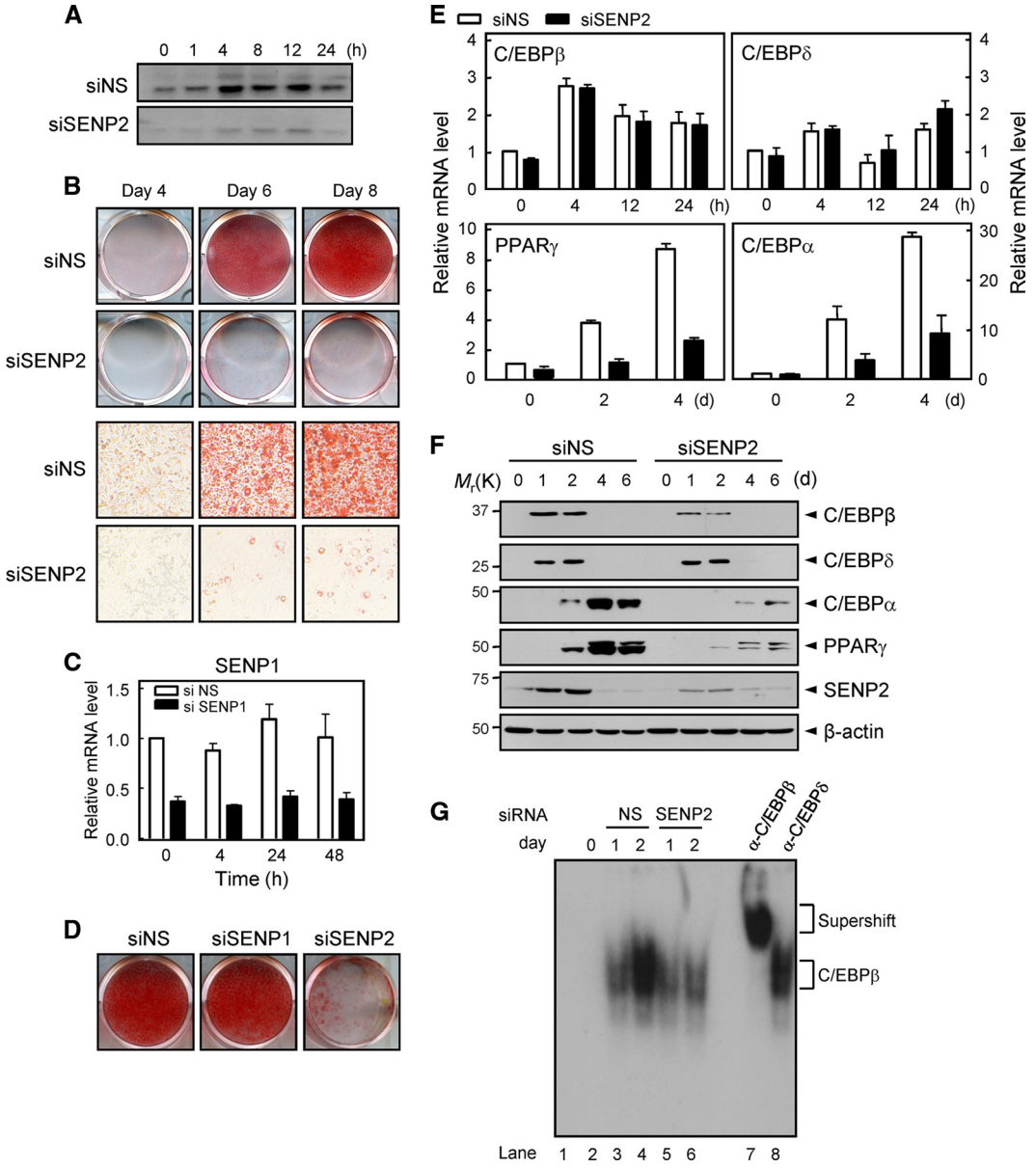

FIG. 3. Effect of SENP2 knockdown on adipocyte differentiation. (A) 3T3-L1 preadipocytes were transfected with siSENP2 (50 nM) or siNS (50 nM, nonspecific siRNA used as a negative control) 1 day before the induction of differentiation. Cells were incubated in differentiation medium for the indicated periods. After incubation, aliquots (10 μg) of total RNAs were subjected to Northern blot analysis with a SENP2 probe. (B) Cells were stained with Oil Red O at the indicated times after DMI stimulation. (C) Total RNAs were isolated from cells at the indicated times after DMI stimulation and then subjected to real-time PCR using primers specific for SENP1. The SENP1 mRNA level seen in the siNS-treated cells immediately after DMI treatment was set to 1.0, and the others are expressed as relative values. Data represent the means ± the standard errors of the means of three independent experiments. (D) Cells transfected with siNS, siSENP1, or siSENP2 were stained with Oil Red O 8 days after DMI treatment. (E) Total RNAs were prepared from cells transfected with siNS or siSENP2 at the indicated times after stimulation with DMI and subjected to real-time PCR using primers specific for C/EBPβ, C/EBPδ, C/EBPα, and PPARγ. The mRNA level seen in siNS-transfected cells immediately after DMI treatment was set to 1.0, and the others are expressed as relative values. Data represent the means ± the standard errors of the means of three independent experiments. (F) Cell lysates were obtained at the indicated times (days) after stimulation with DMI. Immunoblot analysis was then performed using the respective antibodies. Nearly identical results were obtained in at least three independent experiments. (G) Cells transfected with siNS or siSENP2 were incubated with DMI. Nuclear proteins were prepared at the indicated times (days) after stimulation with DMI and subjected to EMSA with a labeled probe containing the C/EBP binding site of the C/EBPα promoter. Antibodies (2 μg) against C/EBPβ and C/EBPδ were used for supershifting.

(lower panels). We then examined the effect of siSENP2 on the protein levels of the transcription factors. Immunoblot analysis reveals that transfection of siSENP2 leads to a dramatic reduction in the protein levels of C/EBPα and PPARγ (Fig. 3F), consistent with the siSENP2 effects on their transcript levels. In addition, siSENP2 transfection resulted in a marked decrease in both the transcript and protein levels of aP2 (data not shown). On the other hand, siSENP2 did not have any effect on

the C/EBPδ protein level, similar to its lack of effect on the C/EBPδ mRNA level. Notably, knockdown of SENP2 caused a marked decrease in the C/EBPβ protein level, unlike its effect on the C/EBPβ mRNA level. These results suggest that the decrease in the C/EBPβ protein level caused by SENP2 knockdown is due to a posttranslational modification that affects the stability of the C/EBPβ protein.

To make clear that treatment of siSENP2 causes a defect in the expression of C/EBPα and PPARγ through reduction of the C/EBPβ protein level, an EMSA was performed with a probe representing a C/EBP binding site in the C/EBPα promoter and nuclear extracts prepared 1 or 2 days after DMI treatment. In accord with previous reports by other researchers (22, 37), C/EBPβ binding was detectable at day 1 and dramatically increased at day 2 in control siRNA (siNS)-treated cells (Fig. 3G, lanes 3 to 4). However, in the case of siSENP2-treated cells, binding activity was markedly reduced (lanes 5 to 6). The result obtained from the supershift with anti-C/EBPβ antibody indicates that C/EBPβ is a major protein that binds to the probe (lanes 7 and 8). Similar results were obtained when a C/EBP binding site in the PPARγ promoter was used as a probe (data not shown). These results clearly showed that the reduction of the C/EBPβ protein level by siSENP2 led to attenuation of the expression of downstream target genes such as those for C/EBPα and PPARγ. Collectively, these findings suggest that SENP2 is required for C/EBPβ stabilization and thereby for the expression of C/EBPα and PPARγ.

C/EBPβ is a substrate of SENP2. As C/EBPβ is a target for sumoylation (10, 20), we determined whether SENP2 can desumoylate C/EBPβ. SUMO1 and C/EBPβ were expressed in COS-7 cells with or without Flag-tagged SENP2 and its catalytically inactive mutant forms in which active-site Cys548 was replaced with Ser (termed C548S) and Arg576 and Lys577 were replaced with Leu and Met, respectively (termed RK576/577LM) (7, 19). As a control, Myc-SENP1 or Flag-SENP3 was also expressed in place of SENP2. SENP2 could remove SUMO1 from SUMO-C/EBPβ conjugates (Fig. 4A). On the other hand, the catalytically inactive mutant SENP2 proteins (C548S and RK576/577LM), SENP1, and SENP3 could not desumoylate C/EBPβ. These results suggest that C/EBPβ is a specific substrate for SENP2.

Next we examined the effect of SENP2 knockdown on the level of endogenous SUMO-C/EBPβ conjugates. 3T3-L1 cells transfected with increasing amounts of siSENP2 were incubated with DMI for 24 h. Cell lysates were then subjected to immunoprecipitation with anti-SUMO1 antibody, followed by immunoblotting with anti-C/EBPβ antibody. Little or no sumoylated C/EBPβ could be detected when siNS was transfected (Fig. 4B). Upon siSENP2 transfection, however, SUMO-C/EBPβ conjugates were evidently accumulated. These results indicate that endogenous SENP2 rapidly removes SUMO from C/EBPβ.

To determine whether desumoylation of C/EBPβ by SENP2 affects C/EBPβ transcriptional activity, COS-7 cells were transfected with C/EBPβ and SENP2 expression vectors and the p21 promoter (containing a C/EBPβ binding site)-Luc reporter vector. As the amount of the SENP2 expression vector was increased, the transcriptional activity of C/EBPβ was also increased (Fig. 4C). In contrast, C548S mutant SENP2 did not affect C/EBPβ transcriptional activity, suggesting that desumoylation of C/EBPβ increases its transcriptional activity.

SENP2 stabilizes the C/EBPβ protein. Our findings that SENP2 knockdown leads to a marked decrease in the C/EBPβ protein level with relatively little effect on its transcript level and that C/EBPβ is a substrate of SENP2 suggested that SENP2 is involved in the control of C/EBPβ protein stability. To verify this possibility, HA-C/EBPβ and SUMO1 were expressed in COS-7 cells with Flag-SENP2 or Flag-C548S. Immunoblot analysis revealed that the level of C/EBPβ protein increased in parallel with the increase in the expression of SENP2 but not with that of catalytically inactive C548S (Fig. 4D). These results indicate that sumoylation causes destabilization of the C/EBPβ protein and that this process can be reversed by SENP2.

To determine whether sumoylation indeed affects the stability of C/EBPβ, we examined the stability of sumoylated C/EBPβ and nonsumoylated C/EBPβ in the presence of cycloheximide, a protein synthesis inhibitor. Sumoylated C/EBPβ was degraded more rapidly than the nonsumoylated form (Fig. 4E). Next, we generated a mutant form of C/EBPβ by replacing Lys133, the SUMO acceptor site, with Arg (termed K133R) (20). We then compared the stability of the sumoylation-defective K133R mutant protein to that of wild-type C/EBPβ upon treatment with cycloheximide. Figure 4F shows that the K133R mutant protein is more stable than C/EBPβ, indicating that sumoylation is responsible for C/EBPβ destabilization. To confirm this finding further, we examined the effect of SENP2 knockdown on the stability of endogenous C/EBPβ during the differentiation of 3T3-L1 cells. Cells transfected with siNS or siSENP2 were incubated with cycloheximide 1 day after DMI treatment, when the C/EBPβ protein level was high. Transfection of siSENP2, but not that of siNS, into 3T3-L1 cells resulted in a marked decrease in the stability of C/EBPβ (Fig. 4G). Taken together, these results indicate that SENP2 plays a critical role in the control of C/EBPβ protein stability.

Sumoylation of C/EBPβ promotes its ubiquitination. Hattori et al. (15) have shown that C/EBPβ is degraded by the ubiquitin-proteasome system. To determine whether sumoylation affects the ubiquitination of C/EBPβ and thereby its degradation by the proteasome, COS-7 cells that had been transfected with vectors expressing Ub and HA-tagged C/EBPβ or K133R were incubated with or without MG132, a specific inhibitor of the proteasome. Without MG132 treatment, ubiquitinated C/EBPβ was hardly detected whether SUMO1 was coexpressed or not (Fig. 5A). In the presence of MG132, however, coexpression of SUMO1 led to a dramatic increase in the level of ubiquitinated C/EBPβ, in addition to the generation of sumoylated C/EBPβ. In contrast, ubiquitination of K133R was hardly observed under any of the conditions described above. In the same context, another sumoylation-defective mutant form of C/EBPβ was generated (E135A) in which the Glu residue in the sumoylation consensus site was replaced with Ala (Fig. 5B). Similar to the K133R mutant form, ubiquitination of C/EBPβ E135A was hardly detected. These results indicate that sumoylation promotes ubiquitination of C/EBPβ and its subsequent degradation by the proteasome.

We next examined the effect of SENP2 overexpression on C/EBPβ ubiquitination. Under conditions in which SUMO1 was coexpressed, SENP2 expression caused a marked reduction in the level of ubiquitinated C/EBPβ concomitantly with

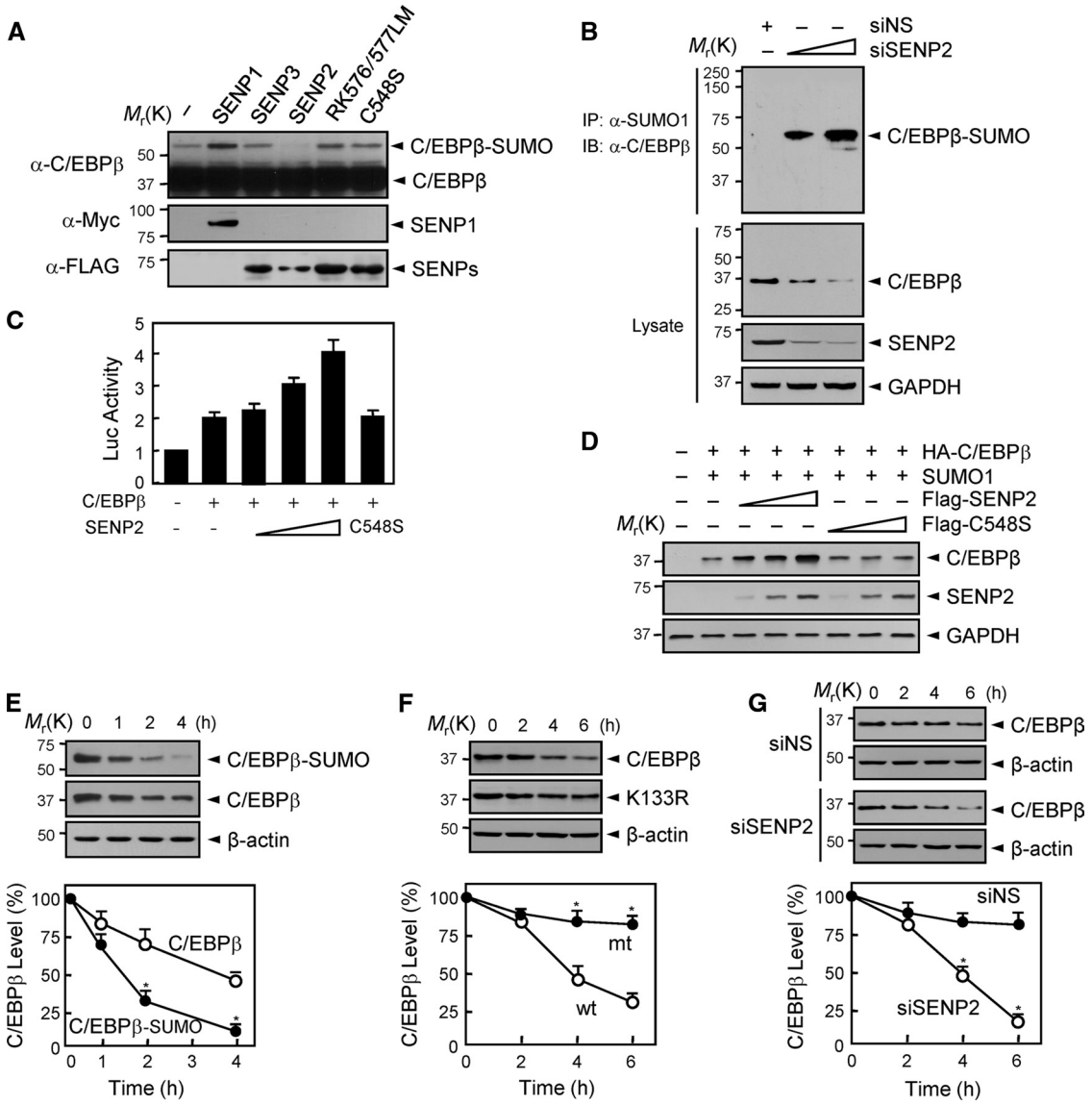

FIG. 4. C/EBPβ is a substrate of and is stabilized by SENP2. (A) COS-7 cells were transfected with expression vectors for C/EBPβ and SUMO-1 and a SENP expression vector (Myc-SENP1 or Flag-tagged SENP2, SENP3, RK576/577LM, or C548S). They were then subjected to immunoblotting with anti-C/EBPβ, anti-Myc, or anti-Flag antibody. (B) 3T3-L1 cells transfected with siNS (100 nM) or siSENP2 (50 or 100 nM) were incubated with DMI for 24 h. They were then subjected to immunoprecipitation (IP) with anti-SUMO1 antibody, followed by immunoblotting (IB) with anti-C/EBPβ antibody. Cell lysates were also directly probed with the respective antibodies. (C) COS-7 cells were transfected with the expression vectors for C/EBPβ (0.02 μg), SENP2 (0.02, 0.05, or 0.1 μg), or SENP2 C548S (0.1 μg), the p21 promoter-Luc reporter vector (0.2 μg), and pCMV-β-gal (0.1 μg). The normalized activity seen without C/EBPβ or SENP2 was set to 1.0, and the others are expressed as relative values. Data represent the means ± the standard errors of the means of five independent experiments. (D) COS-7 cells transfected with the expression vectors for HA-C/EBPβ (0.25 μg), SUMO1 (1 μg), and Flag-SENP2 (0.25, 0.5, or 1 μg) or Flag-C548S (0.25, 0.5, or 1 μg) were incubated for 40 h. Cell lysates were then subjected to immunoblot analysis. (E) COS-7 cells transfected with the expression vectors for SUMO1 and HA-C/EBPβ were incubated with cycloheximide (5 μM) for the indicated periods. After incubation, cell lysates were subjected to immunoblot analysis with anti-C/EBPβ or anti-β-actin antibody. The densities of the C/EBPβ and C/EBPβ-SUMO bands seen immediately after cycloheximide treatment were set to 100%, and the others are expressed as relative values. Data represent the means ± the standard errors of the means of four independent experiments. (F) COS-7 cells transfected with the expression vectors for SUMO1 and HA-C/EBPβ or HA-K133R were incubated with cycloheximide (5 μM) for the indicated periods. Data represent the means ± the standard errors of the means of four independent experiments. (G) 3T3-L1 cells transfected with siSENP2 or siNS were treated with DMI for 24 h and then incubated with cycloheximide for the indicated periods. Data represent the means ± the standard errors of the means of four independent experiments. *, $P < 0.05$. K, 10^3.

the disappearance of sumoylated C/EBPβ (Fig. 5C). When SENP2 was not overexpressed, high-molecular-weight bands, considered to be ubiquitinated C/EBPβ-SUMO conjugates, were also detected with an anti-SUMO antibody after a longer exposure. On the other hand, SENP2 overexpression had little or no effect on the level of ubiquitinated proteins in the whole-cell lysates, indicating that SENP2 does not modulate the ubiquitination system itself. We then examined the effect of SENP2

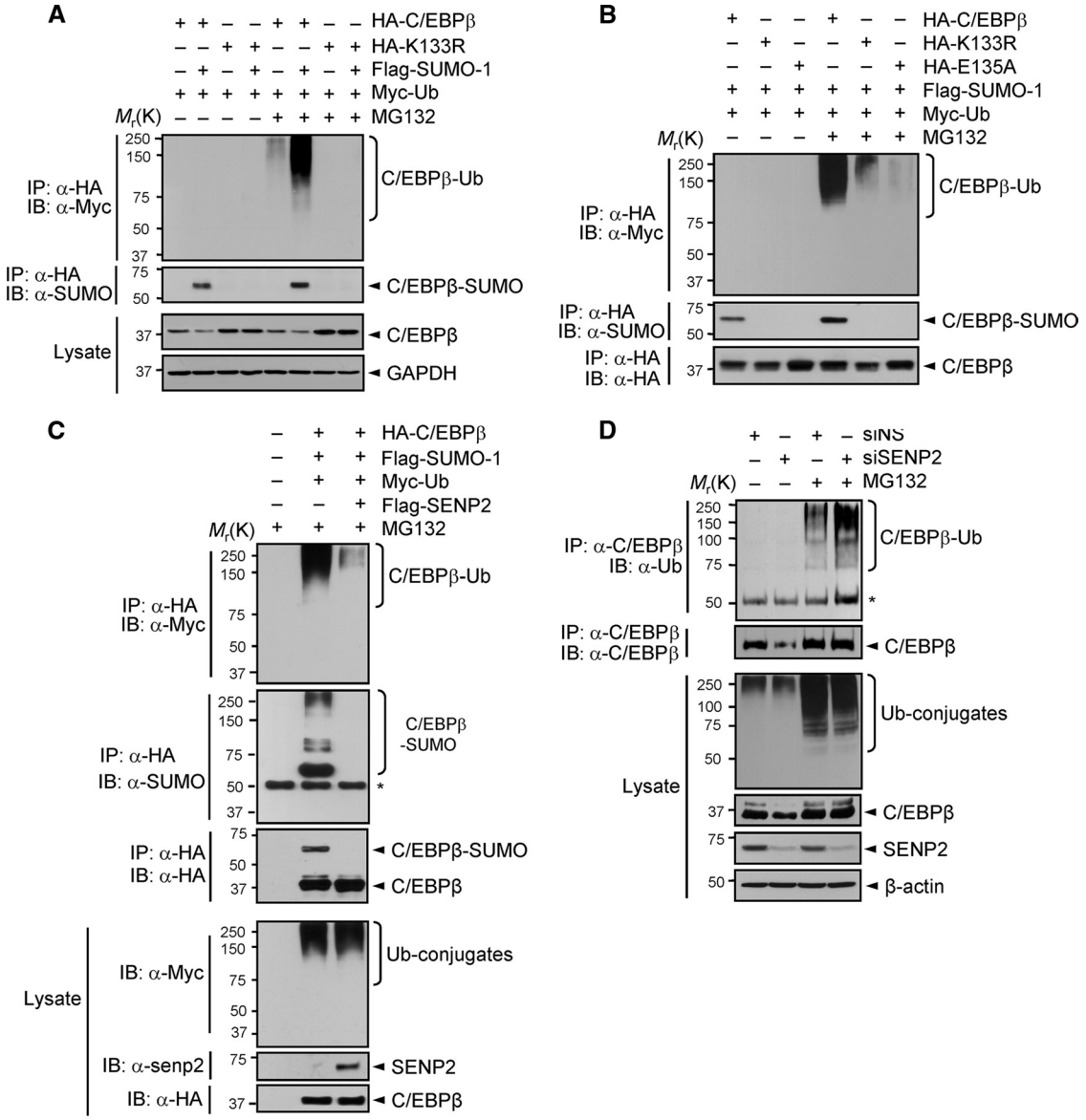

FIG. 5. Sumoylation of C/EBPβ promotes its ubiquitination. (A) COS-7 cells were transfected with the expression vectors for HA-C/EBPβ, HA-K133R, Flag-SUMO1, and Myc-Ub as indicated. After incubation with MG132 (10 μM) for 4 h, cell lysates were subjected to immunoprecipitation (IP) with anti-HA antibody, followed by immunoblotting (IB) with anti-Myc or anti-SUMO1 antibody. (B) COS-7 cells were transfected with the indicated vectors and treated with MG132 as described for panel A. (C) HA-C/EBPβ, Flag-SUMO1, and Myc-Ub were expressed in COS-7 cells with or without Flag-SENP2. The asterisk indicates IgG bands. (D) 3T3-L1 preadipocytes transfected with siSENP2 or siNS were treated with DMI for 24 h and then incubated for 20 h with or without MG132. Cell lysates were then subjected to immunoprecipitation with anti-C/EBPβ antibody, followed by immunoblotting with anti-C/EBPβ or anti-Ub antibody. K, 10^3.

knockdown on the ubiquitination of endogenous C/EBPβ. 3T3-L1 preadipocytes transfected with siSENP2 or siNS were induced with DMI for 24 h. Cells were then incubated with or without MG132 and subjected to immunoprecipitation with anti-C/EBPβ antibody. Without MG132 treatment, ubiquitinated C/EBPβ was hardly detected whether siSENP2 or siNS was transfected (Fig. 5D). In the presence of MG132, however, the level of ubiquitinated C/EBPβ in cells transfected with siSENP2 was much higher than in cells transfected with siNS. On the other hand, neither siSENP2 nor siNS had any effect on the level of ubiquitinated proteins in the whole-cell lysates, again indicating that SENP2 does not modulate the ubiquitination system itself. Collectively, these results demonstrate that SENP2 stabilizes the C/EBPβ protein.

C/EBPβ overexpression reverses siSENP2-mediated inhibition of adipogenesis. To determine whether SENP2-mediated stabilization of C/EBPβ is associated with adipocyte differentiation, we generated 3T3-L1 preadipocytes that stably overexpress C/EBPβ by using a retrovirus harboring a C/EBPβ expression vector (pC/EBPβ). We also generated control cells by infecting a retrovirus harboring an empty vector (pBabe). Northern and immunoblot analyses confirm that C/EBPβ mRNA and its protein product were highly expressed in the stable cells but not in the control cells (Fig. 6A). We then

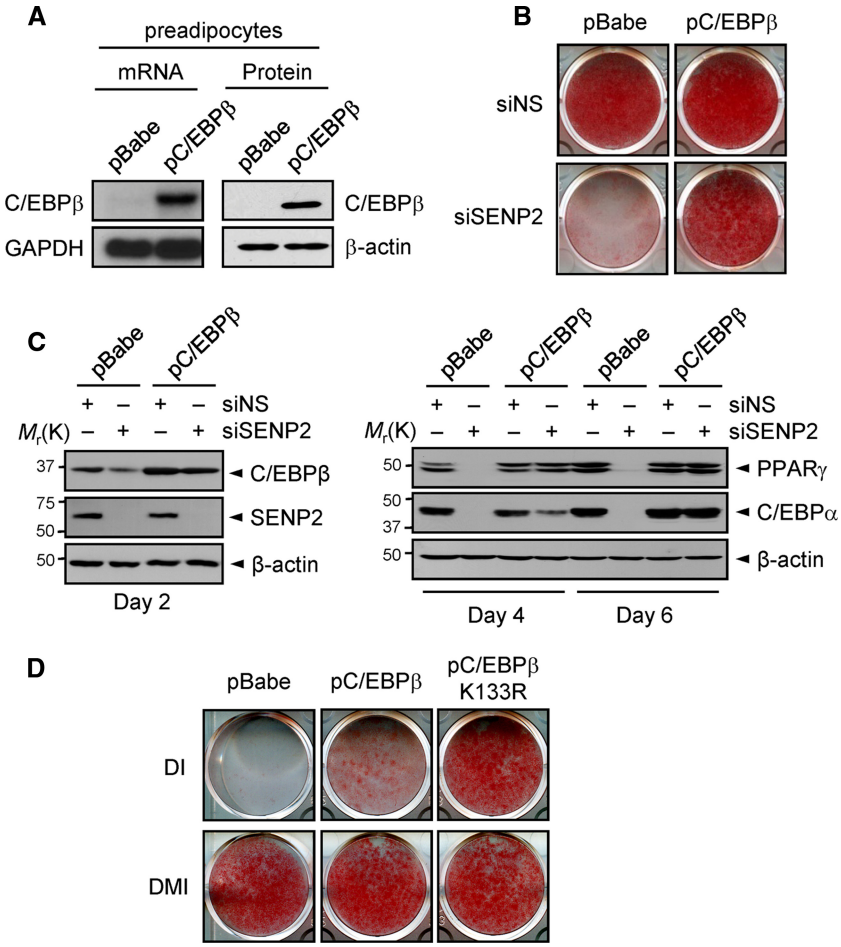

FIG. 6. C/EBPβ overexpression reverses siSENP2-mediated inhibition of adipogenesis. (A) 3T3-L1 preadipocytes were infected with a retrovirus harboring an empty vector (pBabe) or a C/EBPβ expression vector (pC/EBPβ). After selection of cells resistant to puromycin, total RNAs and proteins were prepared and subjected to Northern blot and immunoblot analyses, respectively. (B) Stable cells were transfected with siNS or siSENP2. They were stained with Oil Red O 8 days after DMI treatment. (C) Stable cells transfected with siNS or siSENP2 were incubated with DMI. Cell lysates were prepared at the indicated times after DMI treatment and then subjected to immunoblotting with the respective antibodies. (D) Control (pBabe), C/EBPβ-expressing (pC/EBPβ), and C/EBPβ K133R-expressing (pC/EBPβ K133R) stable cells were incubated with DI or DMI for 2 days and stained with Oil Red O 8 days after treatment. K, 10^3.

examined the effect of C/EBPβ overexpression in the stable cells on adipogenesis under conditions in which SENP2 was knocked down. Oil Red O staining revealed that overexpression of C/EBPβ in the stable cells ameliorated the inhibitory effect of SENP2 knockdown on DMI-induced adipogenesis (Fig. 6B). Consistently, the level of C/EBPβ protein in the stable cells, but not in the control cells, remained elevated even after the knockdown of SENP2 (Fig. 6C, left). Moreover, expression of the adipogenic genes downstream of C/EBPβ, such as C/EBPα and PPARγ, was induced in the C/EBPβ-expressing stable cells but not in the control cells under conditions in which SENP2 was knocked down (Fig. 6C, right). These results indicate that C/EBPβ overexpression could reverse siSENP2-mediated inhibition of adipogenesis. To demonstrate the significance of sumoylation of C/EBPβ further, we used DI (Dex plus insulin) instead of DMI to treat cells, in which the expression of endogenous C/EBPβ and SENP2 was barely induced (45) (Fig. 1D). In control cells, adipocytes were hardly observed 8 days after DI treatment (Fig. 6D). However, the stable cells expressing wild-type C/EBPβ partially rescued the impaired adipogenesis and the cells expressing K133R mutant C/EBPβ underwent adipogenesis at a level similar to that of DMI-treated cells. This result clearly shows that the sumoylation-defective mutant form of C/EBPβ overcomes the SENP2 deficiency-mediated inhibition of adipogenesis better than wild-type C/EBPβ does.

SENP2 is required for adipogenesis *in vivo*. To verify whether SENP2 plays an important role in adipogenesis *in vivo*, we generated retrovirus-mediated stable 3T3-F442A preadipocytes producing shRNAs against SENP2 (shSENP2) in which shRNAs against SENP2 were continuously expressed and SENP2 transcription was significantly knocked down (data not shown). 3T3-F442A preadipocytes (shControl or shSENP2) were implanted into mice. While control preadipocytes were differentiated into adipocytes and formed fat pads 5 weeks after implantation, adipogenesis was significantly inhibited in the SENP2 knockdown stable cells when they were implanted into mice (Fig. 7A). In addition to H&E staining

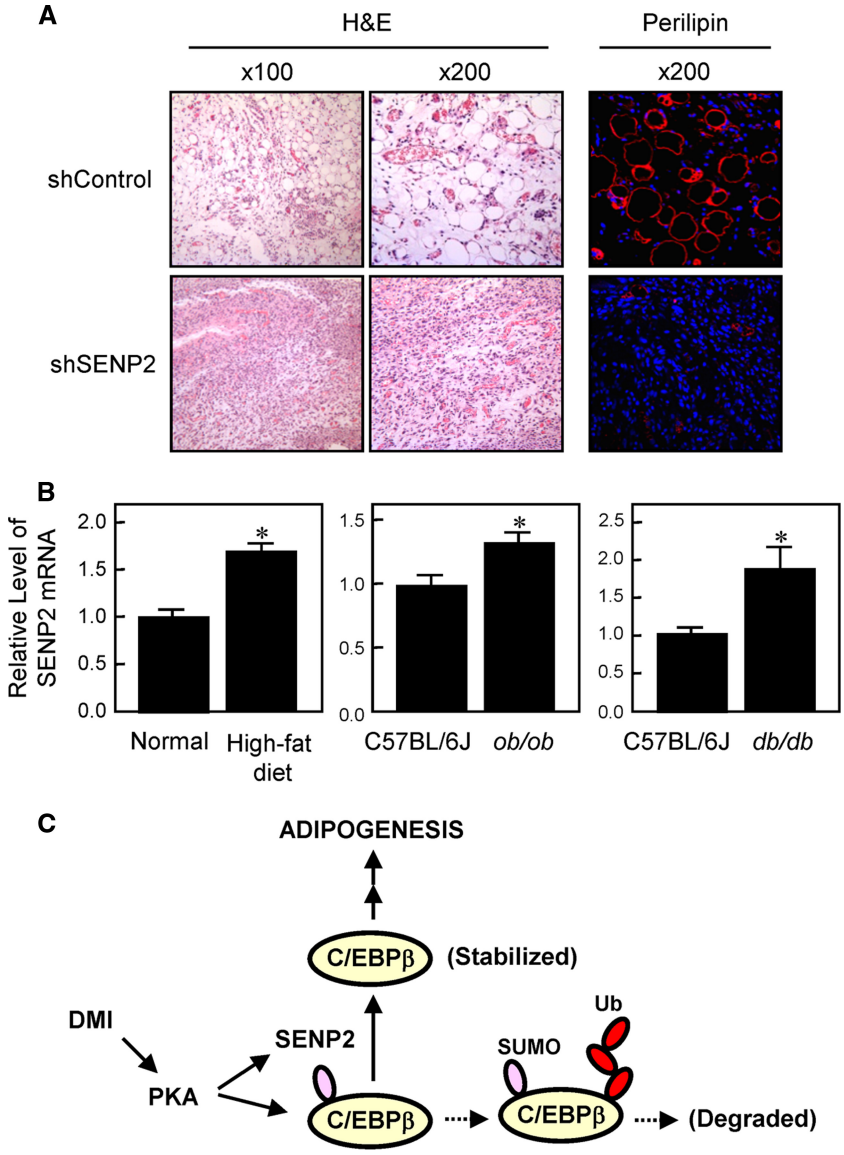

FIG. 7. SENP2 is required for adipogenesis *in vivo*. (A) shControl or stable SENP2 knocked-down 3T3-F442A preadipocytes (shSENP2) were implanted into mice. Implanted cell pads were excised and stained with H&E (left panel) or subjected to immunohistochemistry analysis with antiperilipin antibody (right panel) 5 weeks after implantation. Similar results were obtained with three (shControl) and five (shSENP2) mice. (B) Total RNAs were prepared from white adipose tissues of normally fed ($n = 5$) and high-fat-diet-fed ($n = 5$) mice (left panel), normal (C57BL/6J, $n = 5$) and *ob*/*ob* ($n = 5$) mice (middle panel), and normal ($n = 5$) and *db*/*db* ($n = 5$) mice (right panel). The SENP2 mRNA levels in the samples were then estimated by using real-time PCR. The mean values obtained from the normal mice were set to 1.0, and the others are expressed as relative values. Data represent means ± standard errors. *, $P < 0.05$. (C) Proposed model of the role of SENP2 in adipogenesis.

(Fig. 7A, left), perilipin, a lipid droplet scaffold protein, was detected by immunohistochemistry analysis (Fig. 7A, right). Consistent with the result of H&E staining, perilipin was hardly detected when SENP2 was stably knocked down. These results clearly demonstrated that SENP2 is required for adipogenesis *in vivo*.

Next, we compared the level of SENP2 mRNA in obese mice such as high-fat-diet-fed, *ob*/*ob*, and *db*/*db* mice to that in control mice. Interestingly, the SENP2 transcript levels in obese mice were significantly higher than those in normal mice (Fig. 7B). These results imply a critical *in vivo* function for SENP2 in the control of adipogenesis.

DISCUSSION

In the present study, we show that SENP2 plays an essential role in adipogenesis. Based on these findings, we propose a model for the role of SENP2 in the control of adipogenesis (Fig. 7C). In preadipocytes, expression of C/EBPβ remains uninduced without stimulation. Moreover, the basal C/EBPβ protein level would also be kept low by sumoylation, which promotes ubiquitination and subsequent degradation of C/EBPβ by the proteasome. Upon stimulation, such as by differentiation inducers, expression of SENP2, as well as of C/EBPβ, is induced in a cAMP-dependent fashion. SENP2-

mediated desumoylation and stabilization of C/EBPβ protein further increase its cellular level, leading to induction of the downstream effectors of C/EBPβ, such as PPARγ and C/EBPα, for adipocyte differentiation.

C/EBPβ is considered to be a key regulator of adipocyte differentiation because C/EBPβ is responsible for the expression of its downstream transcription factors such as C/EBPα and PPARγ, and furthermore, the activity of C/EBPβ is finely regulated by posttranslational modifications such as phosphorylation. During the first 2-day period of differentiation, C/EBPβ is sequentially phosphorylated by MAPK, Cdk2, and glycogen synthase kinase 3β (GSK3β) to acquire their C/EBPα and PPARγ promoter DNA-binding activities and thus to induce the expression of the downstream effectors (22, 37). Although the C/EBPβ transcript level was dramatically increased within 1 to 2 h after the treatment of 3T3-L1 preadipocytes with DMI and gradually declined thereafter (Fig. 1B), the C/EBPβ protein level remained elevated at least for the following 2 days (Fig. 3F), in accord with previous reports (38). Likewise, the level of SENP2 protein remained elevated at least for 2 days after DMI treatment. Thus, it appears likely that during the 2-day period, SENP2-mediated stabilization allows C/EBPβ to perform a crucial role in adipogenesis.

The finding that SENP2, but not SENP1 or SENP3, could desumoylate C/EBPβ, suggests that C/EBPβ is a specific substrate for SENP2. Furthermore, expression of SENP2 is induced by the PKA-mediated CREB activation at the early stage of adipogenesis while SENP1 expression is not induced during adipogenesis. These results indicate that each SENP has its specific target and specific physiological function. In addition, it seems likely that transcriptional regulations of SUMO proteases are important links to their specific functions.

Although our data showed that C/EBPβ is a target of SENP2 during adipogenesis, SENP2 can act on several other SUMO-conjugated proteins. Therefore, we cannot exclude the possibility that SENP2 is simultaneously working in the control of other adipogenic factors that are modified by SUMO. Noteworthy, however, was the finding that expression of SENP2 mRNA is increased immediately following the induction of C/EBPβ mRNA expression during the early period of adipogenesis (i.e., about 3 to 4 h after the treatment of 3T3-L1 preadipocytes with DMI). Furthermore, the SENP2 protein level was quite low when the protein levels of transcription factors that are downstream of C/EBPβ, such as C/EBPα and PPARγ, reached a maximum (Fig. 3F). Thus, it appears likely that C/EBPβ serves as a major target of SENP2 for the regulation of adipogenesis in a specific time window.

SENP2 has been shown to participate in downregulation of the Wnt/β-catenin signaling pathway (18, 19). Overexpression of SENP2 decreases the β-catenin protein level, although the direct target involved in this process has not been identified. The β-catenin level is gradually decreased during adipogenesis, and this decrease appears to be required for adipogenesis because a high level of β-catenin induced by Wnt or GSK3β inhibitors efficiently inhibits adipogenesis (2, 35). Therefore, it would be of interest to investigate whether SENP2 is also involved in the downregulation of the Wnt signaling pathway during adipogenesis. Anyhow, our data, specially obtained from C/EBPβ stable cells, indicate that C/EBPβ is a major target of SENP2 for the control of adipogenesis.

Another important finding of this study is elucidation of the mechanism by which sumoylation regulates C/EBPβ activity: sumoylation of C/EBPβ stimulates its ubiquitination and subsequent degradation. This fact may explain why an endogenous sumoylated form of C/EBPβ was hardly observed. There are several examples of sumoylation-mediated ubiquitination, such as PML, HIF-1α, and PEA3 (6, 14, 40). SUMO-dependent recruitment of ubiquitin ligases such as RNF4 and VHL involves the ubiquitination of PML and HIF-1α. Like other posttranslational modifications, the SUMO-dependent ubiquitination pathway may regulate the level of a target protein without any changes in E3 ubiquitin ligase expression. In addition, the number of ubiquitin E3 ligases required could be reduced if it is possible that one E3 ligase targets several proteins on the basis of their sumoylation status. We also briefly examined ubiquitination sites of C/EBPβ. The result showed that N-terminal half domain of C/EBPβ contains major ubiquitination sites and there are more than two lysine residues involved in ubiquitination, one between amino acids 23 and 112 and the other between amino acids 113 and 150 (data not shown). If the C/EBPβ-specific E3 ligase and the lysine residues ubiquitinated are identified, the molecular mechanism of the connection between the sumoylation and ubiquitination of C/EBPβ can be precisely studied. It will be also interesting to study whether sumoylation regulates other C/EBP family members by a similar mechanism.

ACKNOWLEDGMENTS

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