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PIASy-Mediated Sumoylation of SREBP1c Regulates Hepatic Lipid Metabolism upon Fasting Signaling

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SREBP1c is a key transcription factor that regulates *de novo* lipogenesis during anabolic periods. However, the molecular mechanisms involved in the suppression of SREBP1c under nutritional deprivation are largely unknown. In this study, we demonstrate that the small ubiquitin-related modifier (SUMO) E3 ligase, a protein inhibitor of activated STAT Y (PIASy), sumoylates SREBP1c at Lys98, leading to suppression of the hepatic lipogenic program upon fasting-induced signals. In primary hepatocytes, ablation of PIASy stimulated intracellular lipid accumulation through the induction of SREBP1c and its target genes. Given that protein kinase A (PKA) plays important roles in catabolic responses, activated PKA enhances the sumoylation of SREBP1c and potentiates the interaction between SREBP1c and PIASy. Notably, overexpression of PIASy in obese *db/db* mice ameliorated hepatic steatosis, while suppression of PIASy in lean (wild-type) mice stimulated hepatic lipogenesis with increased expression of SREBP1c target genes. Furthermore, PKA-mediated SREBP1c phosphorylation augmented SREBP1c sumoylation, subsequently leading to degradation of SREBP1c via ubiquitination. Together, these data suggest that PKA-induced SREBP1c sumoylation by PIASy is a key regulatory mechanism to turn off hepatic lipogenesis during nutritional deprivation.

The sterol regulatory element-binding proteins (SREBPs) are basic helix-loop-helix-leucine zipper (bHLH-LZ) transcription factors that play key roles in cholesterol and lipid homeostasis (1–4). There are three SREBP isoforms; SREBP1a and SREBP1c are produced from a single gene through the use of alternative promoters and splicing processes, whereas SREBP2 is encoded by a separate gene (5). Initially, the SREBP1c homolog was identified as adipocyte determination- and differentiation-dependent factor 1 (ADD1) because of its expression profiles in adipocytes and fat tissues (6, 7). Unlike most transcription factors, precursor forms of SREBPs are localized in the endoplasmic reticulum (ER) and undergo proteolytic cleavage upon changes in the cellular lipid content to liberate N terminus-containing mature SREBPs into the nucleus (8–10). Among the three SREBPs, SREBP1c primarily regulates the expression of genes for fatty acid synthesis, whereas SREBP2 preferentially modulates the expression of genes for cholesterol synthesis (11).

Lipogenesis actively occurs when the whole-body energy state is high enough to turn on the anabolic pathway. To reflect and accommodate nutritional states, SREBP1c is tightly regulated at both the transcriptional and posttranslational levels. For example, the expression of SREBP1c is promoted by feeding via insulin and/or glucose in liver and fat tissues (12–14). Insulin, a key anabolic hormone, stimulates the expression and activity of SREBP1c through the phosphoinositol-3-kinase (PI3K)- and mammalian target of rapamycin complex 1 (mTORC1)-dependent pathways to mediate insulin action (14–20). Subsequently, activated SREBP1c potentiates most lipogenic genes, including those for fatty acid synthase (FASN), stearoyl coenzyme A (stearoyl-CoA) desaturase 1 (SCD1), acetyl-CoA carboxylase (ACC), and lipoprotein lipase (LPL), to induce *de novo* lipogenesis for the storage of a future energy source (21, 22).

On the contrary, the expression of SREBP1c is sensitively suppressed by fasting or nutritional deprivation (12, 13). Glucagon, a key fasting-induced hormone, represses SREBP1c activity through protein kinase A (PKA) signaling cascades (23, 24). Since PKA is subordinated to glucagon and adrenalin, PKA plays important roles in gluconeogenesis and β-oxidation during nutritional deprivation. However, how hepatic SREBP1c might be repressed during nutritional deprivation has not been thoroughly understood.

Sumoylation dynamically mediates functional changes in target proteins by altering protein-protein interaction and subcellular localization or regulating the ubiquitination of target proteins (25). Although several transcription factors involved in energy homeostasis have been reported to be modified by sumoylation, the underlying mechanisms by which sumoylation is induced to reflect the whole-body energy state are largely unknown. Similar to ubiquitination, the biochemical processes of sumoylation are mediated by the E1, E2, and E3 enzymes (26). The mammalian protein inhibitor of activated STAT (PIAS) protein is a representative SUMO E3 ligase and contains four members: PIAS1, PIAS2, PIAS3, and PIASy (27). Although PIAS proteins were originally isolated during studies of the regulation of STAT signaling (28), they are able to function as SUMO E3 ligases and are involved in the expression of certain genes (29–31). However, it is largely unknown whether PIAS proteins are able to mediate metabolic functions, such as lipid metabolism.

In this study, we have elucidated the molecular mechanism by which PIASy suppresses hepatic lipogenesis through sumoylation of

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SREBP1c upon PKA activation during nutritional deprivation. Activated PKA phosphorylates SREBP1c, which stimulates SREBP1c sumoylation at Lys98. Sequentially, sumoylated SREBP1c is readily degraded by ubiquitination, resulting in decreased hepatic lipid metabolism. Collectively, our data suggest that fasting-induced PKA activation stimulates SREBP1c sumoylation by PIASy, which would be an important turn-off mechanism of hepatic lipogenesis when fasting signals are received.

MATERIALS AND METHODS

Materials. Insulin, H89, *N*-ethylmaleimide (NEM), and cycloheximide (CHX) were purchased from Sigma-Aldrich (St. Louis, MO). Forskolin and MG132 were purchased from Calbiochem (San Diego, CA). A recombinant murine PKA catalytic subunit was purchased from New England BioLabs (Ipswich, MA). PKA inhibitor (PKI) was purchased from Promega (Madison, WI). Antibodies for myc, hemagglutinin (HA), PIASy, and phosphorylated PKA (P-PKA) substrate were from Cell Signaling (Danvers, MA); those for Flag and actin were from Sigma; antibody for SUMO1 was from Enzo Life Sciences (San Diego, CA); antibody for lamin B was from Abcam; and antibody for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was from LabFrontier (Suwon, South Korea). The antibody for SUMO1 was purchased from Enzo Life Sciences (Seoul, South Korea). Polyclonal antibodies against SREBP1 were generated by immunizing rabbits with bacterially produced SREBP1 from LabFrontier.

Plasmids. Flag-SUMO1, Flag-Ubc9, and Flag-PIASy were kindly provided by Kyung Soo Park (Seoul National University). SREBP1c-myc has been described previously (32). Mutants with the K98R, K215R, K225R, K351R, K387R, and E100G mutations were generated from wild-type (WT) SREBP1c by site-directed mutagenesis, and the sequences were confirmed by sequencing.

Animals. All animals studied were 8- to 12-week-old male C57BL/6 or *db/db* mice purchased from Central Lab Animal Inc. (Seoul, South Korea) and maintained on a normal chow diet and a 12-h light/12-h dark cycle in a pathogen-free animal facility. *SREBP1c*$^{-/-}$ mice were kindly provided from J. Horton at the University of Texas Southwestern Medical Center. All animal experiments were approved by the Seoul National University Animal Experiment Ethics Committee.

Preparation of recombinant adenovirus. The adenovirus vector was constructed as previously described (33). The gene for the green fluorescent protein (GFP) was coexpressed from an independent promoter. For *in vivo* experiments, mice were injected through the tail vein with 5×10^9 PFU of adenovirus in 200 μl phosphate-buffered saline (PBS). Empty virus expressing only the gene for GFP served as the control.

Primary mouse hepatocyte cultures. Hepatocytes were isolated as previously described (14). For adenoviral infection, hepatocytes were incubated for 4 h with adenovirus at 10 PFU/cell. The medium was then replaced by fresh medium.

siRNA transfection. Small interfering RNA (siRNA) duplexes for *PIASy* were designed by the siDESIGN Center (Dharmacon, Lafayette, CO) and produced by GenePharma (China). The sequence was 5′-CUA CAGAGGUUGAAGACGA-3′. Primary hepatocytes were transiently transfected with the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol.

Protein extraction, Western blotting, and immunoprecipitation. Whole-cell lysates were prepared with modified radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40 [NP-40], 0.25% sodium deoxycholate) containing 1% SDS, 1 μM phenylmethylsulfonyl fluoride (PMSF), 10 mM NEM, and 0.1% protease inhibitor cocktail. Immunoprecipitation was performed by incubation with the antibody indicated below, followed by incubation with protein A-Sepharose (GE Healthcare, Buckinghamshire, United Kingdom). The beads were washed extensively, and the immunoprecipitates were analyzed by immunoblotting.

RNA preparation and real-time qPCR analysis. As described earlier (34), total RNA was isolated using the TRIzol reagent (Invitrogen). Subsequently, equal amounts of RNA were subjected to cDNA synthesis using RevertAid Moloney murine leukemia virus reverse transcriptase (Fermentas, Glen Burnie, MD). The relative amount of mRNA was evaluated by use of an MyiQ real-time quantitative PCR (qPCR) detection system (Bio-Rad Laboratories, Hercules, CA) and calculated following normalization to the amount of *TBP* mRNA. The primer sequences that were used for the real-time qPCR analyses are described in Table S1 in the supplemental material.

Oil red O staining. Hepatocytes were fixed with 4% paraformaldehyde in PBS, dehydrated with 100% propylene glycol, and then stained with oil red O. Frozen sections from OCT compound-embedded, snap-frozen liver tissue specimens were fixed and stained with oil red O.

Biochemical analysis. The levels of plasma cholesterol and triglycerides were measured using the Infinity triglyceride reagent (Thermo, Melbourne, Australia). Free fatty acid (FFA) levels were measured using colorimetric assays (Half-Micro test; Roche). Plasma insulin levels were quantified by use of an enzyme-linked immunosorbent assay kit (Shibayagi, Japan), according to the manufacturer's instructions.

DuoLink *in situ* PLA. DuoLink *in situ* proximity ligation assay (PLA) analysis was performed according to the manufacturer's instructions (OLink Biosciences, Uppsala, Sweden). Briefly, paraformaldehyde-fixed cells were washed with PBS and blocked with blocking solution. The primary rabbit antibody was applied, and the cells were incubated with plus and minus secondary PLA probes against both rabbit and mouse IgG. The incubation was followed by hybridization and ligation, and then amplification was performed. After mounting with DuoLink mounting medium, the samples were examined using a Zeiss LSM 700 confocal microscope (Carl Zeiss, Jena, Germany).

CHX experiments. HeLa cells were cotransfected with plasmids for WT SREBP1c or K98R SREBP1c. After 24 h, 20 μM CHX was added to each plate, and cells were subsequently harvested at the indicated times. Equal amounts of total proteins from each treatment were taken to perform Western blot analyses.

Cell-based ubiquitination assays. COS-1 cells were transfected with expression plasmids for SREBP1c and HA-ubiquitin (Ub) in the presence or absence of the SUMO1 expression plasmid or 20 μM forskolin. Cells were treated with 10 μM MG132 for 12 h, and cell extracts were prepared using modified RIPA buffer. SREBP1c-myc was immunoprecipitated with anti-Myc antibody (Cell Signaling Technology), and after washing in RIPA buffer, proteins were separated by SDS-PAGE. HA-ubiquitinated SREBP1c was detected by Western blot analysis using HA antibody.

PKA *in vitro* kinase assay. PKA subunit C (PKA-C; 25 units), histidine-tagged SREBP1c (1 μg), and 0.5 μCi of [γ-^{32}P]ATP were mixed in PKA reaction buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 100 μM ATP, 200 μM sodium orthovanadate) in the presence or absence of PKI (5 units) and incubated for 30 min at 30°C. Samples were analyzed by SDS-PAGE, and phosphorylation was visualized by autoradiography. Nonradioactive kinase assays were analyzed by Western blotting with a P-PKA substrate-specific antibody.

Statistical analysis. All the results are presented as means ± standard errors of the means (SEMs) determined from at least three independent experiments. Statistical significance was assessed by the Student *t* test. Differences were considered statistically significant at P values of <0.05.

RESULTS

SREBP1c is a target of SUMO1 modification. Given that SREBP1c is downregulated by fasting (12, 13), we examined whether the stability of the SREBP1c protein is regulated by sumoylation. In the presence of *N*-ethylmaleimide (NEM), the level of nuclear SREBP1 was remarkably reduced (Fig. 1A). Since NEM is a chemical inhibitor of cysteine proteases, including desumoylating enzymes and deubiquitinating enzymes, it appears that these two posttranslational modifications might be involved in the regulation of SREBP1 (35). First, to test whether SREBP1c might be a

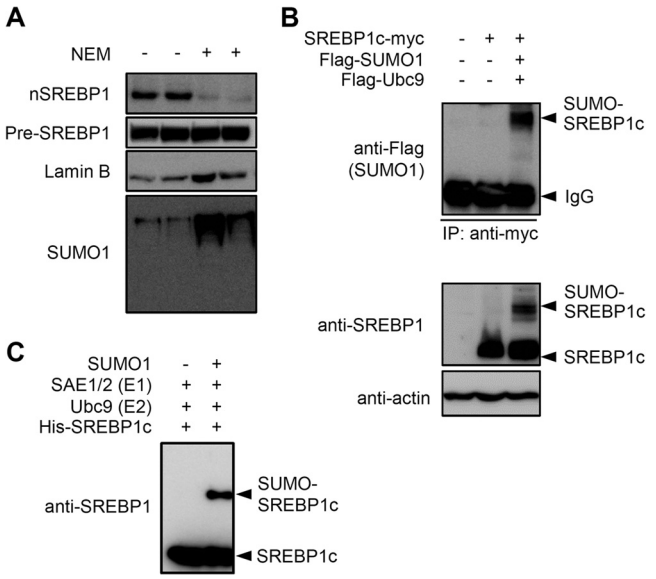

FIG 1 SREBP1c is modified by SUMO1. (A) Nuclear extracts of H4IIE rat hepatoma cells were prepared in the absence or presence of 10 mM NEM and were analyzed by immunoblotting. nSREBP1, nuclear form of SREBP1; Pre-SREBP1, precursor form of SREBP1. (B) HeLa cells were transfected with SREBP1c-myc, Flag-Ubc9, and Flag-SUMO1. Cells were treated with MG132 (20 μM) for 3 h, and immunoprecipitated (IP) SREBP1c-myc was probed for sumoylation using an anti-Flag antibody (top). The levels of SREBP1c protein in total cell lysates are also shown (bottom). (C) An *in vitro* sumoylation assay was performed using a SUMOylation kit (Enzo Life Sciences, San Diego, CA). Affinity-purified His-SREBP1c was incubated with SAE1-SAE2 (E1), Ubc9 (E2), and SUMO1, as indicated, at 30°C for 60 min and then analyzed by immunoblotting.

target for sumoylation, we examined whether SREBP1 would be modified by SUMO. Coexpression of SREBP1c and SUMO1 produced a shifted band whose size corresponded to the estimated size of sumoylated SREBP1c, which was confirmed by analyzing the immunoprecipitated complex with SREBP1c against SUMO1 by immunoblotting (Fig. 1B). In addition, an *in vitro* sumoylation assay with purified recombinant SUMO-conjugating enzymes clearly revealed that SREBP1c protein could be modified by SUMO1 (Fig. 1C). These data indicate that SREBP1c is a target for sumoylation, which is likely to affect SREBP1c stability.

Sumoylation of SREBP1c at Lys98 represses its transcriptional activity. Sumoylation occurs on lysine residues within the consensus sequence ΨKXE (36). To determine a putative sumoylation site(s), SREBP1c was analyzed using SUMOplot software, and five potential sumoylation sites were mapped at Lys98, Lys215, Lys225, Lys351, and Lys387 in rat SREBP1c (Fig. 2A). Each of these five lysine residues was then replaced with an arginine, and these SREBP1c mutants were tested for sumoylation. Interestingly, the mutation at Lys98 abrogated the modification of SREBP1c with SUMO1, whereas the K215R, K225R, K351R, and K387R SREBP1c mutants still exhibited SUMO conjugates to an extent similar to that seen in wild-type (WT) SREBP1c (Fig. 2B). Further, we also noticed that Lys98 of rat SREBP1c and its adjacent residues are well conserved in different species (Fig. 2C). When we assessed SREBP1c sumoylation by *in vitro* recombinant sumoylation assays, sumoylation was not detected in the K98R SREBP1c mutant (data not shown). In order to verify that Lys98 is a real target site for sumoylation, we replaced the conserved glutamic acid with glycine in the ΨKXE motif (E100G), where the E residue in the ΨKXE motif is essential for SUMO conjugation at Lys98. Unlike WT SREBP1c, E100G SREBP1c was not sumoylated (Fig. 2D), which clearly indicates that both the Lys98 and Glu100 residues of SREBP1c are necessary for SREBP1c SUMO modification.

To examine whether sumoylation could influence SREBP1c activity, we compared the transcriptional activity of WT SREBP1c with that of the sumoylation-defective mutant K98R SREBP1c. K98R SREBP1c was more potent than WT SREBP1c in transactivating the *FASN* promoter, and a similar result was obtained with E100G SREBP1c (Fig. 2E). During reporter assays, the level of SREBP1c mutant proteins was normalized to that of WT SREBP1c. Then, to test whether SREBP1c sumoylation could affect the expression of SREBP1c target genes, we expressed WT and K98R SREBP1c in an adenoviral vector (Ad-WT SREBP1c and Ad-K98R SREBP1c, respectively) and overexpressed WT or K98R SREBP1c by infection of mouse primary hepatocytes with Ad-WT SREBP1c and Ad-K98R SREBP1c. Consistent with the results of the reporter assays, Ad-K98R SREBP1c augmented the mRNA levels of SREBP1c target genes, including *FASN*, *SCD1*, and *Elovl6*, which are involved in lipogenesis, to a greater extent than did Ad-WT SREBP1c (Fig. 2F). Together, these data imply that SREBP1c sumoylation at Lys98 could negatively regulate the transcriptional activity of SREBP1c.

PIASy is a SUMO E3 ligase for SREBP1c and regulates hepatic lipogenesis. To gain more insights into the effect of SREBP1c sumoylation by SUMO E3 ligases on lipid metabolism, we first performed knockdown analyses to test whether any SUMO E3 ligase is involved in hepatic lipid metabolism by inducing SREBP1c sumoylation. We tested various SUMO E3 ligases, including four PIAS proteins (PIAS1, PIAS2, PIAS3, and PIASy), a RanBP2 protein, and a Pc2 protein. In mouse primary hepatocytes, *PIASy* siRNA significantly increased intracellular lipid accumulation (Fig. 3A). In addition, elevated lipid accumulation by *PIASy* siRNA was paralleled by the induction of lipogenic genes, such as *SREBP1c*, *FASN*, and *ACC1* (Fig. 3B). These results thus raise the possibility that the PIASy protein plays a role in the regulation of hepatic lipogenesis, probably through SREBP1c sumoylation.

To investigate whether PIASy mediates SREBP1c sumoylation, we carried out cell-based *in vivo* sumoylation assays in mouse primary hepatocytes. As shown in Fig. 3C, PIASy strongly induced SREBP1c sumoylation. Sumoylation is a dynamic and reversible reaction that is also regulated by desumoylating enzymes, such as SUMO-specific proteases (SENPs) (37, 38). When we examined the specificity of SENPs to SREBP1c sumoylation, SENP2 potently decreased SREBP1c sumoylation (Fig. 3D). Consistent with the results presented above, SENP2 augmented the transcriptional activity of WT SREBP1c but did not affect that of K98R SREBP1c (Fig. 3E). Together, these data suggest that PIASy represses the transcriptional activity of SREBP1c via sumoylation, leading to changes in hepatic lipid metabolism.

PIASy knockdown increases hepatic lipogenesis in wild-type mice. The findings that knockdown of PIASy resulted in a significant increase in lipogenic gene expression and lipid accumulation in hepatocytes prompted us to test whether the sumoylation of SREBP1c is crucial for the repression of hepatic *de novo* lipogenesis. In order to address this issue, we suppressed *PIASy* via siRNA

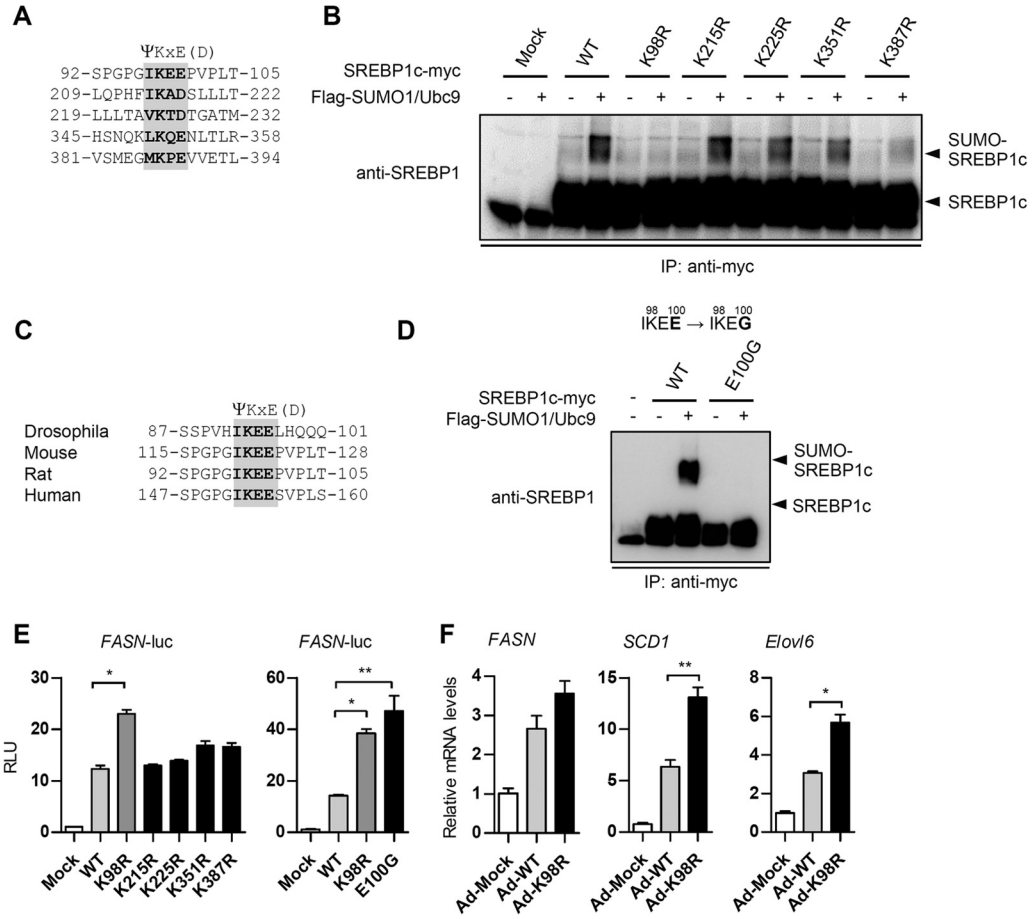

FIG 2 Sumoylation inhibits the transcriptional activity of SREBP1c. (A) Putative SREBP1c sumoylation sites. The shaded column indicates putative sumoylation sites in SREBP1c. (B) HeLa cells were transfected with Flag-SUMO1 and either WT SREBP1c-myc or the K98R, K215R, K225R, K351R, or K387R mutant of SREBP1c-myc. Cells were treated with MG132 (20 μM) for 3 h, and immunoprecipitated SREBP1c-myc was probed for sumoylation using an anti-SREBP1 antibody. (C) Alignment of SREBP1c amino acid sequences from several species with rat SREBP1c sumoylation sites. The shaded column indicates conserved sumoylation sites in SREBPs. (D) HeLa cells were transfected with Flag-SUMO1 and either WT or E100G SREBP1c-myc. Cells were treated with MG132 (20 μM) for 3 h, and immunoprecipitated SREBP1c-myc was probed for sumoylation using an anti-SREBP1 antibody. (E) HeLa cells were cotransfected with a reporter plasmid containing the promoter region of the *FASN* gene (*FASN*-luc) along with expression plasmids encoding either the WT or the mutant form of *SREBP1c*, and luciferase (luc) assays were carried out. Data represent the means ± SEMs of three independent experiments. RLU, relative luciferase units. *, $P < 0.05$ versus WT; **, $P < 0.01$ versus WT. (F) Mouse primary hepatocytes were infected with the empty adenovirus vector (Ad-Mock), Ad-WT SREBP1c (Ad-WT), or Ad-K98R SREBP1c (Ad-K98R) (10 PFU/cell), and the expression of SREBP1c target genes was analyzed by real-time qPCR. Data represent the means ± SEMs of three independent experiments. *, $P < 0.05$ versus WT; **, $P < 0.01$ versus WT.

in wild-type or *SREBP1c*$^{-/-}$ primary hepatocytes. As shown in Fig. 4A and B, *PIASy* knockdown stimulated intracellular lipid accumulation and lipogenic gene expression in wild-type primary hepatocytes but did not cause any increase in lipogenic gene expression or intracellular lipid accumulation in *SREBP1c*$^{-/-}$ primary hepatocytes. These data clearly indicate that PIASy could modulate hepatic lipid metabolism via the regulation of SREBP1c.

Next, we asked whether PIASy might influence hepatic lipogenesis *in vivo*. To address this, adenovirus bearing the *FASN* promoter was injected via the tail vein, along with adenovirus expressing *PIASy* short hairpin RNA (shRNA) (Ad-shPIASy) or control shRNA (Ad-shControl) into lean (C57BL/6) mice. Suppression of *PIASy* increased the level of nuclear SREBP1 in the liver (Fig. 4C). In addition, *PIASy* knockdown slightly decreased the blood glucose level, while fed serum insulin and plasma cholesterol levels and body weight were not significantly altered by PIASy suppression. In contrast, optical *in vivo* imaging analysis showed that Ad-shPIASy elevated the *FASN* promoter activity (more than 3- to 4-fold) compared with that achieved with the Ad-shControl (Fig. 4D). Simultaneously, the *in vivo* suppression of hepatic *PIASy* elevated hepatic lipogenic gene expression (Fig. 4E), as well as the levels of hepatic triglycerides (Fig. 4F), plasma triglycerides, and plasma free fatty acid (FFA) (data not shown), indicating that PIASy suppresses hepatic lipid metabolism through SREBP1c *in vivo*.

PIASy overexpression attenuates hepatic steatosis by reducing lipogenic activity in *db/db* mice. To verify whether PIASy indeed modulates hepatic lipid metabolism via regulation of SREBP1c, reporter assays were carried out. Ectopic *PIASy* expression decreased the transcriptional activity of WT SREBP1c but insignificantly affected the transcriptional activity of K98R SREBP1c (Fig. 5A). Consistently, the expression of mRNA for SREBP1c target genes, such as *FASN*, *SCD1*, and *Elovl6*, was repressed by *PIASy* overexpression (Fig. 5B).

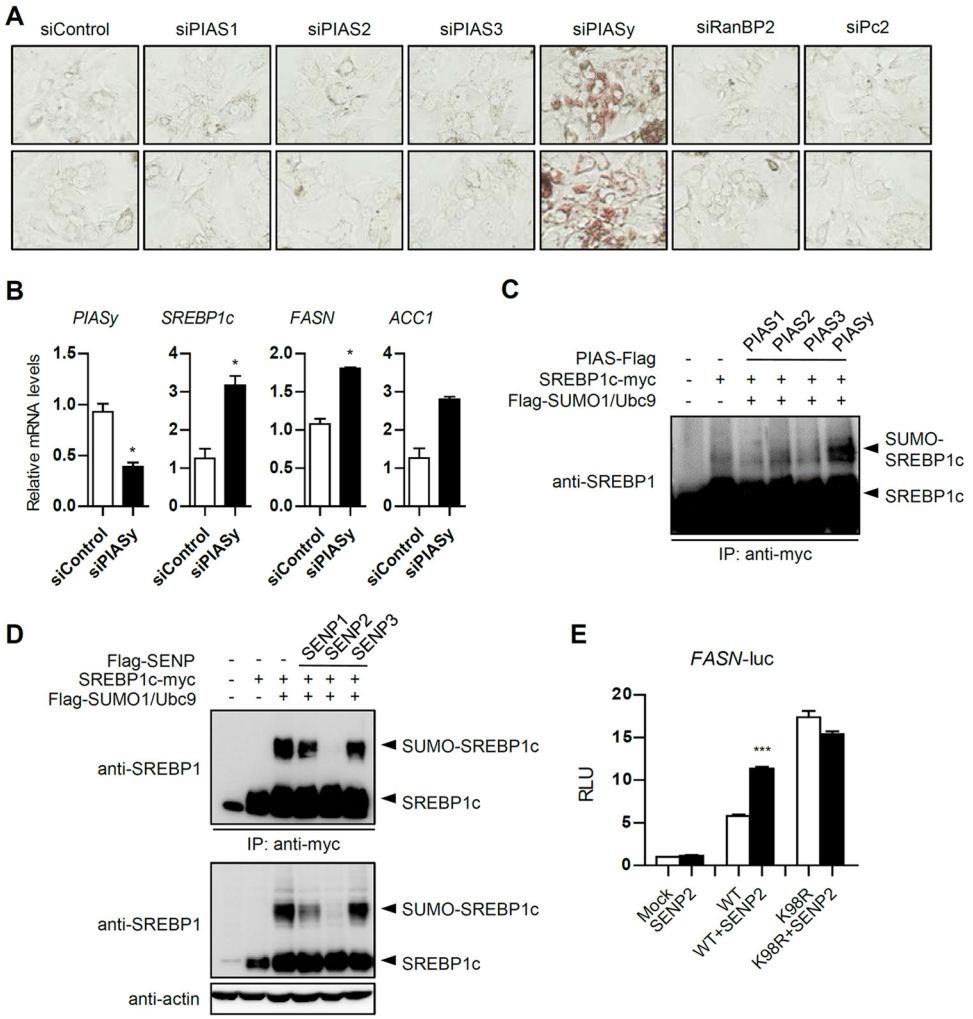

FIG 3 PIASy is involved in the hepatic lipogenic pathway and is a SUMO E3 ligase for SREBP1c. (A) Mouse primary hepatocytes were transfected with various siRNAs for SUMO E3 ligases (siPIAS1, siPIAS2, siPIAS3, siPIASy, siRanBP2, and siPc2) or control siRNA (siControl). After 48 h, mouse primary hepatocytes were stained with oil red O. (B) Mouse primary hepatocytes were transfected with siPIASy or control siRNA. The mRNA level obtained in control siRNA-transfected cells was set to 1.0, and the levels in cells transfected with the other constructs are expressed as relative values. Data represent the means ± SEMs of three independent experiments. *, $P < 0.05$. (C) Mouse primary hepatocytes were cotransfected with the SREBP1c-myc, Flag-SUMO1, Flag-Ubc9, and PIAS isoforms. Cells were treated with MG132 (20 μM) for 3 h, and immunoprecipitated SREBP1c-myc was blotted with an anti-SREBP1 antibody. (D) HeLa cells were transfected with expression vectors for the SREBP1c-myc, Flag-SUMO1, Flag-Ubc9, and Flag-tagged SENP isoforms. Immunoprecipitated SREBP1c-myc was blotted with an anti-SREBP1 antibody (top). The expression level of SREBP1c protein in each cell lysate is also shown (bottom). (E) SENP2 potentiates the transcriptional activity of SREBP1c. HeLa cells were cotransfected with the expression vector for Flag-SENP2 and either WT or K98R SREBP1c-myc, along with the *FASN*-luc reporter vector. The normalized luciferase activity obtained without SREBP1c or SENP2 was set to 1.0, and the activities in cells transfected with the other constructs are expressed as relative values. Data represent the means ± SEMs of three independent experiments. ***, $P < 0.005$.

The finding that PIASy repressed lipogenic gene expression in liver led us to test whether *PIASy* overexpression reverses hepatic steatosis. Since obese and diabetic *db/db* mice exhibit remarkable hepatic steatosis, *PIASy* was overexpressed in *db/db* mice by use of the adenovirus vector. In optical *in vivo* imaging experiments, the adenoviral overexpression of *PIASy* in *db/db* mice greatly decreased hepatic *FASN* promoter activity (Fig. 5C). As shown in Fig. 5D and E, excess hepatic lipid accumulation in *db/db* mice was also reduced by *PIASy* overexpression. Furthermore, *PIASy* overexpression decreased the level of nuclear SREBP1 in the livers of *db/db* mice (Fig. 5F). Accordingly, lipogenic gene expression was attenuated in the livers of *db/db* mice in which *PIASy* was overexpressed (Fig. 5G). Taken together, these results explicitly suggest that PIASy could alleviate hepatic steatosis by reducing lipogenic gene expression in *db/db* mice.

Sumoylation of SREBP1c is enhanced by PKA activation. SREBP1c is sensitively regulated by nutritional hormones, such as insulin and glucagon, to maintain lipid homeostasis, which adapts to changes in energy states (13, 15, 21). Thus, it is plausible to speculate that SREBP1c sumoylation might be modulated by nutritional changes. To address this, we examined whether SREBP1c sumoylation is altered by glucagon and forskolin, a PKA activator. Although the basal level of endogenous SREBP1c sumoylation was quite low, glucagon promoted SREBP1c sumoylation in primary hepatocytes (Fig. 6A). Furthermore, it is notable that both glucagon and forskolin increased SREBP1c sumoylation (Fig. 6B and

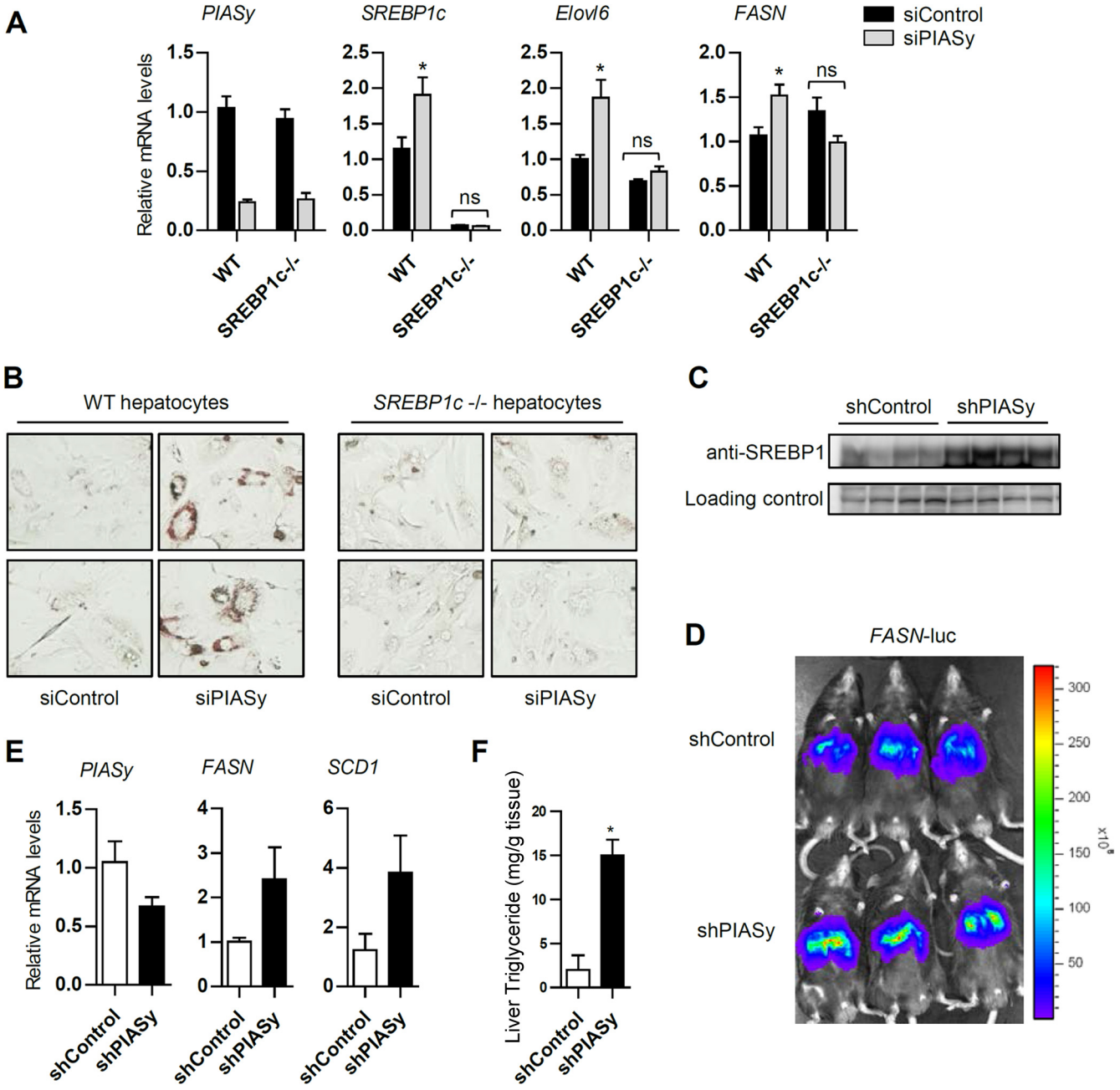

FIG 4 *PIASy* knockdown increases lipogenic activities in mouse primary hepatocytes and liver. (A) Wild-type and *SREBP1c*$^{-/-}$ mouse primary hepatocytes were transfected with siPIASy or control siRNA (siControl). Total RNAs were prepared and subjected to real-time qPCR using primers specific for *PIASy*, *SREBP1c*, *Elovl6*, and *FASN*. The mRNA level obtained in scrambled siRNA-transfected cells was set to 1.0, and the levels in cells transfected with the other constructs are expressed as relative values. Data represent the means ± SEMs of three independent experiments. *, $P < 0.05$; ns, not significant. (B) Wild-type and *SREBP1c*$^{-/-}$ mouse primary hepatocytes were transfected with siPIASy or control siRNA. After 48 h, mouse primary hepatocytes were stained with oil red O. (C) Expression levels of hepatic SREBP1 were examined by immunoblotting. (D) C57BL/6 mice were injected with Ad-*FASN*-luc and Ad-shControl or Ad-shPIASy. The effect of hepatic PIASy knockdown on *FASN*-luc activity was measured by optical *in vivo* imaging analysis. (E) Transcript levels of SREBP1c target genes were measured in C57BL/6 mouse liver injected with Ad-shControl or Ad-shPIASy. (F) The total liver triglyceride content was measured from 100 mg liver tissue. Data represent the means ± SEMs ($n = 5$ mice for each treatment). For *PIASy* shRNA, the asterisk indicates a $P < 0.05$ versus the control.

C). In addition, treatment with H89, a PKA inhibitor, attenuated the effect of both glucagon and forskolin on SREBP1c sumoylation (Fig. 6B and C). To explore these results at the cellular level, we investigated SREBP1c sumoylation via *in situ* proximity ligation assay (PLA), which allowed us to monitor protein modifications and, indirectly, physical interactions. As shown in Fig. 6D and E, SREBP1c sumoylation was detected in endogenous and exogenous SREBP1c protein from mouse primary hepatocytes and HeLa cells, respectively. Although PLA-positive signals (shown in red) indicated relatively weak SREBP1c sumoylation in primary hepatocytes, the mean number of PLA-positive red clusters per cell was augmented by forskolin, whereas these signals

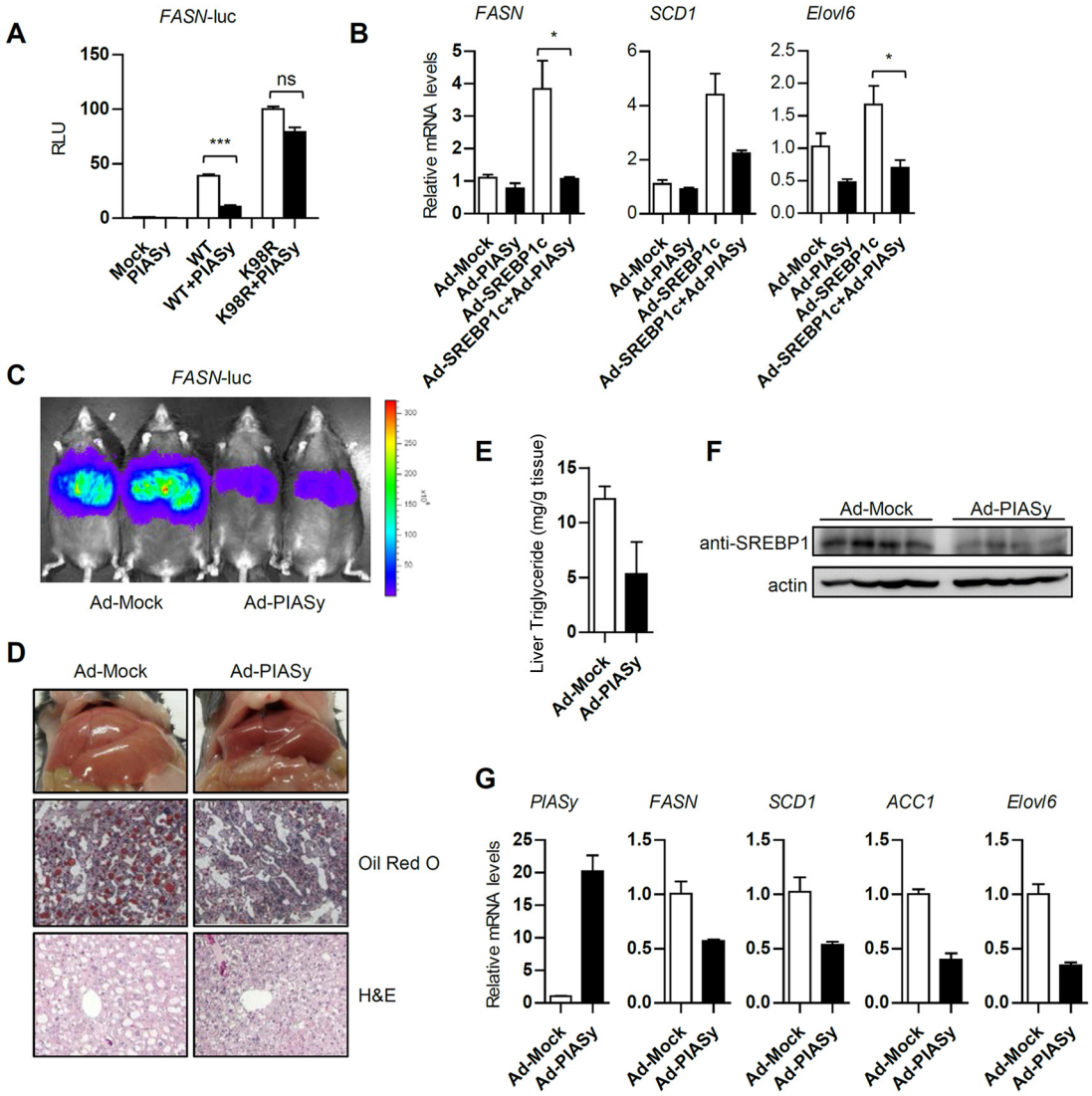

FIG 5 PIASy mediates the transcriptional repression of SREBP1c, and *PIASy* overexpression decreases lipogenic activity in *db/db* mice. (A) HeLa cells were transfected with Flag-PIASy and either WT or K98R SREBP1c-myc, along with the *FASN*-luc reporter vector. The luciferase activity obtained without SREBP1c or PIASy was set to 1.0 relative luciferase unit (RLU), and the activities of the other constructs are expressed as relative values. Data represent the means ± SEMs of three independent experiments. ***, $P < 0.005$; ns, not significant. (B) Mouse primary hepatocytes were infected with Ad-SREBP1c, Ad-PIASy, or the mock-infected adenovirus vector (Ad-Mock), as indicated. The mRNA level observed in mock vector-transfected cells was set to 1.0, and the levels for the other constructs are expressed as relative values. Data represent the means ± SEMs of three independent experiments. *, $P < 0.05$. (C) *db/db* mice were injected with Ad- *FASN*-luc and the mock-infected adenovirus vector or Ad-PIASy. The effect of hepatic *PIASy* overexpression on *FASN*-luc activity was measured by optical *in vivo* imaging analysis in *db/db* mice. (D) Representative hematoxylin-eosin (H&E) and oil red O staining of liver sections from mice injected with the mock-infected adenovirus vector or Ad-PIASy. (E) The total liver triglyceride content was measured from 100 mg liver tissue. (F) Expression levels of hepatic SREBP1 were examined by immunoblotting. (G) Transcript levels of SREBP1c target genes were measured in *db/db* mice injected with the mock-infected adenovirus vector or Ad-PIASy. Shown are the mean mRNA levels of SREBP1c target genes from the liver.

were decreased by H89 pretreatment (Fig. 6D and data not shown).

Further, to examine whether the interaction between SREBP1c and PIASy is affected by PKA activity, we performed PLA in HeLa cells transfected with SREBP1c and PIASy. As shown in Fig. 6F, forskolin enhanced PLA signals, while H89 greatly repressed PLA signals. These data imply that sumoylation of SREBP1c is regulated by nutritional state; in particular, fasting signals with PKA activation seem to promote SREBP1c sumoylation.

Sumoylation of SREBP1c regulates its protein stability via ubiquitination. In the nucleus, the SREBP1c protein is unstable and rapidly degraded via ubiquitin-dependent proteolytic cleavage (39). To investigate the effect of sumoylation on SREBP1c protein stability, the steady-state levels of either unmodified or SUMO-modified SREBP1c proteins were examined in the presence of cycloheximide, a protein synthesis inhibitor. When the half-life of the unmodified form of the SREBP1c protein was compared with that of the sumoylated form of the SREBP1c protein, sumoylated SREBP1c revealed decreased protein stability, as judged by a shortening of its half-life from ~6 h to ~1 h (Fig. 7A and B). Furthermore, sumoylated SREBP1c produced from coexpression of SREBP1c and SUMO1 was rarely detected in the ab-

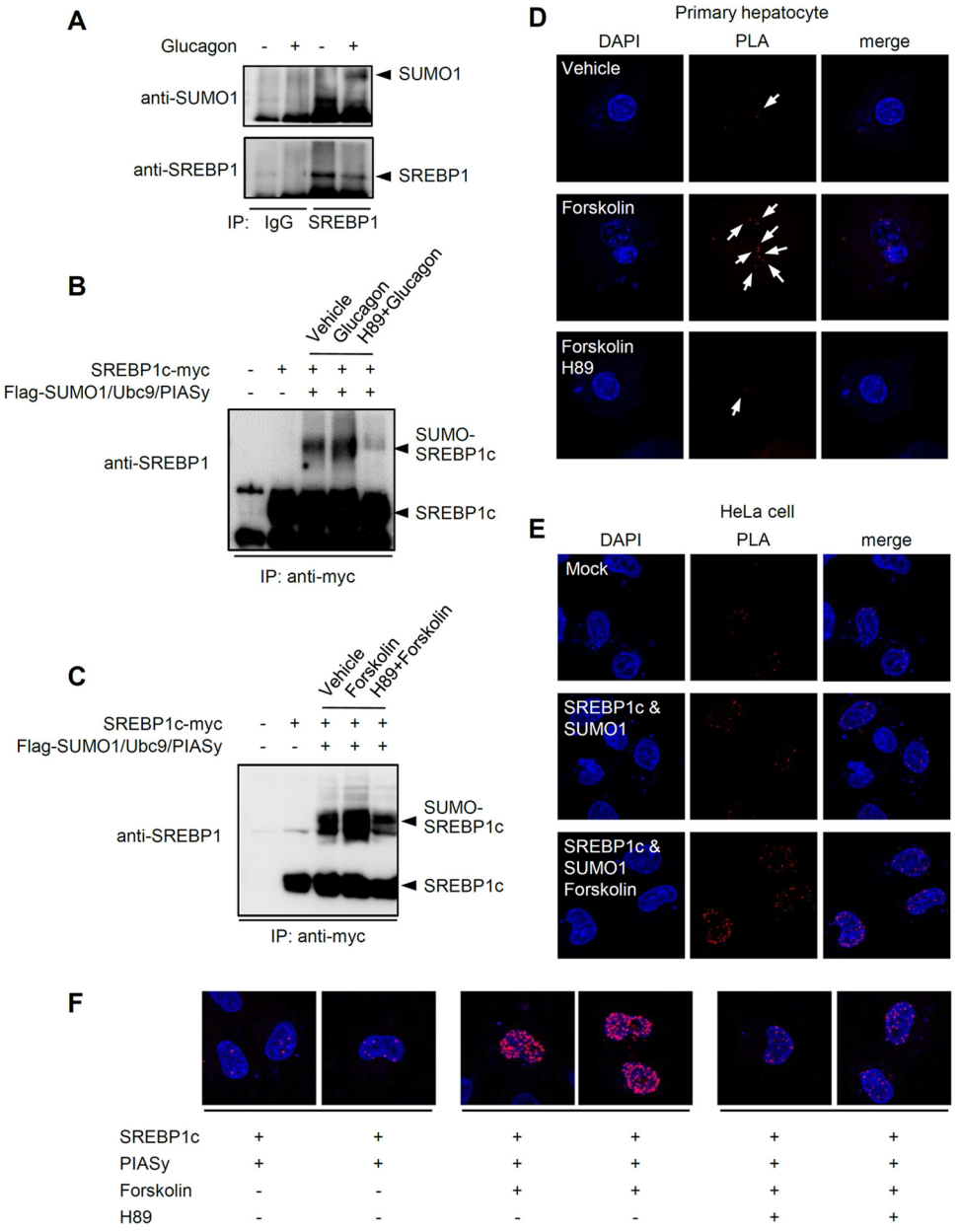

FIG 6 Sumoylation of SREBP1c is increased by PKA activation. (A) Mouse primary hepatocytes were treated with glucagon (100 nM) and MG132 (20 μM) and were immunoprecipitated with anti-SREBP1 antibody. Immunoprecipitated SREBP1 was probed with anti-SUMO1 antibody or anti-SREBP1 antibody. (B) Mouse primary hepatocytes were cotransfected with SREBP1c-myc, Flag-SUMO1, and Flag-Ubc9 and treated with glucagon (100 nM) and MG132 (20 μM) for 3 h. Immunoprecipitated SREBP1c-myc was analyzed by immunoblotting. (C) HeLa cells were cotransfected with SREBP1c-myc, Flag-SUMO1, and Flag-Ubc9, pretreated with H89 (20 μM) for 30 min, and then treated with forskolin (20 μM) and MG132 (20 μM) for 3 h. Immunoprecipitated SREBP1c-myc was analyzed by immunoblotting. (D) PLA was performed to detect the *in vivo* sumoylation of SREBP1c. As indicated, mouse primary hepatocytes were treated with forskolin alone or were pretreated with H89 before forskolin treatment. The positive signals were analyzed using confocal microscopy. Arrows, physical interaction between SREBP1c and SUMO1. (E) HeLa cells were cotransfected with SREBP1c-myc and Flag-SUMO1. After transfection, cells were treated with forskolin or vehicle and the PLA assay was performed. DAPI, 4′,6-diamidino-2-phenylindole. (F) HeLa cells were cotransfected with SREBP1c-myc and Flag-PIASy. After transfection, cells were treated with forskolin or pretreated with H89 and the PLA assay was performed.

sence of the proteasome inhibitor MG132 but was readily detectable in the presence of MG132, indicating that the level of sumoylated SREBP1c might be further regulated by a proteasome-dependent mechanism. Therefore, we studied whether SREBP1c sumoylation might induce SREBP1c degradation via the ubiquitination-proteasome pathway. Moreover, in primary hepatocytes, treatment with forskolin reduced the nuclear SREBP1 level, which was reversed by H89 pretreatment (data not shown), implying that PKA-dependent sumoylation would be involved in the proteasome-dependent proteolysis of SREBP1c.

To directly address the question of whether SREBP1c sumoylation affects SREBP1c ubiquitination and, thereby, degradation by the proteasome, cells were cotransfected with plasmids expressing Ub-HA and WT or K98R SREBP1c-myc with or without

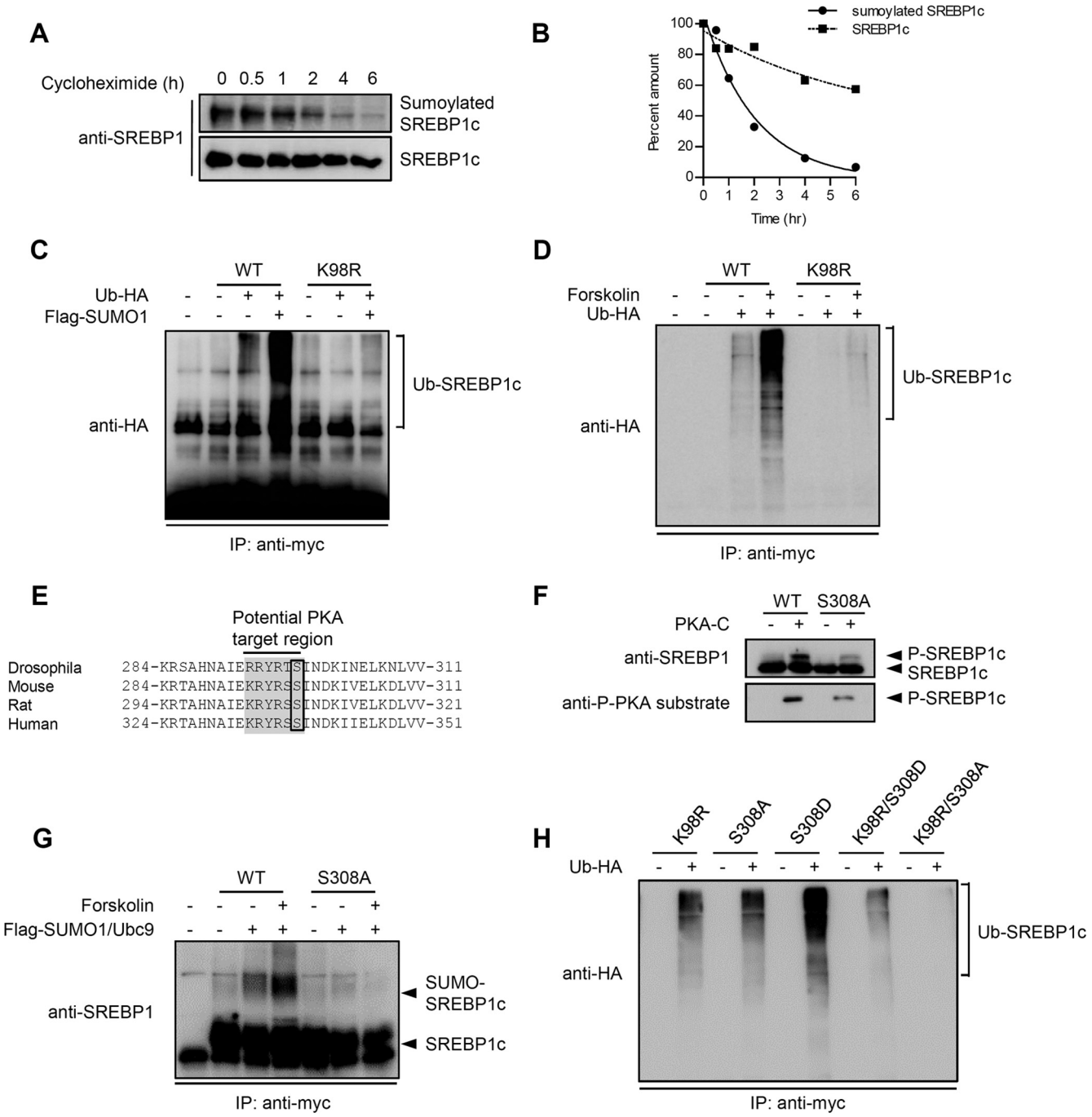

FIG 7 PKA-dependent phosphorylation of serine 308 of SREBP1c is important in sumoylation-dependent ubiquitination. (A) HeLa cells were transfected with either WT or K98R SREBP1c-myc and treated with or without cycloheximide (20 μM) for the indicated periods. Cell lysates were subjected to immunoblot analysis. (B) Quantification of the band intensities in panel A. (C) COS-1 cells were cotransfected with either WT or K98R SREBP1c-myc, Flag-SUMO1, and Ub-HA, as indicated. Cells were treated with 10 μM MG132 for 12 h, and cell lysates were subjected to immunoprecipitation with anti-Myc antibody, followed by immunoblotting with anti-HA antibody. (D) COS-1 cells were cotransfected with either WT or K98R SREBP1c-myc and Ub-HA, as indicated. Cells were treated with 10 μM MG132 for 12 h, the cells were treated with forskolin (20 μM) for 4 h, and then cell lysates were subjected to immunoprecipitation, followed by immunoblotting. (E) The shaded column indicates one of the potential PKA phosphorylation sites of SREBP1c. The boxed column indicates the conserved Ser residue in SREBPs. (F) Purified WT or S308A His-SREBP1c proteins were incubated with recombinant PKA-C for 30 min. The reaction mixtures were then subjected to SDS-PAGE and immunoblot analysis with anti-phospho-PKA (anti-P-PKA) substrate antibody or SREBP1 antibody. (G) Mouse primary hepatocytes were cotransfected with either WT or S308A SREBP1c-myc, Flag-SUMO1, and Flag-Ubc9, and the cells were treated with forskolin or vehicle, as indicated. (H) COS-1 cells were cotransfected with K98R, S308A, S308D, K98R/S308D, or K98R/S308A SREBP1c-myc and Ub-HA, as indicated. Cells were treated with MG132 (10 μM) for 12 h, and cell lysates were subjected to immunoprecipitation, followed by immunoblotting.

SUMO1. As shown in Fig. 7C, the level of ubiquitinated SREBP1c was greatly enhanced in the presence of SUMO1. In contrast, ubiquitination of K98R SREBP1c was barely detected, indicating that SREBP1c sumoylation promotes SREBP1c ubiquitination and its subsequent degradation by the proteasome. Next, when the effect of PKA activation on SREBP1c ubiquitination was investigated, forskolin markedly increased SREBP1c ubiquitination (Fig. 7D). However, this forskolin-induced SREBP1c ubiquitination was suppressed in K98R SREBP1c. In order to confirm whether sumoylation could affect SREBP1c ubiquitination, cells in which PIASy was suppressed were treated with or without forskolin and the level of SREBP1c ubiquitination was examined. PIASy knockdown via siRNA greatly reduced the ubiquitination of SREBP1c with forskolin (data not shown). These data suggest that the stability of the SREBP1c protein is controlled by sumoylation and that sumoylated SREBP1c is rapidly degraded via ubiquitination, which is augmented by PKA.

Sumoylation of SREBP1c is augmented by Ser308 phosphorylation upon PKA. It has been reported that both ubiquitination and sumoylation are modulated by phosphorylation (40, 41). Because forskolin could promote the sumoylation and ubiquitination of SREBP1c, we tested whether PKA causes sumoylation and ubiquitin-dependent SREBP1c reduction through SREBP1c phosphorylation. A previous study has shown that SREBP1c phosphorylation by PKA negatively regulates SREBP1c transcriptional activity (23). Since Ser308 and its adjacent amino acid residues in the SREBP1c protein are well conserved in several species (Fig. 7E), we performed *in vitro* kinase assays to test whether Ser308 of SREBP1c is a potential phosphorylation residue for PKA. As shown in Fig. 7F, recombinant PKA phosphorylated WT SREBP1c but phosphorylated S308A SREBP1c less. Furthermore, when the sumoylation levels of the WT or S308A SREBP1c protein were assessed, the extent of sumoylation was lower in S308A SREBP1c than in WT SREBP1c. Consistent with these results, in primary hepatocytes, forskolin-dependent SREBP1c sumoylation was further elevated in WT SREBP1c but not in S308A SREBP1c (Fig. 7G). Similarly, the level of ubiquitination of WT SREBP1c was potentiated by forskolin, as opposed to that of S308A SREBP1c (data not shown). These data imply that SREBP1c sumoylation might be regulated by phosphorylation at Ser308 of SREBP1c by PKA.

We next examined the level of ubiquitination in various SREBP1c mutants, including the sumoylation-deficient (K98R), PKA-dependent phosphorylation-defective (S308A), and phosphorylation-mimetic (S308D) mutants and double mutants of these mutants (K98R/S308D and K98R/S308A). Among these, the S308D mutant showed the strongest SREBP1c ubiquitination, whereas the S308A and K98R mutants exhibited lower levels of ubiquitination (Fig. 7H). Although the S308D mutant showed robust ubiquitination, the corresponding double mutant (K98R/S308D) exhibited decreased ubiquitination. Further, the K98R/S308A double mutant was hardly ubiquitinated, implying that phosphorylation at Ser308 and sumoylation at Lys98 appear to be crucial for the ubiquitination of SREBP1c. Taken together, these results suggest that SREBP1c that has undergone PKA-dependent phosphorylation readily undergoes sumoylation, ubiquitination, and proteasome-dependent degradation during nutritional deprivation.

K98R and S308A SREBP1c mutant proteins restore the suppressive effects of fasting on hepatic lipogenesis in wild-type mice. To explore the idea of whether the suppressed lipogenic activity of SREBP1c during nutritional deprivation might be associated with phosphorylation-dependent sumoylation of the SREBP1c protein, we analyzed sumoylation-deficient and phosphorylation-defective SREBP1c mutants in fasted livers of wild-type mice that were injected with adenovirus expressing WT SREBP1c, K98R SREBP1c, or S308A SREBP1c. The levels of ectopically expressed SREBP1c proteins and mRNA were not significantly different between each SREBP1c-overexpressing group (Fig. 8A and B). As shown in Fig. 8C, sumoylation-deficient K98R SREBP1c and PKA-dependent phosphorylation-defective S308A SREBP1c potently stimulated hepatic lipogenic gene expression compared to the level for WT SREBP1c. Accordingly, the levels of triglycerides were increased in liver in which K98R or S308A SREBP1c was overexpressed (Fig. 8D). These results indicate that phosphorylation (at Ser308)-dependent sumoylation (at Lys98) of SREBP1c might suppress the transcriptional activity of SREBP1c upon nutritional deprivation.

DISCUSSION

SREBP1c plays a key role in hepatic lipogenesis; therefore, it should be dynamically fine-tuned in response to various nutritional and hormonal changes to prevent unnecessary lipid synthesis. Chronic activation of SREBP1c with increased lipogenic activity contributes to the development and progression of several pathological conditions, such as fatty liver, obesity, and diabetes (42). In this regard, SREBP1c has received much attention as a therapeutic target against lipid dysregulation and related diseases. Although most studies have focused on the mechanisms of transcriptional activation of SREBP1c, the modification or stability control of nuclear SREBP1c protein upon nutritional deprivation has been poorly understood. In this study, we elucidated the molecular mechanism involving PKA-mediated SREBP1c phosphorylation, which promotes PIASy-dependent SREBP1c sumoylation and its degradation via ubiquitination, eventually turning off hepatic lipid metabolism upon fasting signaling.

It is of interest to note that SREBP1c sumoylation by PIASy is stimulated by PKA because the detailed mechanism of how sumoylation is induced by specific signaling cascades has rarely been reported. Several lines of evidence in the present study can be used to propose the idea that activated PKA would provoke SREBP1c phosphorylation, sumoylation, and ubiquitin-dependent degradation, as follows. First, the physical interaction between SREBP1c and PIASy was promoted by the PKA activator forskolin, which eventually led to the stimulation of SREBP1c sumoylation. On the contrary, H89, a PKA inhibitor, reduced the physical interaction between SREBP1c and PIASy. In addition, compared to WT SREBP1c, the PKA phosphorylation-defective S308A mutant of SREBP1c showed a reduced interaction with PIASy (data not shown). Second, in primary hepatocytes, SREBP1c sumoylation was increased by forskolin and decreased by H89. Third, SREBP1c sumoylation promoted its rapid degradation via ubiquitination in a PKA-dependent manner. Lastly, S308A SREBP1c was neither efficiently sumoylated nor ubiquitinated. Thus, these data suggest that sumoylation-mediated SREBP1c ubiquitination seems to be sensitively regulated by PKA activity as part of the catabolic cascade.

Although PIASy appears to be involved in the regulation of several metabolism-related proteins, such as peroxisome proliferator-activated receptor (PPAR), AMPK, and SIRT1 (43–45), the

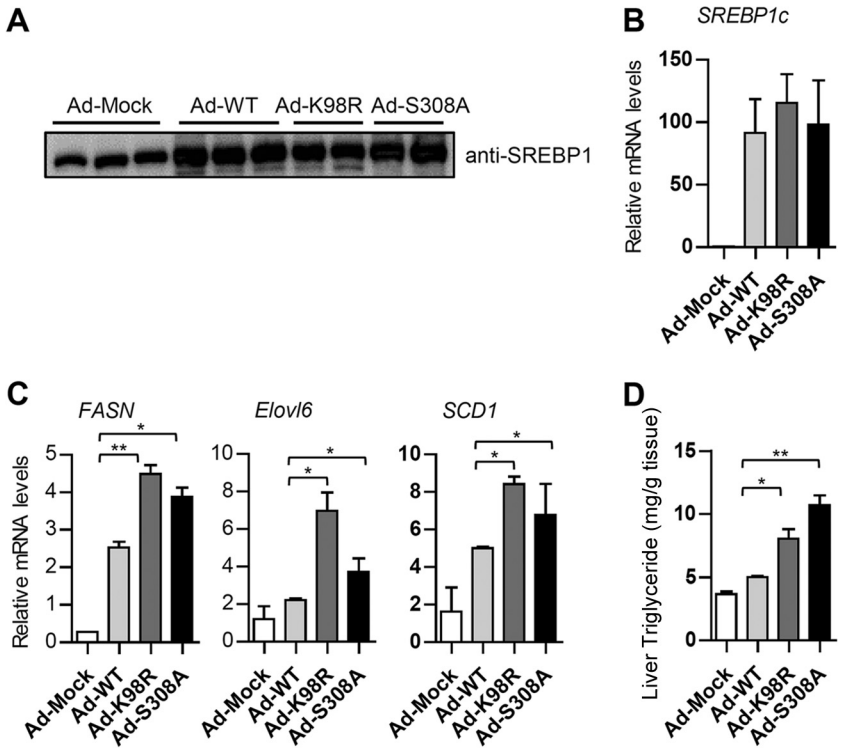

FIG 8 K98R and S308A SREBP1c restore the suppressive effects of fasting on hepatic lipogenesis in wild-type mice. Wild-type mice were injected with the indicated adenovirus expressing WT, K98R, or S308A SREBP1c. After 3 days, the mice were fasted for 3 h, and then they were sacrificed. (A) The expression levels of each SREBP1c protein were analyzed by immunoblotting. (B) The expression level of each SREBP1c mRNA was analyzed by real-time qPCR. (C) Levels of SREBP1c target gene expression. (D) The total liver triglyceride content was measured from 100 mg liver tissue. *, $P < 0.05$ versus WT; **, $P < 0.01$ versus WT.

specific role of PIASy in hepatic lipid metabolism has not been clearly elucidated. In this work, our data suggest that PIASy suppresses hepatic lipid metabolism through SREBP1c sumoylation. In hepatocytes, the level of SREBP1c target gene expression and intracellular lipid accumulation was markedly enhanced by *PIASy* knockdown via siRNA. However, in *SREBP1c*$^{-/-}$ hepatocytes, *PIASy* suppression did not enhance lipogenic gene expression. These results clearly indicate that SREBP1c is essential for the PIASy-dependent repression of hepatic lipogenesis. In accordance with these findings, *in vivo PIASy* knockdown in WT lean mice increased hepatic triglyceride levels, and this was accompanied by elevated lipogenic gene expression. In contrast, *PIASy* overexpression prominently alleviated hepatic steatosis in *db/db* mice. Taken together, both the *in vivo* and *ex vivo* data support the idea that PIASy appears to be a crucial factor in the regulation of hepatic lipid metabolism by mediating SREBP1c sumoylation.

It is well-known that nuclear SREBP1c is unstable and rapidly degraded (39). However, the underlying mechanism(s) that regulates SREBP1c stability upon nutritional deprivation is poorly understood. We failed to monitor the sumoylation or ubiquitination of endogenous SREBP1c protein from liver tissue upon fasting, presumably due to its low abundance and rapid degradation. Previously, it has been reported that SREBP1a and SREBP2 are negatively regulated by sumoylation (46, 47). Nonetheless, pulse-chase experiments and *in vitro* ubiquitination assays demonstrated that sumoylation does not affect the degradation of SREBP1a. However, we have clearly demonstrated that sumoylation of SREBP1c modulates SREBP1c protein stability via ubiquitin-dependent proteolysis. Here, we identified that Lys98 is a target residue for sumoylation. However, we cannot exclude the possibility that Lys98 of SREBP1c would be a target for ubiquitination as well. Although it remains to be elucidated which ubiquitin E3 ligase(s) is involved in the SUMO-mediated ubiquitination of SREBP1c, our results strongly support the idea that SREBP1c sumoylation can induce the degradation of SREBP1c in a proteasome-dependent manner.

Cross talk between various posttranslational modifications may represent a concerted and coordinated regulatory circuit to fine-tune the physiological functions of proteins. Since SREBP1c is modified by various posttranslational modifications, including phosphorylation, acetylation, sumoylation, and ubiquitination, interplay among these modifications could precisely control lipogenesis under specific nutritional conditions. It has been reported that SREBPs are acetylated by p300 and CBP, thereby increasing protein stability (48). Moreover, recent studies have shown that SIRT1 deacetylates the SREBP protein, causing it to become unstable, during fasting (49, 50). Although it is yet unknown whether sumoylation and acetylation might work together to fine-tune SREBP1c activity in the presence of different signaling cues, we cannot rule out the possibility that these two posttranslational modifications collaborate with each other to modulate SREBP1c activity to accommodate various nutritional and hormonal changes, which should be clarified in future work.

Here, we provide another regulatory layer for SREBP1c by revealing that SREBP1c sumoylation might repress hepatic lipogenic pathways. During nutritional deprivation, activated PKA

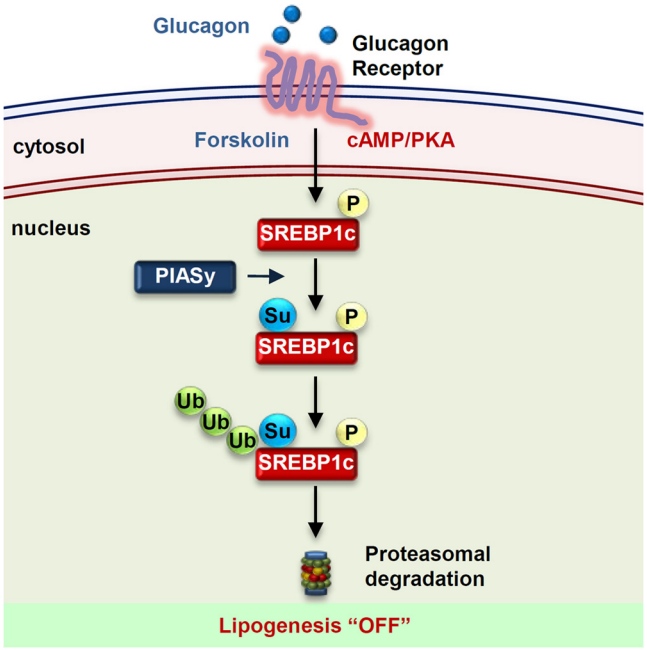

FIG 9 Proposed model of SREBP1c sumoylation in hepatic lipid metabolism. Under fasting conditions, glucagon activates adenylyl cyclase; thereby, increased cyclic AMP (cAMP) activates PKA. PKA phosphorylates Ser308 of SREBP1c, which promotes sumoylation of SREBP1c by PIASy. Sumoylated SREBP1c is readily modified by ubiquitin, leading to decrease hepatic lipogenic activity. Su, SUMO; P, phosphorylation.

promotes the phosphorylation, sumoylation, and ubiquitin-dependent degradation of SREBP1c, subsequently leading to repression of lipogenic activity to prevent unwanted lipid synthesis (Fig. 9). Our study is the first report to demonstrate the role of PIASy in SREBP1c sumoylation and the importance of its regulatory cascade in the regulation of hepatic lipid metabolism under catabolic conditions. Collectively, these data pave the way toward understanding the molecular mechanisms that link metabolic signaling processes with sequential modifications of SREBP1c, resulting in the fine-tuning of energy homeostasis.

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