Sumoylated SnoN Represses Transcription in a Promoter-specific Manner*

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The transcriptional modulator SnoN controls a diverse set of biological processes, including cell proliferation and differentiation. The mechanisms by which SnoN regulates these processes remain incompletely understood. Recent studies have shown that SnoN exerts positive or negative regulatory effects on transcription. Because post-translational modification of proteins by small ubiquitin-like modifier (SUMO) represents an important mechanism in the control of the activity of transcriptional regulators, we asked if this modification regulates SnoN function. Here, we show that SnoN is sumoylated. Our data demonstrate that the SUMO-conjugating E2 enzyme Ubc9 is critical for SnoN sumoylation and that the SUMO E3 ligase PIAS1 selectively interacts with and enhances the sumoylation of SnoN. We identify lysine residues 50 and 383 as the SUMO recognition sites in SnoN. Analyses of SUMO “loss-of-function” and “gain-of-function” SnoN mutants in transcriptional reporter assays reveal that sumoylation of SnoN contributes to the ability of SnoN to repress gene expression in a promoter-specific manner. Although this modification has little effect on SnoN repression of the plasminogen activator inhibitor-1 promoter and only modestly potentiates SnoN repression of the p21 promoter, SnoN sumoylation robustly augments the ability of SnoN to suppress transcription of the myogenesis master regulatory gene myogenin. In addition, we show that the SnoN SUMO E3 ligase, PIAS1, at its endogenous levels, suppresses myogenin transcription. Collectively, our findings suggest that SnoN is directly regulated by sumoylation leading to the enhancement of the ability of SnoN to repress transcription in a promoter-specific manner. Our study also points to a physiological role for SnoN sumoylation in the control of myogenin expression in differentiating muscle cells.

In recent years, the c-ski novel related (sno) gene products have garnered much interest in developmental and cancer biology. SnoN, a sno gene alternatively spliced product, is a protein of ~80 kDa that is expressed in diverse vertebrate species, including human, mouse, and chicken (1, 2). SnoN shares a 100-amino acid homology region at its N terminus with the related proteins c-Ski and Dach (3). The C-terminal region of SnoN has a coiled-coiled predicted structure that promotes SnoN-SnoN or SnoN-c-Ski dimerization (4, 5). SnoN localizes mainly in the nucleus where it interacts with different transcriptional factors and coregulators to control gene expression (1, 2).

SnoN regulates a diverse set of biological processes. SnoN is amplified in several tumors, including melanomas, breast, and esophageal carcinomas (6, 7). Consistent with the interpretation that amplified SnoN exerts oncogenic effects, overexpression of SnoN in fibroblasts induces malignant transformation and anchorage-independent growth (8, 9). Interestingly, disruption of the sno gene has revealed that SnoN expressed at its physiological levels may paradoxically exert tumor-suppressive properties in cells (10). SnoN+/− heterozygote mice have higher rates of spontaneous and carcinogen-induced tumors as compared with the wild-type controls (10). Consistent with these results, sno+/− mouse embryonic fibroblasts display increased rates of cell proliferation as compared with the wild-type control (10). These results support the idea that SnoN exerts distinct tumor-promoting or suppressor effects depending on the level of SnoN within the cells. At endogenous concentrations, SnoN may operate as a tumor suppressor, whereas overexpressed SnoN may stimulate neoplastic transformation. Whereas much of the emphasis on SnoN function has been on its role in cell proliferation and transformation, SnoN also has interesting functions in cell differentiation. In particular, SnoN appears to modulate the differentiation of skeletal muscle cells (2, 8, 11). In the nervous system, SnoN promotes the growth of axons in differentiated neurons (12). Thus, the emerging picture is that SnoN acts as a versatile transcriptional modulator that affects different biological responses. As a result, there is great interest in elucidating the mechanisms that regulate SnoN function. To date, these mechanisms remain incompletely understood.

Recent data have shown that SnoN may act via the transforming growth factor (TGF)4 β-Smad signaling pathways to regulate cellular responses (13–15). The receptor-regu-

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4 The abbreviations used are: TGF, transforming growth factor; R-Smad, receptor-regulated Smad; co-Smad, common partner Smad4; CREB, cAMP response element-binding protein; SUMO, small ubiquitin-like modifier; CMV, cytomegalovirus; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; FBS, fetal bovine serum; NEM, N-ethylmaleimide; HA, hemagglutinin; hpRNA, hairpin RNA.
lated Smad (R-Smad) subfamily and the common partner Smad4 (co-Smad4) play a major role in transducing the signal from the TGF-β cell surface receptors to the nucleus (16–18). In the nucleus, the ligand-phosphorylated-R-Smad in complex with co-Smad4 binds to Smad binding elements within promoters of TGF-β-responsive genes, and in collaboration with distinct transcriptional coregulators, promotes or blocks the transcription of these genes (19). For example, together with the transcriptional coactivators p300 and CREB-binding protein, the Smad complex induces gene expression (18, 20).

SnoN associates with the TGF-β-R-Smad2 and Smad3, as well as the co-Smad4 (13, 21). When overexpressed, SnoN antagonizes the ability of TGF-β to induce transcription and inhibit cell proliferation supporting the idea that SnoN is a proto-oncogene (13, 15, 21, 22). SnoN inhibition of TGF-β-dependent transcription is thought to occur at least in part via SnoN recruitment of a histone deacetylase complex to promoters of TGF-β-responsive genes (15, 23). However, we have recently found that, at physiological concentrations, SnoN mediates TGF-β-induced transcription and inhibition of cell proliferation in specific cells (24). These findings suggest that, under certain circumstances, SnoN activates rather than represses TGF-β-induced transcription. Interestingly, a number of transcriptional modulators, including MEF2A and the Smad interacting protein SIP1, can activate or repress transcription depending on the post-translational modification status of the protein. Sumoylation has emerged as a key modification in regulating transcription factors such as MEF2A, SIP1, and Sp3 that have both transcriptional activator and repressive functions (25–29). In view of these observations, we reasoned that SnoN might also be regulated by protein sumoylation.

Sumoylation of substrates occurs by the covalent attachment of the carboxyl group of the C-terminal glycine residue of SUMO to a lysine ε-amino group of the substrate (30). Sumoylation of substrates is catalyzed by the SUMO-activating E1 enzyme Aos1/Uba2, the SUMO-conjugating E2 enzyme Ubc9, and a SUMO E3 ligase (30). SUMO E3 ligases, which catalyze the addition of SUMO to target proteins, are divided into four categories: the conjugating E3s Aos1, Uba2, the SUMO-conjugating E2 enzyme Ubc9, and a SUMO E3 ligase (30).

In this study, we show that SnoN is covalently modified by SUMO. Sumoylation of SnoN occurs at the distinct sites of lysines 50 and 383. The sumoylation of SnoN is stimulated by the SUMO-conjugating E2 enzyme Ubc9. In addition, we demonstrate that the SUMO E3 ligase PIAS1 interacts with and promotes SnoN sumoylation. The sumoylation of SnoN potentiates significantly the ability of SnoN to repress transcription in a promoter-specific manner. In particular, we find that sumoylation of SnoN enhances the ability of SnoN to regulate the promoter activity of a muscle-differentiating gene. Our data suggest that sumoylation may have relevance for SnoN function in cell differentiation. Together, these results suggest that sumoylation of SnoN represents a mechanism contributing to the functional diversity of SnoN.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Mammalian expression plasmids (pCMV5B) containing cDNA encoding FLAG-tagged SnoN and Myc-tagged SnoN proteins have been described previously (15). The FLAG-tagged PIAS1, PIAS3, PIASα, and PIASγ plasmids were generous gifts from Dr. Ke Shuai (University of California). CMV-based plasmids containing cDNA encoding mouse PIAS1 SUMO ligase mutant PIAS1 (C350S) protein, human SnoN sumoylation mutant proteins, SnoN (K50R), SnoN (K383R), and SnoN (K50/383R), were constructed using the PCR-based site-directed mutagenesis method. To express a SUMO-SnoN fusion protein that is resistant to cleavage by the C-terminal SUMO hydrolases, SUMO1 lacking the C-terminal diglycine motif was amplified by PCR and cloned upstream of SnoN (25). The PIAS1 RNA interference vector pU6/pia1-EGFP encoding PIAS1 hairpin RNAs (PIAS1 hRNA) and enhanced green fluorescent protein (EGFP) under the control of the U6 and CMV promoters, respectively, was generated using the pU6/EGFP vector by a previously described strategy (24). The PIAS1 RNA interference vector was designed to specifically target the 21-nucleotide region “CTATGCCTATGACTTACAAG” in PIAS1 mRNA. This region displayed no significant homology to any other gene by BLAST search. The 3TP-lux luciferase reporter construct was described previously (31). Luciferase reporter constructs for p21 promoter (p21-lux) (32) and myogenin promoter (myogenin-p-lux) (33) were generously provided by Dr. Phyllis LuValle (University of Florida) and Dr. Michael Underhill (University of Western Ontario, Canada), respectively. All constructs were confirmed by restriction digests and/or DNA sequence analyses (University of Calgary Core Sequencing Facility).

**Cell Lines and Transfections**—Human kidney epithelial 293T cells were cultured in Dulbecco’s modified Eagle’s medium with high glucose and l-glutamine (Invitrogen) containing 10% fetal bovine serum (FBS, Invitrogen). The human hepatoma HepG2 and the mink lung epithelial Mv1Lu cell lines were cultured in minimum essential medium (Invitrogen) containing 1% nonessential amino acids (Invitrogen) and 10% FBS. The mouse myoblast C2C12 cells were maintained in Dulbecco’s modified Eagle’s medium containing high glucose, l-glutamine, and sodium pyruvate (Invitrogen) supplemented with 10% FBS (growth medium). The 293T and HepG2 cells were transfected using the calcium phosphate method. The C2C12 and Mv1Lu cells were transfected using FuGENE 6 according to the manufacturer’s instructions (Roche Applied Science).

**Immunoprecipitation and Immunoblotting**—Transfected cells were lysed using an established procedure (24, 34, 35). Briefly, cells were lysed in TNTE buffer containing 0.5% Triton X-100 plus protease and phosphatase inhibitors as described previously (24). In addition, 20 mM N-ethylmaleimide (NEM) isopeptidase inhibitor or an equivalent volume of the vehicle where indicated was included in the lysis buffer. Cell lysates were centrifuged at 15,000 × g for 10 min at 4 °C. 10% of each supernatant was removed for protein determination and protein expression analyses. The remainder of the supernatant was subjected to mouse anti-FLAG (M2, Sigma) or rabbit anti-SnoN (H-317, Santa Cruz Biotechnology, Santa Cruz, CA) immunoprecipitations as described previously (36). In the case of double anti-FLAG immunoprecipitation, 90% of the immunocomplexes from the first immunoprecipitations were eluted in 1% SDS to denature proteins complexes. The eluates were diluted in lysis buffer to reduce the SDS concentration to 0.1% and subjected to a second anti-FLAG immunoprecipitation.
Sumoylation of SnoN Regulates Its Function

The protein content of each cell lysate or immunoprecipitation sample was resolved by SDS-PAGE, and visualized by Western blotting coupled to enhanced chemiluminescence (ECL, Amersham Biosciences) using mouse anti-HA (16B2, Covance), mouse anti-FLAG, rabbit anti-SnoN, mouse anti-myogenin (F5D, Developmental Studies Hybridoma Bank at the University of Iowa), rabbit anti-Actin (Sigma), or mouse anti-GFP antibody (B34, Covance) as primary antibodies, and horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (Amersham Biosciences) as the secondary antibodies as described previously (24). A VersaDoc 5000 Imager (Bio-Rad) was used to detect and quantify, respectively, the ECL signals.

Luciferase Reporter Assays—Cells were seeded in 24-well plates at a density of $2 \times 10^4$ cells per well and 1 day later were cotransfected with the luciferase reporter constructs 3TP-lux, p21-lux, or myogenin-p-lux (0.1 µg/well), CMV-β-galactosidase vector (0.05 µg/well), and various SnoN, PIAS1, or PIAS1 RNA interference constructs as specified in the figure legends. In experiments involving the 3TP-lux and p21-lux reporters, 1 day post-transfection, cells were incubated in 0.2% FBS-containing medium in the presence or absence of 100 pm TGF-β (R&D Systems, Minneapolis, MN) for 16–18 h at 37 °C before lysing. For the myogenin-p-lux reporter study, C2C12 cells were maintained in 10% FBS containing Dulbecco’s modified Eagle’s medium until 40 h post-transfection at which time myogenin was initiated by incubating cells in 2% horse serum containing Dulbecco’s modified Eagle’s medium (differentiation medium) for 2–4 days before lysing. The cell lysates were assayed for luciferase activity as described previously (24). Arbitrary luciferase activity, expressed in relative light units, was normalized to β-galactosidase activity to control for variations in transfection efficiency. Each experimental condition was carried out in triplicates, and the data are presented as mean ± S.E.). Experiments were repeated on independent batches of cells at least three times.

RESULTS

SnoN Is Modified by Sumoylation—SnoN has positive and negative transcriptional regulatory functions. However, the mechanisms by which SnoN produces these distinct transcriptional activities remain to be identified. Because sumoylation can influence the function of transcription factors, we considered the possibility that this modification may represent one mechanism that regulates the transcriptional function of SnoN. To test this idea, we first asked if SnoN can be conjugated to SUMO in cells. We expressed FLAG-tagged SnoN alone or together with HA-tagged SUMO in human hepatoma HepG2 cells. Cell lysates prepared in the absence or presence of the general SUMO-protease inhibitor NEM were immunoprecipitated with FLAG antibodies followed by immunoblotting with antibodies to the HA tag. SUMO coexpression with SnoN in cells resulted in the appearance of NEM-sensitive slower mobility protein species in the SUMO blots of the SnoN immunoprecipitation (Fig. 1A, compare lanes 3 to 7). The SUMO immunoreactive protein bands were absent in the samples from cells expressing SnoN or SUMO alone (Fig. 1A, lanes 2, 4, 6, and 8). Two reproducible NEM-sensitive SUMO-conjugated protein species displayed apparent molecular masses of 100 and 120 kDa (SnoN is 80 kDa) (Fig. 1A, lane 7). Because SUMO-conjugated species have apparent molecular masses that exceed the unmodified substrate by ~20 kDa or greater, our data suggest that the SUMO-conjugated protein bands in the SnoN immunocomplexes represent SUMO-conjugated forms of expressed SnoN (30, 37–39). Consistent with this conclusion, the slower mobility protein species were detected when SnoN immunocomplexes were immunoblotted with the SnoN antibody (bottom panel of Fig. 1A). Our quantitative analysis indicated that on average the SUMO-modified SnoN represents ~17% of the unmodified SnoN in HepG2 cells. This finding is consistent with the idea that only a small percentage of a given protein is sumoylated at steady state (40). This low stoichiometry is thought to reflect two possibilities that are mutually not exclu-
gating E2 enzyme termed Ubc9. The activated SUMO is then transferred from Ubc9 to lysine residues of specific substrates, a reaction that is catalyzed by a SUMO E3 ligase. A SUMO E3 ligase promotes sumoylation of distinct substrates through the SUMO ligase complexing with a specific substrate and Ubc9.

To demonstrate that the SUMO pathway controls SnoN sumoylation, we first asked if the SUMO E2 enzyme Ubc9 affects SnoN sumoylation in cells. We assessed SnoN sumoylation in NEM-treated lysates of 293T cells transiently coexpressing SUMO, SnoN, and a wild-type or a catalytically inactive mutant Ubc9 protein in which cysteine 93 is mutated into serine (42, 43). Expression of wild-type Ubc9 protein in cells coexpressing SnoN produced a robust increase in the total amount of SUMO-conjugated SnoN protein bands in SnoN immunocomplexes (Fig. 2A, panel i, compare lanes 2 and 4). In contrast, expression of the catalytically inactive Ubc9 did not enhance but rather decreased SnoN sumoylation detected in the SnoN immunocomplexes (Fig. 2A, panel i, compare lanes 2, 4, and 5, and see Fig. 2A, panel ii) suggesting that this mutant of Ubc9 acts a dominant negative to inhibit SnoN sumoylation by the endogenous Ubc9. Together these data demonstrate that the SUMO E2 enzyme Ubc9 is critical in the sumoylation of SnoN.

Next we asked if SnoN modification by SUMO is modulated by SUMO E3 ligases, because these enzymes are believed to play a role in specifying substrates for sumoylation. We first asked if known SUMO E3 ligases interact with SnoN. Members of the PIAS family of SUMO E3 ligases associate with and promote the sumoylation of a number of transcriptional regulators (30, 44). We thus investigated whether SnoN interacts with one or more members of the PIAS family of SUMO E3 ligases. We carried out coimmunoprecipitation studies in 293T cells expressing SnoN alone or together with PIAS1, PIAS3, PIASy, or PIASxα. We found that SnoN robustly forms a physical complex with PIAS1. In contrast, SnoN interacted weakly with other PIAS family members (Fig. 2B, panel i, compare lane 4 to lanes 5 and 6, and data not shown). Quantitative analysis of PIAS-associated SnoN further confirmed that SnoN interacts more efficiently with PIAS1 compared with other members of PIAS family of SUMO E3 ligases (Fig. 2B, panel ii, and data not shown). These data suggest that SnoN associates selectively with PIAS1.

Our finding that SnoN interacts with the PIAS1 protein raised the possibility that SnoN sumoylation may be regulated by this SUMO ligase. We examined the effect of PIAS1 expression on SnoN sumoylation in 293T cells. These experiments revealed that expression of PIAS1 together with SnoN enhances the sumoylation of SnoN (Fig. 2C, panel i). The PIAS1-induced increase in SnoN sumoylation was confirmed by quantifying the density of total SUMO-conjugated bands relative to unmodified SnoN in the SnoN immunoprecipitates (Fig. 2C, panel ii). These data show that PIAS1 enhances SnoN sumoylation by more than 50%, indicating that PIAS1 acts as a SUMO ligase that regulates SnoN sumoylation. Taken together, our results suggest that PIAS1 represents a SUMO E3 ligase that stimulates the SUMO modification of SnoN.

Lysine Residues 50 and 383 Are Sites of Sumoylation on SnoN—Following the characterization of SnoN as a substrate for the SUMO pathway, we next sought to identify sites of
sumoylation on SnoN. SUMO is conjugated to proteins via an isopeptide bond between the carboxyl group of the SUMO C-terminal glycine residue and the ε-amino group of a lysine residue in the substrate. Conjugation of the SUMO moiety to proteins occurs on the substrate’s lysine residues found within a ψ/KX(E/D) consensus motif, where ψ is a large hydrophobic residue and X is any amino acid (30).

By sequence gazing, we found that SnoN contains two SUMO consensus motifs that are conserved in different species, including human, mouse, and chicken SnoN (Fig. 3A). To test if one or both lysine residues corresponding to residues 50 and 383 of human SnoN represent sites of sumoylation, we constructed expression vectors encoding human SnoN with either lysine 50 (K50R), 383(K383R), or both residues (KdR) replaced with arginine (Fig. 3B). We analyzed the effects of these mutations on SnoN sumoylation status in 293T cells (Fig. 3C). These experiments revealed that mutation of lysine 50 to arginine in SnoN abrogated the 100-kDa molecular mass SUMO-conjugated SnoN band suggesting this protein represents lysine 50-monosumoylated SnoN (Fig. 3C, lane 2). By contrast, the density of the 120-kDa SUMO-conjugated SnoN protein species decreased drastically upon mutation of lysine 383 indicating that lysine 383-sumoylated SnoN represents the second SUMO-conjugated protein (Fig. 3C, lane 3). Consistent with these results, mutation of both lysines 50 and 383 led to complete disappearance of both SUMO-conjugated protein bands in SnoN immunoprecipitates (Fig. 3C, lane 4). SUMO1, which we used in the current study, lacks a SUMO consensus motif. Therefore, SUMO1 is not believed to efficiently form polySUMO1 chains (30, 38, 45, 46). This suggests that lysine 383-sumoylated SnoN may represent a mono-sumoylated SnoN species.

The slower mobility of lysine 383-sumoylated SnoN on SDS-PAGE as compared with that of lysine 50-sumoylated SnoN might be explained by the differences in relative positioning of lysine 50 and 383 relative to the full-length SnoN protein. Lysine 383 is situated approximately in the middle of the SnoN protein, a 684-amino acid protein. Previous studies have shown that conjugation of SUMO molecule to a

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**FIGURE 2. Regulation of SnoN sumoylation by the SUMO pathway.** A, panels i and ii: the SUMO-conjugating enzyme Ubc9 is critical for SnoN sumoylation; panel i, SnoN sumoylation was determined in 293T cells transfected with pcMV5B plasmids encoding FLAG-tagged SnoN, HA-tagged SUMO, wild-type, or cysteine 93 to alanine (C93S) mutant HA-tagged Ubc9 (HA/Ubc9) as described in Fig. 1A. Expression of FLAG/SnoN and HA/Ubc9 constructs in cell lysates were confirmed by immunoblotting with anti-FLAG and anti-HA antibodies, respectively. A, panel ii: the bar graph shows the density of total sumoylated SnoN normalized to the amount of unmodified SnoN in the SnoN-immunoprecipitation (α-Flag IP). B, panels i and ii: SnoN interacts selectively with the SUMO ligase PIAS1; panel i, 293T were transfected with pcMV5B encoding Myc-tagged SnoN (Myc/SnoN), alone or together with FLAG-tagged PIAS1, -3, or -γ. NEM-containing lysates were subjected to anti-FLAG immunoprecipitation (α-Flag IP), and the PIAS-interacting SnoN protein was determined by anti-SnoN immunoblotting. Expression of SnoN and PIAS proteins were confirmed by immunoblotting the cell lysates with SnoN and FLAG antibodies, respectively. Blotting for actin (α-actin) in cell lysates was used as a loading control. Panel ii, the bar graph shows the density of PIAS-interacting SnoN normalized to the amount of PIAS protein in the FLAG-immunoprecipitation and Myc/SnoN expression in the respective transfection. In C, Panels i and ii, PIAS1 increases SnoN sumoylation; Panel i, 293T cells were transfected with a pcMV5B plasmid containing Myc-tagged SnoN alone or together with a plasmid expressing FLAG-tagged PIAS1. SUMO-conjugated SnoN was determined by SnoN immunoblotting of SnoN-immunoprecipitation from NEM-containing lysates (upper panel). Expression of PIAS1, SnoN, and actin proteins in cell lysates were confirmed as described in B, panel i. C, panel ii: the bar graph depicts the density of the two SUMO-conjugated SnoN protein bands normalized to the level of unmodified SnoN protein in the SnoN immunoprecipitation in the presence and absence of FLAG/PIAS1. We found that in three independent experiments PIAS1 reproducibly led to an increase in SnoN sumoylation. The blots shown in A–C are from a representative experiment that was repeated at least two or three times.

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lysin residue situated in the center of a protein may produce a branching effect leading to a larger apparent molecular weight of the modified protein than expected when analyzed by SDS-PAGE (46–48). In contrast, lysine 50 is closer to the N terminus of the protein, thus explaining why this sumoylated-SnoN species behaves on SDS-PAGE more like a linear SUMO-SnoN species. Interestingly, SnoN immunoblotting of the SnoN immunocomplexes revealed a similar profile of SUMO-conjugated SnoN whether the wild-type, K50R, K383R, or the KdR mutant SnoN protein was expressed alone or together with HA-SUMO1 (Fig. 3, lanes 1–4 versus lanes 5–8). These data suggest that lysines 50 and 383 in SnoN are also sites of conjugation by endogenous SUMO. In summary, our data indicate that SnoN is sumoylated and that lysines 50 and 383 are required for SnoN sumoylation.

**Role of Sumoylation on SnoN Repression of TGF-β-stimulated Transcription**—Having demonstrated that SnoN is sumoylated on lysines 50 and 383, we next asked if modification of SnoN by SUMO alters the transcriptional function of SnoN. SnoN is an important regulator of TGF-β-mediated transcriptional responses. When overexpressed, SnoN inhibits TGF-β-dependent transcriptional and biological responses (1, 2). SnoN inhibition of TGF-β-dependent biological responses contributes to the role of SnoN as an oncogene (22). In view of these observations, we investigated the role of sumoylation in the ability of overexpressed SnoN to inhibit the TGF-β signaling pathway. We first determined the effect of sumoylation on the ability of overexpressed SnoN to repress TGF-β-induced expression of the 3TP-lux reporter gene, which contains TGF-β-responsive elements from the promoter of the plasminogen activator inhibitor-1 gene driving the expression of the firefly luciferase gene (31). We transfected HepG2 or Mv1Lu cells with the 3TP-lux reporter gene and a β-galactosidase expression plasmid, the latter to serve as an internal control for transfection efficiency, together with an expression plasmid encoding wild-type SnoN or the sumoylation-deficient mutant SnoN (KdR). Expression of wild-type SnoN decreased the basal activity and the TGF-β-induced 3TP-lux reporter activity in both HepG2 (Fig. 4A) and Mv1Lu cells (data not shown). Loss of SUMO mutation in SnoN led to only 15% reduction in SnoN-dependent repression of TGF-β-induced 3TP-lux activity in both HepG2 and Mv1Lu cells (Fig. 4A and data not shown). In addition to using a “loss-of-function” approach of SnoN sumoylation, we used a “gain-of-function” strategy to test if sumoylated SnoN is more potent in repressing TGF-β-dependent 3TP-lux activity in these cells. To do this, we expressed a SnoN protein that is fused to a non-cleavable SUMO moiety in cis. This approach has been successfully used to characterize the function of sumoylation of transcriptional regulators (25, 28, 49). Surprisingly, we found that SnoN fused to SUMO (SUMO-SnoN) only slightly increased the ability of SnoN to repress TGF-β-dependent 3TP-lux reporter gene expression (Fig. 4B). Together these data suggest that SnoN sumoylation has very little or no impact on the ability of SnoN to inhibit TGF-β-induction of the plasminogen activator inhibitor-1 promoter.

We next tested the consequence of sumoylation of SnoN on a TGF-β-responsive promoter distinct from the plasminogen activator inhibitor-1 promoter. The p21 cyclin-dependent kinase inhibitor gene is a TGF-β-responsive gene that is important in TGF-β-inhibition of cell proliferation (50). We used a p21-lux reporter in which the luciferase gene is controlled by a 2.3-kb fragment of the p21 promoter containing TGF-β-responsive elements (33). We compared the effect of wild-type SnoN, “SUMO loss-of-function” mutant SnoN (KdR) and the “SUMO gain-of-function” SUMO-SnoN fusion protein on the response of the p21 promoter to TGF-β stimulation in HepG2 cells (Fig. 4C). Overexpression of wild-type SnoN inhibited the basal and TGF-β-induced p21-promoter-driven luciferase activity (Fig. 4C). The SUMO loss-of-

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**FIGURE 3. Lysine residues 50 and 383 are sites of sumoylation in SnoN.** A, sequence alignment of the human, mouse, and chicken SnoN protein showing the regions that contain the two conserved SUMO-consensus motifs. B, a schematic representation of the human SnoN protein with the lysine to arginine SnoN mutants generated in this study to determine potential sumoylation sites on SnoN. C, identification of lysine sites of sumoylation in SnoN. Lysates of 293T cells coexpressing HA-tagged SUMO and FLAG-tagged wild-type SnoN (WT), or SnoN in which lysine 50 (K50R), lysine 383 (K383R), or both lysines (KdR) were mutated into arginine were subjected to anti-FLAG immunoprecipitation (α-FLAG IP), followed by anti-HA (upper panel) or anti-SnoN (lower panel) immunoblotting. The results shown in C are from a representative experiment that was repeated three times.
Sumoylation of SnoN Regulates Its Function

**FIGURE 4. Role of SnoN sumoylation on SnoN repression of TGF-β-induced transcription.** SnoN sumoylation has a small or no effect on the ability of SnoN to repress TGF-β induction of the 3TP-lux reporter activity (A and B). A, lysates of HepG2 cells, transiently transfected with the 3TP-lux reporter and β-galactosidase constructs together with two concentrations of pCMV5B plasmids encoding a wild-type SnoN (SnoN (WT)) or a SUMO loss-of-function SnoN mutant where lysines 50 and 383 were changed into arginine residues (SnoN (KdR)), and either left untreated or treated for 20 h with 100 pM TGF-β, were subjected to luciferase and β-galactosidase assays (see “Experimental Procedures”). The basal (- TGF-β) and TGF-β-induced (+ TGF-β) luciferase values are normalized to the β-galactosidase values (relative light units), and the normalized data are expressed relative to the normalized luciferase data in lysates of control cells grown in the absence of TGF-β. B, effect of SUMO gain-of-function SnoN protein on basal and TGF-β-dependent 3TP-lux activity. HepG2 cells were transfected with the 3TP-lux reporter and β-galactosidase constructs together with two concentrations of pCMV5B plasmids encoding a wild-type SnoN (SnoN (WT)) or a SUMO gain-of-function SUMO-SnoN fusion protein (SUMO-SnoN) and processed as described in A. C, SnoN sumoylation modestly enhances SnoN repression of the TGF-β-responsive p21 promoter. HepG2 cells were transfected with the p21-p-lux reporter and β-galactosidase constructs together with a mammalian expression plasmid encoding wild-type SnoN (SnoN (WT)), a SUMO loss-of-function SnoN (KdR) mutant (SnoN (KdR)), or the SUMO gain-of-function SUMO-SnoN fusion (SUMO-SnoN), and processed as described in A. D, lysates of HepG2 cells transfected with a mammalian expression vector encoding wild-type SnoN (WT), SUMO loss-of-function SnoN mutant (KdR), the SUMO gain-of-function SUMO-SnoN fusion protein (SUMO), or the control vector (–) were immunoblotted with anti-SnoN and anti-actin antibodies to confirm protein expression. The upper two protein bands in the SUMO-SnoN fusion lane (lane 4) are full-length SUMO-SnoN, and the lower two bands represent degradation products. The results shown in A–C represent the mean (+ S.E.) of a triplicate from a representative experiment that was independently repeated at least three times.

function SnoN mutant was only modestly less effective than wild-type SnoN in repressing the p21 promoter gene. Conversely, the SUMO gain-of-function SUMO-SnoN fusion protein repressed TGF-β-dependent p21 promoter activity more effectively than wild-type SnoN (Fig. 4C). In control experiments, we found that wild-type SnoN, SnoN (KdR), and SUMO-SnoN were expressed at equivalent levels (Fig. 4D, compare lanes 2–4). These data suggest that sumoylation may potentiate the ability of overexpressed SnoN to suppress TGF-β-dependent p21 transcription. Together, these results raise the possibility that SnoN sumoylation enhances SnoN repression of transcription in a promoter-specific manner.

**SnoN Sumoylation Controls Differentiation-induced Myogenin Promoter Activity—**The finding that sumoylation of SnoN may contribute to SnoN-dependent transcriptional repression in a promoter-specific manner led us to consider how SnoN sumoylation might contribute to SnoN function. Beyond the control of cell proliferation, SnoN has been implicated in muscle differentiation (2, 8). We investigated the role of SnoN sumoylation in SnoN function in muscle differentiation. We used mouse C2C12 myoblast cells in these studies as this cell line serves as a well characterized model for skeletal muscle differentiation. When grown in low serum containing medium, C2C12 cells express muscle markers and regulators, including the transcription factor myogenin and adopt a skeletal muscle phenotype (51–53). We employed a luciferase reporter construct driven by a 1.14-kb promoter of the mouse myogenin gene (myogenin-p-lux), a myogenesis master regulatory gene, as a marker for muscle differentiation (32). We first designed studies to determine the role of increasing SnoN expression on the luciferase activity of the myogenin-p-lux reporter construct. Luciferase and β-galactosidase assays were carried out on lysates of cells that were transiently transfected with the reporter and the SnoN expression constructs and either left in regular growth medium or switched to muscle differentiating medium (see “Experimental Procedures” for details). Myogenin-
dependent transcription increases by several folds when cells are induced to undergo muscle differentiation (Fig. 5A). At lower concentrations, SnoN produced little change in the myogenin-promoter driven luciferase activity. However at higher doses, SnoN appeared to inhibit differentiation-induced myogenin promoter activity by 75%. These data show that SnoN inhibits muscle-dependent activation of the myogenin promoter in a dose-dependent manner.

Next, we asked if SnoN sumoylation plays a role in the ability of SnoN to inhibit the myogenin promoter activity. We investigated the effects of the SUMO loss-of-function and SUMO gain-of-function SnoN mutants on the myogenin-p-lux transcriptional activity in undifferentiated or muscle-differentiated C2C12 cells. In these experiments, we selected a concentration of wild-type SnoN producing sub maximal inhibition of the myogenin promoter (see Fig. 5A). In contrast to wild-type SnoN, which inhibited the myogenin promoter, the sumoylation-deficient SnoN (KdR) mutant did not significantly reduce the activity of the myogenin promoter despite equivalent level of protein expression (Fig. 5C, compare lanes 2 and 3), suggesting that sumoylation of SnoN at lysines 50 and 383 might contribute to SnoN-dependent inhibition of the myogenin promoter activity (Fig. 5B). Consistent with this interpretation, the SUMO gain-of-function SUMO-SnoN protein robustly inhibited the luciferase activity as compared with the wild-type SnoN control (Fig. 5B), although this mutant seems to express at similar concentrations compared with the wild-type SnoN (Fig. 5C, lane 4 versus 2). Therefore, these data suggest that sumoylation of SnoN enhances the ability of SnoN to repress the myogenin promoter. In separate experiments, just as in HepG2 and Mv1Lu cells SnoN, sumoylation had little or no effect on the ability of SnoN to repress TGF-β-induced PIAS1 promoter activity in C2C12 cells (data not shown). These data suggest that the differential effects of SnoN sumoylation on repression of distinct promoters are unlikely to be explained by cell-type differences. Taken together, the results of the transcriptional assays support the idea that sumoylation may provide a mechanism that regulates SnoN repression of specific promoters.

We next determined the impact of SnoN sumoylation on the expression of endogenous myogenin. We tested the effect of expression of wild-type SnoN (WT), SUMO loss-of-function SnoN (KdR), or SUMO gain-of-function SUMO-SnoN fusion protein on endogenous myogenin expression in C2C12 cells under growth or muscle differentiating conditions (Fig. 6). Immunoblotting analyses revealed that both wild-type SnoN and SUMO-SnoN blocked the ability of differentiating medium to induce the expression of endogenous myogenin (Fig. 6, compare lanes 3 and 5 to 2). In contrast, the SUMO loss-of-function SnoN (KdR, lane 4) failed to significantly alter myogenin expression. Together, these results suggest sumoylation of SnoN contributes to the ability of SnoN to suppress myogenin gene expression. Because myogenin is a key player in muscle differentiation, our data point to a regulatory role for SnoN sumoylation in myogenesis.

PIAS1 Suppresses Myogenin Promoter Transcription—Next, we investigated the role of the SnoN E3 SUMO ligase PIAS1, at its endogenous levels, on myogenin transcription. We used a DNA template-based method of RNA interference to acutely

![FIGURE 5. Sumoylation of SnoN promotes SnoN transcriptional repression of the muscle-specific myogenin promoter activity.](image)
knockdown PIAS1 in cells. Expression of PIAS1 hairpin RNAs (hpRNAs) significantly reduced the expression of PIAS1 protein in 293T cells (Fig. 7A, panel i). We found PIAS1 knockdown decreased SnoN sumoylation thus further supporting the idea that PIAS1 is a SnoN SUMO ligase (Fig. 7A, panel ii). Expression of PIAS1 hpRNAs in C2C12 cells significantly increased the activity of the myogenin promoter (Fig. 7B). These data suggest that PIAS1 knockdown derepresses myogenin promoter transcription in differentiating C2C12 cells.

In addition to the RNA interference approach, we also determined the consequence of disrupting endogenous PIAS1 activity by a dominant negative method. In these studies, we tested the effect of a PIAS1 SUMO ligase mutant in which cysteine 350 was converted to serine (PIAS1(CS)) on myogenin promoter transcription (see “Experimental Procedures”). PIAS1(CS) does not bind Ubc9 but still associates with substrates leading to inhibition of substrates sumoylation by endogenous PIAS1 protein (54). We found that PIAS1(CS), when compared with control vector or wild-type PIAS1, enhanced myogenin promoter-mediated transcription in differentiating C2C12 cells (Fig. 7C). These results suggest that endogenous PIAS1 represses the myogenin promoter. Collectively, based on two distinct approaches, we conclude that endogenous PIAS1 suppresses myogenin transcription in differentiating muscle cells. Taken together, our data suggest that PIAS1 stimulates the sumoylation of SnoN and thereby inhibits the expression of myogenin in differentiating muscle cells.

**DISCUSSION**

In this study, we identify sumoylation as a critical mechanism that regulates the function of the transcriptional modulator SnoN. We find that SnoN undergoes the covalent modification of sumoylation in several distinct cell types. The SUMO E3 ligase PIAS1 appears to play a key role in the regulation of SnoN sumoylation, because among the PIAS proteins, PIAS1 selectively interacts with SnoN and thereby enhances the conjugation of SnoN with SUMO. We identify lysines 50 and 383 as the sites of SnoN modification by SUMO. Sumoylation enhances the ability of SnoN to repress transcription in a promoter-specific fashion. Our data suggest that PIAS1 promotes the sumoylation of SnoN and thereby represses the expression of myogenin in differentiating muscle cells. The selective effect of SnoN sumoylation on SnoN-dependent repression of a muscle-spe-
cific master regulatory gene suggests this modification may control SnoN function in cell differentiation. Our findings support the view that sumoylation may provide a basis for SnoN to regulate different functions in diverse cell types.

In addition to modification by SUMO, SnoN is ubiquitinated by two distinct ubiquitin ligase pathways (15, 36, 55). Sumoylation and ubiquitination of a number of transcriptional regulators, including IkappaBalpha and PCNA, oppose each other as these modifications compete for the same lysine residues (56, 57). In such cases, sumoylation decreases the ubiquitin-dependent rate of turnover of these substrates. In the case of SnoN, sumoylation occurs on lysine residues 50 and 383, whereas ubiquitination occurs on lysine residues 440, 446, and 449, suggesting that sumoylation and ubiquitination regulate SnoN function independently of each other. Consistent with this interpretation, we observed no significant differences in the ubiquitination status or half-life of wild-type and the sumoylation-deficient SnoN mutant protein (data not shown).

Growing evidence indicates that the majority of SUMO substrates are transcriptional factors and coregulators that activate or repress gene expression. Sumoylation of some transcriptional factors, including the beta-catenin-activated transcription factor TCF-4, and the heat shock factors HSF1 and HSF2, appears to increase the transcriptional activity of target substrates (58–62). However, in most cases, SUMO modification favors transcriptional repression by the substrates (63, 64). In this study, we found that sumoylation of SnoN potentiates the ability of SnoN to repress transcription. Surprisingly, sumoylation enhances significantly the ability of SnoN to repress myogenin promoter-driven reporter gene activity. In contrast, SnoN sumoylation has a very modest effect on TGF-beta-responsive p21-promoter activity and has little or no effect on the plasminogen activator inhibitor-1 promoter. These data support the idea that SnoN sumoylation plays a role in the ability of SnoN to repress transcription in a promoter-specific manner. This conclusion is supported by several recent studies focusing on the consequence of sumoylation on the function of different transcriptional regulators (27, 46, 65). For example, sumoylation was found to potentiate the ability of the basic Kruppel-like factor BKLF to repress a glucocorticoid-responsive element, whereas this modification did not affect BKLF-dependent inhibition of the A gamma-globin promoter activity (46). Similarly, sumoylation of the transcriptional factor SIP1 was found to relieve SIP1-mediated repression of the E-cadherin promoter activity while having no effect on the ability of SIP1 to repress TGF-beta-responsive elements or activate vitamin D3-responsive elements driven reporter genes (27). It will be interesting in future studies to investigate the extent by which sumoylation contributes to the ability of SnoN to repress the activity of promoters of other SnoN-regulated genes.

Our finding that sumoylation can potentiates SnoN-dependent repression of the transcriptional activity of the myogenin promoter indicates that SUMO-conjugated SnoN may negatively regulate muscle differentiation. Under this scenario, expression of the SUMO loss-of-function SnoN mutant would be predicted to promote C2C12 cells to acquire the skeletal muscle phenotype. Conversely, expression of the SUMO gain-of-function SnoN mutant should block or reduce the muscle differentiation program in C2C12 cells. Therefore, it will be important to characterize the role of SnoN sumoylation in muscle differentiation.

The consequences of sumoylation on the function of transcriptional factors and coregulators occur via distinct mechanisms (30, 64). In some cases, sumoylation regulates the function of substrates via changes in substrate protein stability or their subcellular localization (30). Our results indicate that sumoylation of SnoN does not appreciably alter the stability or the subcellular distribution of SnoN (data not shown). Sumoylation may also directly regulate transcription, although the precise mechanism by which this occurs remains to be investigated (64). In summary, we have uncovered that the transcriptional modulator SnoN is regulated by sumoylation. This modification enhances the ability of SnoN to suppress transcription of specific genes. These findings suggest that sumoylation may contribute to the functional diversity of SnoN in different cell types.

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