Recent progress has been made on the role of oncoproteins c-Ski and related SnoN in the control of cellular transformation. c-Ski/SnoN potently repress transforming growth factor-β (TGF-β) antiproliferative signaling through physical interaction with signal transducers called Smads. Overexpression of c-Ski/SnoN also induces skeletal muscle differentiation, but how c-Ski/SnoN function in myogenesis is largely unknown. During our investigation on the role of sumoylation in TGF-β signaling, we inadvertently found that SnoN is modified by small ubiquitin-like modifier-1 (SUMO-1). Here, we biochemically characterize SnoN sumoylation in detail and report the physiological function of the modification. Sumoylation occurs primarily at lysine 50 (Lys-50). PIAS1 and PIASx proteins physically interact with SnoN to stimulate its sumoylation, thus serving as SUMO-protein isopeptide ligases (E3) for SnoN sumoylation. SnoN sumoylation does not alter its metabolic stability or its ability to repress TGF-β signaling. Notably, loss of sumoylation in the Lys-50 site (via a Lys-to-Arg point mutation) potently activates muscle-specific gene expression and enhances myotube formation. Our study suggests a novel role for SUMO modification in the regulation of myogenic differentiation.

Ski and SnoN are structurally and functionally related nuclear proto-oncoproteins implicated in the control of cell proliferation and differentiation. Overexpression of Ski/SnoN induces oncogenic transformation, and high levels are associated with many human cancers. Ski/SnoN induce cellular transformation by interacting with Smad2, -3, and -4 to repress TGF-β signaling and Smad-mediated growth control (1).

Ski/SnoN also initiate terminal skeletal muscle differentiation in quail embryo cells (2, 3). The paradoxical property of Ski/SnoN on transformation and differentiation is not understood, although Ski family proteins may regulate the choice between proliferation and terminal differentiation. In humans and mice, loss of Ski leads to reduced skeletal muscle mass (4, 5), whereas Ski overexpression in mice produces muscle hypertrophy (6). Furthermore, Ski transactivates expression of muscle-specific genes (7).

Posttranslational modification is a common mechanism for regulating protein function. For example, Ski/SnoN are targets of ubiquitination and proteasome degradation (8–10). Ubiquitin-like molecules such as SUMO-1 (small ubiquitin-like modifier) can also modify eukaryotic proteins and alter protein function via changes in protein stability, transcriptional activity, or subcellular localization (reviewed in Ref. 11). Covalent attachment of SUMO to substrates requires Aos1/Uba2 (E1 enzyme) and Ubc9 (E2 enzyme). In addition, three types of SUMO E3 ligases, namely PIAS proteins, RanBP2, and Pc2, are likely to contribute to substrate specificity and efficiency (11).

Here, we provide evidence that SnoN is SUMO-1 modified, define the sumoylation sites, and identify the E3 ligases required for SnoN sumoylation. We show that sumoylation does not regulate the ability of SnoN to repress TGF-β signaling and that mutation of the major sumoylation site of SnoN enhances its ability to promote myogenesis.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—cDNAs for SnoN, SnoN(K1–5R), SnoN(K6–8R), and SnoNΔ89–92 (9) were subcloned as N-terminally His-tagged proteins in pXF2RH. Point mutants and SnoNΔ50 (deletion of the first 50 amino acids) were obtained by PCR. SnoN from pXF2RH was subcloned into pXF2F or pXF3H to generate FLAG and HA-tagged SnoNs, respectively. FLAG-SUMO-1, HA-Smad3, and HA-PIAS plasmids were described previously (12, 13). pXF2RH, pXF2F, and pXF3H are derived from the cytomegalovirus-driven vector pRK5 (Genentech).
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**Ni-NTA Precipitation, Immunoprecipitation, and Western Blotting**—Immunoprecipitation and Ni-NTA precipitation were performed as described (14). Precipitated proteins were subjected to Western blotting using anti-SnoN (H-317; Santa Cruz Biotechnology) or anti-SUMO antibodies (Zymed Laboratories Inc.). Endogenous SnoN sumoylation was assayed as described (14).

**In Vitro Sumoylation Assay**—Production of recombinant SUMO-1, Uba2/Aos1, Ubc9, and PIAS proteins and the in vitro sumoylation assay were performed as described (14). Recombinant SnoNs were generated by purification of bacterially expressed GST fusion proteins and cleaved from GST by PreScission protease (Amersham Biosciences). SUMO conjugates were analyzed by anti-SnoN or anti-SUMO Western blotting.

**GST Binding Assay**—35S-labeled PIAS, Smad3, and GFP proteins were generated using the TNT® kit (Promega). Translation products were precleared by GST on glutathione-Sepharose (Amersham Biosciences) and incubated with glutathione-Sepharose-bound GST-SnoN. After washing, GST-SnoN binding products were analyzed by SDS-PAGE and autoradiography.

**Transcription Reporter Assays**—Plasmid SBE-luc (containing the luciferase gene under the control of Smad-binding elements (SBEs)), p21-luc, and cyclin A-luc were used to assay TGF-β-induced transcription in HaCaT cells. Transfections, TGF-β treatment, and reporter assays were carried out as described (12). Plasmid 4R-TK-luc, containing 4xE-boxes activated by myogenic transcription factors, was used to assay the myogenic potential of SnoN in C2C12 cells. C2C12 cells were cultured in Dulbecco’s modified Eagle’s medium/10% fetal bovine serum and transfected with 4R-TK-luc, SnoN, and/or MyoD using Lipofectamine (Invitrogen), and after 24 h, shifted to Dulbecco’s modified Eagle’s medium/2% horse serum (differentiation medium) for 48 h. Assays were performed in triplicate, and values were normalized against β-galactosidase activity.

**SnoN Stability Assays**—F-SnoN-transfected HEK293T cells were pulse-labeled for 30 min with 400 μCi ml−1[35S]methionine/cysteine and then chased in regular medium for the indicated periods of time (see Fig. 5C). Cells were harvested, and SnoN was purified by anti-FLAG immunoprecipitation and subjected to SDS-PAGE and autoradiography. Bands were quantified using NIH image (version 1.62), and values were graphed using Microsoft Excel.

In an additional SnoN stability test, HEK293T cells were transfected with FLAG-SnoN or FLAG-SnoN(K50R) and FLAG-SUMO-1 and co-transfected with HA-Smad3 where indicated. 48 h after transfection, cells were treated with 20 μM MG132 for 4 h where appropriate. Cell lysates were analyzed for FLAG-SnoN and HA-Smad3 levels by anti-FLAG and anti-HA Western blotting respectively.

**RESULTS AND DISCUSSION**

**SnoN Is Sumoylated at Lys-50 and Lys-383 in HEK293T Cells**—To determine whether SnoN is SUMO-1 modified, HEK293T cells were transfected with His-SnoN and FLAG-SUMO-1. His-SnoN proteins were precipitated with Ni-NTA-agarose and analyzed by Western blotting.

**FIGURE 1.** SnoN is sumoylated at Lys-50 and Lys-383 in HEK293T cells. A, HEK293T cells were transfected with His-SnoN and FLAG-SUMO-1. His-tagged proteins were precipitated with Ni-NTA agarose. SnoN sumoylation was determined by anti-SnoN or anti-SUMO immunoblotting (IB). B, endogenous SnoN sumoylation was assessed as in A. K50R and K383R represent Lys to Arg amino acid substitution at Lys-50 and Lys-383, respectively, whereas K50R/K383R carries both mutations. K1–5R encodes Lys to Arg substitutions at Lys-383, Lys-407, Lys-423, Lys-427, and Lys-432, and K6–8R encodes Lys to Arg substitutions at Lys-440, Lys-446, and Lys-449. Cand D, endogenous SnoN sumoylation in HEK293T cells. Antibodies used for immunoprecipitation (IP) and immunoblotting (IB) are indicated. Note that only Lys-50 sumoylation was detected on endogenous SnoN.
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SUMO antibody (Fig. 1A, lower panel) and two protein species in addition to free SnoN using anti-SnoN antibody (Fig. 1A, upper panel). As SUMO-1 often migrates at 20 kDa, the 100–120-kDa bands likely represent conjugation of one or two SUMO-1 molecules to SnoN. About 30% of SnoN was sumoylated under these conditions, and the smaller SnoN-SUMO product appears to be the predominant species. In addition, His-SnoN translocated in the absence of SUMO-1 appears to be modified by endogenous SUMO, as judged by two protein species in addition to free SnoN (Fig. 1A, upper panel, lane 1). These proteins are marginally smaller than corresponding modified SnoN in lane 3 due to the higher molecular weight of exogenous FLAG-tagged-SUMO-1. The additional species in lane 1 are not detected by αSUMO blotting, likely as this antibody is less sensitive than αSnoN.

The sequence around SnoN Lys-50 and Lys-383 matches the sumoylation consensus motif ϕKXE (ϕ = a hydrophobic amino acid; X = any amino acid). Thus, we created SnoN mutants where these lysines, individually or together, were mutated to similarly charged arginine. Mutation of Lys-50 (SnoN[K50R]) abolished the smaller SnoN-SUMO conjugate (Fig. 1B, lane 4), whereas mutation of Lys-383 (SnoN[K383R]) abolished the larger SnoN-SUMO conjugate (Fig. 1B, lane 5). Simultaneous mutation at both sites (SnoN[K50R/K383R]) completely abolished SnoN sumoylation (Fig. 1B, lane 6). These data suggest that SnoN is SUMO-modified predominantly at Lys-50, and to a lesser extent, at Lys-383.

We also tested two other SnoN mutants that had three or five lysine residues mutated. SnoN(K1–5R) contains lysines 383, 407, 423, 427, and 432 mutated to arginine (9), and not surprisingly, resulted in loss of the SnoN-SUMO species corresponding to Lys-383 but not Lys-50 (Fig. 1B, lane 7). SnoN(K6–8R) carries mutation at lysines 440, 446, and 449, which represent the acceptor lysines for ubiquitin (9). These mutations did not disrupt SnoN sumoylation, suggesting that SUMO and ubiquitin are accepted by different lysine residues in SnoN.

PIAS1, xα, and xβ Promote SnoN Sumoylation—We next analyzed SnoN SUMO modification in an in vitro sumoylation assay using recombinant SnoN, SUMO-1, E1, E2, and ATP. No SnoN sumoylation was observed in the absence of E3 ligase (Fig. 2A, lane 3). We then tested two types of SUMO E3 ligase, including all five PIAS family members (1, 3, xα, xβ, and y) and RanBP2, in SnoN sumoylation. Anti-SnoN Western blotting detected free SnoN and ~100-kDa SUMO-conjugated SnoN in the presence of PIAS1, xα, and xβ (Fig. 2A, lanes 4, 6, and 7).

We also analyzed the SnoN sumoylation sites in vitro. The presence of PIASxβ (Fig. 2B), PIASxα-, or PIAS1 (data not shown)-induced sumoylation of wild-type SnoN and SnoN(K383R) but not SnoN(K50R) or SnoN(K50R/K383R). These data suggest that SnoN is modified at Lys-50 in the presence of PIAS1, xα, and xβ. Sumoylation at Lys-383 was undetectable in vitro, suggesting that an additional cellular factor is critical for SnoN sumoylation at Lys-383 and that Lys-50 is the major SnoN sumoylation site.

To confirm the importance of PIAS E3 ligases in SnoN sumoylation, we tested the effect of PIAS1, xα, and xβ on SnoN sumoylation in vivo. PIASxβ enhanced Lys-50 sumoylation (Fig. 2C, lanes 7 and 8), and PIAS1 and PIASxα, but not PIAS3, similarly influenced SnoN sumoylation (data not shown). Our data suggest that PIAS1, xα, and xβ function as SnoN-SUMO E3 ligases (Fig. 2; data not shown). This is similar to our findings that these PIASs are E3 ligases for Smad4 (13) and CtBP1 (14).

SnoN Interacts with PIAS Proteins—As SUMO E3 ligases bind to their substrates to promote sumoylation, we determined whether SnoN and PIAS proteins physically interact. Immunoprecipitation revealed that SnoN physically interacts with PIAS1 and PIAS3 (Fig. 3A) as well as PIASxα, PIASxβ, and PIASy (Fig. 3B). A GST-SnoN fusion protein was able to bind all five PIAS proteins and the positive control Smad3 (Fig. 3C, lanes 1–6) but not GFP (Fig. 3C, lane 7). Thus, SnoN and all PIAS proteins specifically interact, although...
only PIAS1, αx, and αβ exhibited SnoN SUMO E3 ligase activity (Fig. 2A). The SnoN interaction with PIAS3 and PIASy may have an alternative function to sumoylation, such as altering the ability of SnoN to repress TGF-β-mediated transcription, given that PIASs are also transcriptional regulators independently of sumoylation (11).

Sumoylation Does Not Alter SnoN-mediated Repression of TGF-β Signaling or SnoN Stability—SnoN is a critical repressor of TGF-β signaling. As sumoylation often alters the transcriptional properties of target proteins, we were interested in determining whether SUMO modification of SnoN affects its ability to repress TGF-β signaling. We first used SBE-luc, a TGF-β-responsive synthetic reporter gene dependent on Smad activation, to compare the effects of SBE-luc reporter (A), p21-luc (B), or cyclin A-luc (C) and the indicated plasmids. TGF-β treatment and luciferase assay are described under “Experimental Procedures.” WT, wild type. D, SnoN and SnoN(K50R) interact with Smad3 in vivo in HEK293T cells. HEK293T cells were transfected with FLAG-SnoN and HA-Smad3. SnoN-bound Smad3 was detected by anti-FLAG immunoprecipitation (IP) and anti-HA immunoblotting (IB). WCL, whole cell lysate. E, free SnoN and SnoN-SUMO conjugates bind to Smad3 in vitro. In vitro sumoylation was performed on wild-type recombinant SnoN in the presence of PIASxβ. SnoN sumoylation products were assessed for their ability to bind to GST-Smad3, with assay products analyzed by anti-SnoN and anti-Smad3 immunoblotting.
wild-type SnoN and SnoN(K50R) as Lys-50 appears to be the major SnoN sumoylation site (Fig. 1A). TGF-β-induced ~9-fold increase in SBE-luc activity in HaCaT cells, and SUMO-1 further increased TGF-β-induced activity (Fig. 4A) as reported previously (12). SnoN and SnoN(K50R) repressed TGF-β-induced SBE-luc activity to a similar extent in the presence or absence of SUMO-1 (Fig. 4A). We next compared the effects of SnoN and SnoN(K50R/K383R) on the activity of natural TGF-β-responsive p21 and cyclin A promoters. TGF-β-induced ~2-fold increase in p21-luc activity, and SnoN and SnoN(K50R/K383R) were able to repress this to a similar extent (Fig. 4B). They were also able to block TGF-β-induced repression of cyclin A-luc equally (Fig. 4C). In addition, we found that Smad3 interacts with both SnoN and SnoN(K50R) in vivo (Fig. 4D). Furthermore, Smad3 retains its ability to bind to SnoN-SUMO conjugate in addition to free SnoN (Fig. 4E). As SnoN binds directly to Smads to inhibit their activity (1), the data in Fig. 4 suggest that sumoylation of SnoN is unlikely to alter its ability to repress TGF-β signaling.

We next examined the effect of Smad3 on the stability of SnoN and SnoN(K50R). Previous studies showed that Smad2/3 accelerate SnoN degradation through recruitment of the E3 ligase Smurf2 or the anaphase-promoting complex (8–10). We transfected HEK293T cells with HA-SnoN or HA-SnoN(K50R) in the presence of FLAG-SUMO-1 (to ensure a high level of sumoylation on wild-type SnoN) and compared wild type and SnoN(K50R) in the presence or absence of Smad3. Co-transfection with Smad3 promoted degradation of SnoN as expected (Fig. 5A, compare lane 3 with lane 1) and also degradation of SnoN(K50R) (Fig. 5A, compare lane 7 with lane 5). The proteasome inhibitor MG132 blocks protein degradation via the ubiquitin-proteasome pathway; thus, an increase or restoration of protein levels in its presence provides proof that the protein undergoes ubiquitin-mediated degradation. The Smad3-induced decrease in both SnoN and SnoN(K50R) was partially restored by the addition of the proteasome-inhibitor MG132 (Fig. 5A, lanes 4 and 8). Most significantly, under the above conditions, a similar trend was observed for the SnoN-SUMO(K50) conjugate (wild-type SnoN-transfected cells only) and the SnoN-SUMO(K383) conjugate (SnoN- and SnoN(K50R)-transfected cells). Taken together, these data suggest that wild-type SnoN, SnoN(K50R), and the SnoN-SUMO conjugates (at both Lys-50 and Lys-383) are all equally turned over in the cell by the proteasome degradation pathway in the presence of Smad3.

### Loss of SnoN Lys-50 Sumoylation Enhances Its Myogenic Potential

—Overexpression of Ski/SnoN in muscle precursor cells induces myogenic marker gene expression and formation of multinucleated myotubes typical of differentiation (2, 3). We sought to determine whether SnoN sumoylation may regulate its role in myogenesis. As TGF-β inhibits myogenesis (15, 16), we confirmed that sumoylation did not alter SnoN-mediated...
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Expression of SnoN or SnoN(K383R) resulted in higher expression of MHC than vector alone, and cells expressing SnoN(K50R) expressed ~1.9 times more MHC at days 3 and 4 of differentiation than those expressing SnoN or SnoN(K383R) (Fig. 6E). This agrees with the higher level of MHC myotube staining in cells expressing SnoN(K50R) than SnoN (Fig. 6B). The trend for MHC protein levels was apparent for myogenin, although myogenin was not initially observed until day 3 (Fig. 6E, top panel). If Lys-50 sumoylation negatively impacts myogenesis, deletion of the first 50 amino acids of SnoN (SnoNΔ50) might also promote myogenesis similar to SnoN(K50R). Cells expressing SnoNΔ50 showed a level of MHC and myogenin similar to cells expressing SnoN(K50R), ruling out that the increased myogenic potential of SnoN(K50R) is simply due to the presence of an arginine at amino acid position 50 (Fig. 6E). Thus, although SnoN enhances myogenesis, SnoN(K50R) and SnoNΔ50 further stimulate myogenic marker expression at days 3 and 4 of differentiation.

FIGURE 7. Exogenous and endogenous SnoN is sumoylated in physiologically relevant C2C12 myoblasts. A, C2C12 cells were transfected with His-SnoN and FLAG-SUMO-1. K50R and K383R represent Lys to Arg amino acid substitution at Lys-50 and Lys-383, respectively, whereas K50R/K383R carries both mutations. His-tagged proteins were precipitated with Ni-NTA agarose, and SnoN sumoylation was determined by anti-SnoN or anti-SUMO immunoblotting (IB). WT, wild type. B, endogenous SnoN was immunoprecipitated from C2C12 cell lysates with anti-SnoN antibody, or rabbit IgG was used as a control. Antibody-bound proteins were analyzed by anti-SnoN and anti-SUMO Western blotting. IP, immunoprecipitation; WCL, whole cell lysate. C, endogenous SUMO was immunoprecipitated from C2C12 cell lysate with anti-SUMO antibody, or rabbit IgG was used as a control. Antibody-bound proteins were analyzed by anti-SnoN and anti-SUMO Western blotting. D, the ratio of sumoylated SnoN decreases during differentiation. C2C12 cells were transfected with His-SnoN and FLAG-SUMO. Cells were harvested 24 h after transfection, or after further culture in differentiation medium for the indicated number of days. His-tagged proteins were precipitated with Ni-NTA agarose. SnoN sumoylation was determined by anti-SnoN immunoblotting. Days Diffn, days differentiation; NiNTA ppt, Ni-NTA precipitation.

FIGURE 6. Loss of SnoN Lys-50 sumoylation promotes C2C12 cell myogenesis. A, C2C12 cells were transfected with 4xRTK-luc, SnoN, and/or MyoD. Luciferase activity was measured 48 h after transfection. WT, wild type. B, differentiated C2C12 cells were fixed, permeabilized, and stained with anti-MHC antibody followed by fluorescein isothiocyanate-conjugated goat anti-mouse antibody. 4',6-Diamidino-2-phenylindole (DAPI)-containing mounting medium was used to highlight cell nuclei. 4',6-Diamidino-2-phenylindole staining of nuclei demonstrates an even number of cells in each field and highlights multinucleated myotubes when merged with αMHC staining in the right panel. C, MHC-positive myotubes were counted in 20 random vision fields, and the mean of the mean number of myotubes from three independent biological experiments is graphed. D, multinucleated myotubes were counted in 20 random fields, and the mean for SnoN and SnoN(K50R) is shown as a ratio relative to the mean number of multinucleate myotubes for vector. E, C2C12 cells were transfected with FLAG-SnoN or empty vector. Cells were allowed to differentiate before harvest at the indicated days. Lysates were analyzed by anti-myogenin, anti-MHC, anti-FLAG, and anti-actin immunoblotting. MHC and myogenin levels, normalized to actin, were quantified using NIH image version 1.62. DM, differentiation medium; WT, wild type.

repression of TGF-β signaling in C2C12 cells using the SBE-luc reporter (supplemental Fig. S2). To investigate the role of SnoN sumoylation in muscle differentiation, we expressed SnoN or SnoN(K50R) cDNA in C2C12 myoblasts and compared their effects in myogenic differentiation.

In myogenic reporter assays, which can monitor the activation of myogenic transcription factors such as MyoD, SnoN(K50R) induced a significantly higher 4xRTK-luc activity than SnoN and SnoN(K383R) (Fig. 6A). The K50R mutant is also more potent in its synergistic effect with MyoD than SnoN (presumably a mix of free and SUMO-modified forms) in inducing 4xRTK-luc reporter expression. In addition, SnoN(K50R) mutated at residues 89–92 (SnoN(K50R/89–92)), the SnoN amino acids required for SnoN-Smad3 interactions, enhances 4xRTK-luc activity more than wild-type SnoN and similarly to SnoN(K50R) (supplemental Fig. S2). These data suggest that SnoN is more myogenic when it cannot be sumoylated at Lys-50 and that the increased myogenic potential of SnoN(K50R) does not depend on SnoN-Smad3 binding and thus may be independent of TGF-β signaling.

C2C12 cells differentiate into multinucleated myotubes when cultured in differentiation medium for several days. After 5 days in differentiation medium, cells were fixed and stained for MHC, a late marker of differentiation predominantly expressed in myotubes. SnoN induced a higher degree of MHC staining and myotube formation than vector, consistent with reports that SnoN promotes myogenesis (3). Expression of SnoN(K50R) resulted in twice as many MHC-stained myotubes and twice as many multinucleated myotubes than for SnoN-expressing cells (Fig. 6, B–D). These morphological data further suggest that loss of sumoylation enhances the myogenic potential of SnoN.

We next determined whether endogenous MHC and myogenin (muscle-specific proteins) levels were affected by SnoN(K50R) during C2C12 differentiation. MHC was expressed at day 2 of differentiation and increased at days 3 and 4 (Fig. 6E).
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Finally, to confirm that a function for SnoN sumoylation in myoblasts is physiologically relevant, we sought to determine whether SnoN was sumoylated in C2C12 cells. C2C12 cells were co-transfected with His-SnoN and FLAG-SUMO-1. His-SnoN proteins were precipitated with Ni-NTA-agarose and analyzed by Western blotting. As for HEK293T cells in Fig. 1, co-transfection of SnoN and SUMO-1 in C2C12 cells resulted in detection of a protein of ~100 kDa using anti-SUMO antibody (Fig. 7A, lower panel) and of a protein species in addition to free SnoN using anti-SnoN antibody (Fig. 7A, upper panel). Mutation of Lys-50 (SnoN(K50R); SnoN(K50R/K383R)) completely abolished the SnoN-SUMO conjugate (Fig. 7A, lanes 4 and 6). The SnoN sumoylation at Lys-383 we detected in HEK293T cells (Fig. 1) was barely detectable in C2C12 cells, suggesting that SnoN is SUMO-modified predominantly at Lys-50 in C2C12 cells. To further demonstrate the physiological relevance of SnoN sumoylation in C2C12 cells, we immunoprecipitated endogenous SnoN from C2C12 cell lysates using an anti-SnoN antibody. Western blot analysis of products using anti-SUMO antibody revealed that SnoN is SUMO-modified at the endogenous level (Fig. 7B). No band was detected in the IgG lane. Conversely, we immunoprecipitated endogenous free SUMO and SUMO-conjugated proteins from C2C12 cell lysates using anti-SUMO antibody. Western blot analysis of products using anti-SnoN antibody reveals that SnoN is a SUMO-conjugated protein in C2C12 cells (Fig. 7C). No band was detected in the IgG control lane.

The data in Fig. 6 strikingly suggest that ablation of the SnoN Lys-50 sumoylation site enhances its ability to promote C2C12 cell differentiation. A proportion of SnoN proteins exist as SnoN-SUMO conjugates in C2C12 cells (Fig. 7B) and may need to be desumoylated at Lys-50, by unknown SUMO proteases, to have full myogenic potential. To address whether SnoN sumoylation fluctuates during skeletal muscle differentiation, C2C12 cells were transfected with His-SnoN and FLAG-SUMO. Cells were harvested 24 h after transfection or differentiation for 1 or 2 days. His-tagged proteins were precipitated with Ni-NTA agarose, and SnoN sumoylation was determined by immunoblotting. As shown in Fig. 7D, SnoN sumoylation slightly decreases during differentiation, consistent with the finding that SnoN(K50R) has greater myogenic potential than wild-type SnoN. The significance of the decrease in SnoN-SUMO at day 2 of differentiation is partially masked by the fact that there is a greater level of total SnoN in this lane due to sample overloading.

Taken together, the data in Fig. 7 suggest that SnoN is sumoylated at Lys-50 in C2C12 cells and that our data showing a negative role for SnoN sumoylation in C2C12 cell myogenesis are physiologically relevant. This notion is consistent with Ski being more myogenic than SnoN (3) as the Lys-50 sumoylation site is located in the N-terminal 69-amino-acid-long extension of SnoN, which is absent in c-Ski (17). As sumoylation is inhibitory, SnoN(K50R) and SnoNA50 may promote myogenesis similarly to Ski.

Our current study describes the SUMO modification on oncoprotein SnoN and provides the underlying mechanism of how SUMO modification selectively regulates cell differentiation but not the transforming activity (the ability to inhibit TGF-β/Smad signaling) of SnoN. Our finding that SnoN is SUMO-modified is one example of how SnoN activity, as opposed to stability, is posttranslationally regulated. We ruled out the possibility that SnoN(Lys-50) promotes myogenesis through altered acetylation or increased SnoN stability. Under the conditions suitable for p53 acetylation, SnoN is apparently not acetylated (supplemental Fig. S1). With regard to ubiquitination, the major site of SnoN sumoylation (Lys-50) and the ubiquitin acceptor sites are independent, and Lys-50 sumoylation does not indirectly regulate SnoN stability as wild-type SnoN, SnoN(K50R), and SnoN-SUMO conjugates are similarly degraded by the ubiquitin-mediated proteasome pathway (Fig. 5). Although the Lys-50 sumoylation site is close to the Smad3-interacting region (amino acids 89–92) of SnoN (9) and TGF-β inhibits muscle differentiation via Smad3, which interacts with and inhibits myogenic MyoD (16), SnoN sumoylation had no effect on its ability to interact with Smad3 or repress TGF-β signaling (Fig. 4 and supplemental Fig. S2). Thus, SnoN sumoylation must influence myogenesis by a TGF-β-independent mechanism. Further analysis revealed that sumoylation appears not to regulate the subcellular localization of SnoN (supplemental Fig. S3) or its interaction with MyoD (supplemental Fig. S4). Whether SnoN sumoylation indirectly influences assembly of the MyoD-containing transcription activator complex on chromatin awaits further investigation.

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Addendum—While this manuscript was in preparation, a similar study reported that SnoN sumoylation negatively regulates myogenin transcription in differentiating muscle cells (18), which agrees with our conclusion.

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