Small Ubiquitin-like Modifier (SUMO) Protein-specific Protease 1 De-SUMOylates Sharp-1 Protein and Controls Adipocyte Differentiation*

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Background: It is unknown whether SENP1 controls adipocyte differentiation.

Results: SENP1 de-SUMOylates Sharp-1 and promotes PPARγ expression and adipogenesis.

Conclusion: SENP1 regulates adipocyte differentiation.

Significance: SENP1 is a novel regulator in adipocyte differentiation.

Adipocyte differentiation is regulated by a transcriptional cascade that mainly includes CCAAT/enhancer-binding protein family members and the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ). Here we show the defects in adipocyte differentiation as well as PPARγ expression in Senp1−/− mouse embryonic fibroblast cells induced by adipogenic stimuli. We further determine that SENP1 is a specific de-SUMOylation protease for Sharp-1, a repressor for PPARγ transcription and adipogenesis. SENP1 enhances adipogenesis through de-SUMOylation of Sharp-1, which then releases Sharp-1 repression of PPARγ expression and adipocyte differentiation. These results reveal SENP1 as a novel regulator in adipogenesis.

Adipocyte differentiation is regulated by a transcriptional cascade that mainly includes CCAAT/enhancer-binding protein (C/EBP) family members (i.e. C/EBPα, C/EBPβ, and C/EBPδ) and the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ). This transcriptional cascade directs the extensive programming of gene expression required to convert preadipocytes to mature adipocytes (1–3). C/EBPs are early adipogenic transcription factors that are induced within hours of initiation of adipogenesis. C/EBPβ is directly binds to the PPARγ2 promoter and activates PPARγ2 expression (4). SENP1 and SENP regulates the expression of another principal adipogenic transcription factor, C/EBPα (5).

PPARγ mainly coordinates with C/EBPα in a positive feedback loop to promote the terminal differentiation of adipocytes (6). It is well documented that the activity of C/EBPs and PPARγ is tightly controlled by distinct regulators in adipocyte differentiation. These regulators include transcriptional coactivators and corepressors, Wnt and FGF signaling, as well as protein posttranslational modification enzymes (3). Sharp-1, a basic helix-loop-helix transcription factor, is one of the transcriptional corepressors modulating C/EBPs and PPARγ activity. Sharp-1 binds to class B E-box sites with high affinity to repress the transcription of target genes and also associates with distinct corepressors, including HDAC1, SIRT1, and the histone methyltransferase G9a, to inhibit gene transcription (7). Taneja and co-workers (8) found that Sharp-1 interacts with and inhibits the transcriptional activity of both C/EBPβ and C/EBPα by retaining HDAC1 and G9a at the C/EBP regulatory sites on the C/EBPα and PPARγ2 promoters to inhibit their expression and, thus, adipogenesis, identifying Sharp-1 function as a negative regulator during adipogenesis.

SUMO (also called SENTrin) is a novel ubiquitin-like protein that can covalently modify a large number of proteins (9, 10). SUMO modification has now emerged as an important regulatory mechanism in many signaling pathways through alteration of the function of target proteins (9, 11, 12). SUMOylation is catalyzed by activating (E1), conjugating (E2), and ligating (E3) enzymes. It is reversed by a family of Sentrin/SUMO-specific proteases (SEPNs) (9, 12). In mammalian cells, six SENPs are identified. These six SENPs have substrate specificity and different cellular localization and tissue distribution (9, 12). The SENPs mediating deconjugation play a crucial role in determining protein SUMOylation status (9, 13–17). Interestingly, many transcriptional regulators in adipocyte differentiation are shown as SUMOylated proteins, suggesting that SUMOylation has emerged as a novel regulation mechanism in adipogenesis (18–20).

In this study, Senp1−/− MEF cells show defects in adipocyte differentiation and PPARγ expression induced by adipogenic stimuli. A mechanism study found that SENP1 de-SUMOylates Sharp-1 and releases Sharp-1 inhibition of PPARγ expression.
and adipogenesis. These results reveal a role of SENP1 in control of Sharp-1 activity and adipocyte differentiation.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The Sharp-1-HA, and pGL3.0-PPARγ plasmids were provided by Dr. Bing Sun (Institute Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai, China) and Dr. Zhaoyuan Hou (Shanghai Jiao Tong University School of Medicine, Shanghai, China), respectively. FLAG-SUMO1, FLAG-SENP1, FLAG-SENP1mutant, RGS-SENP1, and RGS-SENP1mutant have been described previously (18–20). We used antibodies against FLAG (M2, Sigma), HA (Covance), RGS (Qiagen), SUMO1 (Cell Signaling Technology), and Sharp-1 (Santa Cruz Biotechnology).

**Mutagenesis**—To mutate potential SUMOylation residues from lysine to arginine in Sharp-1, the QuikChange™ site-directed mutagenesis kit (Stratagene) was used. The primers for generating Sharp-1m (K240R)-HA were 5’-CCGCGGAGGCGGCTAGGCGGAGGACACCC-3’ and 5’-GGTGGCTGCTCTGTGCCTG-3’. Cells were transfected with expression plasmids. The cells were treated with 30 μl of Opti-MEM/10% FBS (Invitrogen) and 1% antibiotics until reaching full confluence. Two days later (day 2), differentiation was induced by addition of insulin (5 μg/ml, Sigma), dexamethasone (1 μm, Sigma), isobutyl-1-methylxanthine (0.5 mM, Sigma), and rosiglitazone (1 μM, Sigma). On day 2, the medium was replaced with the same medium containing 5 μg/ml insulin and 1 μM rosiglitazone. This medium was changed every 2 days until the end of differentiation. Oil Red O (Sigma) staining and quantification were performed as described previously (8).

**Luciferase Assay**—MEF and 3T3-L1 cells were cultured in DMEM (Hyclone) supplemented with 5% FBS (Invitrogen) and 1% antibiotics until reaching full confluence. The generation of Senp1+/+ and Senp1−/− MEF cells has been described previously (18). MEF, 293T, and MEF cells were transfected with expression plasmids. The cells were transiently transfected with expression plasmids. The cells were incubated for 24 h before luciferase was assayed using the Dual-Luciferase reporter assay system (Promega). Luciferase activity was used as an internal control.

**Statistical Analysis**—Error bars indicate mean ± S.D. Statistical analysis was performed using Student’s t test, and p < 0.01 was considered to be statistically significant.

**RESULTS**

**SENP1 Deficiency Decreases Adipogenesis in Senp1−/− MEF Cells**—To determine whether SENP1 is involved in the regulation of adipogenesis, we first monitored the expression of SENP1 during adipogenesis. MEF cells were induced to differentiate by dexamethasone, isobutyl-1-methylxanthine, insulin, and rosiglitazone (referred to as “DMIR”). Analysis of the messenger RNA of SENP1 showed that SENP1 expression increased in the early stage (peak at day 2 after induction) and then went down to normal levels (Fig. 1A) during differentiation induced by DMIR. This result indicates that SENP1 might be involved in the regulation of the initiation event of adipogenesis. We further compared the adipogenic ability of Senp1+/+ and Senp1−/− MEF cells. Senp1+/+ and Senp1−/− MEF cells were induced by the adipocyte differentiation stimulant DMIR for 8 days. The differentiated adipocytes were stained with Oil Red O. As shown in Fig. 1, B and C, there was less red staining in Senp1−/− MEF cells than that in the Senp1+/+ control. The lipid droplet formation in Senp1−/− MEF cells was also much less than that in Senp1+/+ MEF cells. These data suggest an essential role of SENP1 in adipocyte differentiation.
**SENP1 Regulates Adipocyte Differentiation**

**Adipogenically Related Genes Are Down-regulated in Senp1−/− MEF Cells during Adipocyte Differentiation**—To understand the molecular basis in SENP1 regulation of adipogenesis, we analyzed the expression of adipogenically related genes in Senp1+/+ and Senp1−/− MEF cells induced by DMIR. The expression of C/EBPα/β and PPARγ, the master regulators and transcription factors during adipogenesis, was down-regulated significantly in Senp1−/− MEF cells compared with Senp1+/+ MEFs (Fig. 2, A−C). We also examined the expression of aP2, adiponectin, and LPL, which are indicators of the differentiated adipocyte and PPARγ target genes, and found that SENP1 deficiency significantly reduced the expression of these genes during adipogenesis (Fig. 2, D−F). These data reveal an essential role of SENP1 in the regulation of C/EBPα/β and PPARγ expression in adipocyte differentiation.

**SENP1 Is a Positive Regulator of PPARγ Transcription**—Because SENP1 functions in the initiation of adipogenesis, we determined PPARγ expression in MEF cells at the first day of induction by DMIR. As shown in Fig. 3A, PPARγ mRNA increased abruptly in Senp1+/+ MEFs after induction. However, only a mild increase in PPARγ expression was detected in the induced Senp1−/− cells, suggesting that SENP1 is essential for PPARγ expression in the initiation stage of adipocyte differentiation. By using PPARγ promoter-driven luciferase, we confirmed the role of SENP1 in promoting PPARγ transcription (Fig. 3B). Importantly, the SENP1 catalytic mutant could not activate PPARγ expression, suggesting that de-SUMOylation activity is essential for SENP1 regulation of PPARγ transcription. To further demonstrate the de-SUMOylation activity of SENP1 in the regulation of the expression of adipogenically related genes, we generated SENP1 wild-type- or SENP1 catalytic mutant-transfected Senp1−/− MEF cells. SENP1 up-regulated the expression of PPARγ and its targets aP2, adiponectin, and LPL, as shown in SENP1-transfected cells. However, the mutation of the catalytic domain abolished the SENP1 induction of these genes (Fig. 3C). These data suggest that SENP1 is a positive regulator of PPARγ expression through de-SUMOylation.

**SENP1 De-SUMOylates Sharp-1**—At the initiation of adipogenesis, C/EBP binds directly to the PPARγ promoter and induces PPARγ. PPARγ also turns on the expression of C/EBP and then further induces PPARγ expression. This self-reinforcing regulatory loop is critical for PPARγ function in the initia-
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SUMOylation Contributes to Sharp-1 Repression of PPARγ Transcription—To test whether SUMOylation could modulate Sharp-1 activity in PPARγ expression, we compared the effect of Sharp-1 or the Sharp-1 SUMOylation mutant on PPARγ transcription. As shown in Fig. 5A, SUMOylation-deficient Sharp-1 showed much less repressive activity in PPARγ transcription than the Sharp-1 wild type, suggesting that SUMOylation could enhance Sharp-1 repression of PPARγ expression. This phenotype was further confirmed by coexpression of SENP1, which reduced the Sharp-1 repression of PPARγ luciferase activity (Fig. 5, A and B). Importantly, the SENP1 catalytic mutant could not affect Sharp-1 repression (Fig. 5B), suggesting that SENP1 regulation of PPARγ transcription is through de-SUMOylation of Sharp-1.

Deficiency in SUMOylation Reduces Sharp-1 Suppression of Adipogenesis—To determine whether SUMOylation has an effect on Sharp-1 regulation of adipogenesis, we generated Sharp-1 wild-type or Sharp-1 SUMOylation mutant-transfected 3T3-L1 cell lines. We first measured the expression of aP2 and PPARγ in these cells on day 2 after DMIR induction. Overexpression of Sharp-1 markedly reduced the expression of these genes compared with the vector control. However, Sharp-1 repression of these genes was reduced significantly in Sharp-1 SUMOylation mutant cells (Fig. 6A). We then stained these cells with Oil Red O on day 6 after DMIR induction. The Sharp-1 wild-type cells showed less red staining compared with the vector. However, the Sharp-1 SUMOylation mutant had more red color than the Sharp-1 wild type. The lipid droplet formation in the Sharp-1 cells was also much less than that in the vector cells and in Sharp-1 mutant cells were more than in the Sharp-1 wild type (Fig. 6B). To further confirm the contribution of Sharp-1 SUMOylation in SENP1 regulation of adipogenesis, we stably transfected the Sharp-1 wild-type and the Sharp-1 SUMOylation mutant into Senp1<sup>−/−</sup> MEF cells and compared their ability for adipogenesis. As shown in Fig. 6C, the Senp1<sup>−/−</sup> cells showed less red staining compared with the vector-Senp1<sup>−/−</sup> cells. However, Sharp-1<sup>−/−</sup> cells had more red color than Sharp-1<sup>−/−</sup> cells. The lipid droplet formation in the Sharp-1-Senp1<sup>−/−</sup> cells was also much less than in the vector-Senp1<sup>−/−</sup> cells. However, in Sharp-1 mutant cells, it was more than in Sharp-1-Senp1<sup>−/−</sup> MEF cells. These data suggest that SUMOylation contributes to Sharp-1 repression of adipogenesis.

DISCUSSION

In this study, we reveal the role of SENP1 as a novel positive regulator in adipogenesis on the basis of the following evidence. First, we observed a phenotype of Senp1<sup>−/−</sup> MEF cells showing defects in adipogenesis. Second, SENP1 can enhance the expression of PPARγ, a master regulator of adipogenesis. Third, SENP1 can de-SUMOylate Sharp-1, a repressor for PPARγ transcription as well as for adipogenesis. Therefore, we propose a model in which SENP1 enhances adipogenesis
through de-SUMOylation of Sharp-1, which then releases Sharp-1 repression of PPARγ function as well as adipocyte differentiation.

It is well documented that adipogenesis is controlled by a tightly regulated transcriptional cascade where the transcription factors activate or repress the expression of each other in a sequential manner. C/EBP and PPARγ, as master transcription factors in adipogenesis, play a critical role in regulation of the expression of adipocyte differentiation-related genes. Adipogenic stimuli activate C/EBP, which directly binds to the PPARγ promoter and induces PPARγ. PPARγ also turns on the expression of C/EBP and then further induces PPARγ expression. This self-reinforcing regulatory loop is critical for PPARγ function in adipogenesis. Recently, many regulators have been identified that control C/EBP and PPARγ activity. Sharp-1, a member of the transcriptional repressor subfamily of basic helix-loop-helix transcription factors, has been shown to be one of the negative regulators in control of C/EBP and PPARγ activity. Taneja and co-workers (8) reported that Sharp-1, as a corepressor, inhibits C/EBP activity by retaining HDAC1 and G9a on the C/EBPα and PPARγ2 promoter to inhibit PPARγ expression and adipogenesis. Furthermore, Sharp-1 has been shown to be a SUMOylated protein. However, it is unknown whether SUMOylation contributes to Sharp-1 suppression of adipogenesis. In this study, we show that SUMOylation enhances Sharp-1 repression of PPARγ expression and adipocyte differen-
SENP1 Regulates Adipocyte Differentiation

A

B

C

| Senp1+/+ | Vector | Sharp-1 | Sharp-1m |
|---------|--------|---------|----------|
| Senp1-/- | Vector | Sharp-1 | Sharp-1m |

FIGURE 6. Deficiency in SUMOylation reduces Sharp-1 suppression of adipogenesis. A, expression of PPARγ and aP2 in Sharp-1 and Sharp-1m-transfected 3T3-L1 cells was measured on day 2 after DMIR treatment. *, p < 0.01; Student’s t test; differences between Sharp-1 versus control or Sharp-1 versus Sharp-1m. B, vector control, Sharp-1, or Sharp-1 mutant-transfected 3T3-L1 cells were stained with Oil Red O on day 6 after DMIR treatment. C, Senp1+/+ MEF cells and vector, Sharp-1, or Sharp-1 mutant-transfected Senp1+/+ MEF cells were stained with Oil Red O on day 8 after DMIR treatment.

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