SUMO Modification Reverses Inhibitory Effects of Smad Nuclear Interacting Protein-1 in TGF-β Responses*

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Sisi Liu‡*, Jianyin Long§*, Bo Yuan‡, Mingjie Zheng‡, Mu Xiao‡, Jianming Xu‡, Xia Lin‡, and Xin-Hua Feng‡*‡‡ From the ‡Life Sciences Institute, Innovation Center for Cell Signaling Network, Zhejiang University, Hangzhou, Zhejiang 310058, China, the §Michael E. DeBakey Department of Surgery, Baylor College of Medicine, Houston, Texas 77030, and the ¶Department of Molecular & Cellular Biology, Baylor College of Medicine, Houston, Texas 77030

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SNIP1 (Smad nuclear interacting protein 1) is a transcription repressor for the TGF-β and NF-κB signaling pathways through disrupting the recruitment of co-activator p300. However, it is unclear how the functions of SNIP1 in the TGF-β signaling pathway are controlled. Our present studies show that SNIP1 is covalently modified by small ubiquitin-like modifier (SUMO) in vitro and in vivo at three lysine sites: Lys5, Lys30, and Lys108, with Lys30 being the major SUMO modification site. SUMOylation of SNIP1 is enhanced by SUMO E3 ligase PIAS proteins and inhibited by SUMO proteases SENP1/2. Furthermore, we find that SUMOylation of SNIP1 attenuates its inhibitory effect in TGF-β signaling because the SUMO-conjugated form of SNIP1 exhibits impaired ability to disrupt the formation of Smad complex and the interaction between p300 and Smads. Subsequently, SUMOylation of SNIP1 leads to the loss of SNIP1-mediated inhibition on expression of the TGF-β target genes PAI-1 and MMP2 and eventually enhances TGF-β-regulated cell migration and invasion.

The TGF-β signaling pathway regulates a variety of cellular processes such as cell growth, apoptosis and differentiation, and controls a diversity of developmental progresses and the pathogenesis of many diseases. A family of intracellular proteins named Smads is the key signaling mediator for TGF-β pathway. In canonical TGF-β signaling, the ligand binds to the type II receptor, which recruits and phosphorylates the type I receptor. Then the type I receptor phosphorylates Smad2/3, which forms complexes with Smad4, leading to the nuclear translocation of the Smad complex. Inside the nucleus, the Smad complex binds to DNA, recruits transcription co-activators p300/CBP, and induces expression of the TGF-β target genes (1, 2). It has been well established that TGF-β signaling enhances the expression of genes related to cell cycle arrest, extracellular matrix production, epithelial-mesenchymal transition, immunity evasion, and eventually induces the tumor metastasis (3–5).

SNIP1 (Smad nuclear interacting protein 1), a widely expressed nuclear protein, was originally identified as a Smad-interacting protein from a yeast two-hybrid screen (6). Structurally, SNIP1 contains an N-terminal nuclear localization signal and a C-terminal forkhead-associated domain. Functional studies have demonstrated that SNIP1 interacts with the TGF-β/BMP signaling key component Smads and NF-κB signaling transcription factor p65/RelA via its N-terminal domain and works as a transcription repressor by disrupting the recruitment of the transcription co-activator p300/CBP (6–8). SNIP1 was also found to play important roles in tumorigenesis because when using SNIP1 specific knockdown assays, SNIP1 was shown to regulate cyclin D1 expression and cell cycle progression (9) and modulate ATR checkpoint kinase pathway (10). Moreover, SNIP1 directly interacts with c-Myc oncoprotein and enhanced the transcriptional activity of c-Myc (11). Thus, SNIP1 plays important roles in multiple cellular processes. However, how the activity of SNIP1 itself is regulated remains to be elucidated. Recently, SNIP1 was found to be SUMOylated in a SUMO1* overexpressed system (12). However, the regulation and function of SNIP1 SUMOylation have not been investigated.

SUMOylation is a posttranslational modification that covalently conjugates small ubiquitin-like modifier (SUMO, also called Sentrin) to target proteins on lysine residues. SUMOylation usually utilizes the consensus acceptor motif KXX(D/E), where K is a hydrophobic amino acid (13, 14). This modification involves a cascade of enzymatic reactions, in a similar manner to ubiquitination. These enzymes include E1 SUMO-activating enzyme (heterodimer of SAE1 and SAE2), E2-conjugating enzyme Ubc9, and an E3 ligase, which promotes the transfer of SUMO from Ubc9 to specific proteins (14, 15). The PIAS (protein inhibitor of activated STAT) family proteins, nucleoporin

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1 Present address: Section of Nephrology, Dept. of Emergency Medicine, University of Texas M. D. Anderson Cancer Center, Houston, TX 77030.

2 To whom correspondence may be addressed: Life Sciences Institute, Zhejiang University, Hangzhou, Zhejiang 310058, China. E-mail: fenglab@zju.edu.cn.

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3 The abbreviations used are: SUMO, small ubiquitin-like modifier; BMP, bone morphogenetic protein; CBP, CREB-binding protein; MMP2, matrix metalloproteinase 2; PAI-1, plasminogen activator inhibitor type 1; SBE, Smad binding element; Ni-NTA, nickel-nitrilotriacetic acid; qRT-PCR, quantitative real time PCR.
protein RanBP2, polycomb protein Pc2, and NSE2 (a component of the SMC5-SMC6 complex involved in DNA double-strand breaks by homologous recombination) have been identified as SUMO E3 ligases (13, 14). Whereas the SENP (Sentrin-specific protease) family proteins are responsible for the removal of SUMO from substrates (16, 17). The functional consequences of protein SUMOylation vary because it has been linked to a variety of cellular activities, including protein stability, protein-protein interaction, subcellular localization, transcriptional regulation, DNA repair, and genome integrity (13, 14).

In this study, we showed that SNIP1 is modified by SUMOylation, and the SUMOylation occurs at three lysine residues, Lys5, Lys30 and Lys108, of which Lys30 is the major SUMO acceptor site. We also found that PIAS proteins enhance SNIP1 SUMOylation, whereas SENP proteins inhibit SNIP1 SUMOylation. Furthermore, we found that SUMO modification attenuated SNIP1 activity because the SUMO-modified form of SNIP1 lost its inhibitory effect in TGF-β signaling in comparison to wild-type SNIP1. Mechanistically, we revealed that opposite to wild-type SNIP1, SUMOylated SNIP1 lost the destabilizing activity on the Smad complex and the ability to inhibit the interaction between Smad2/3 and p300, thereby resulting in constitutively active TGF-β signaling and enhanced TGF-β-induced cell migratory/invasive responses. Thus, our novel studies provide insights into how SUMOylation regulates SNIP1 as a Smad-associated factor in fine-tuning TGF-β signaling.

Results

SNIP1 Is Modified by SUMOylation—We have been interested in how Smad activity is regulated by other molecules and pathways. Here we focused on SNIP1 protein. SNIP1 was originally identified as a Smad-interacting protein that inhibits the interaction of Smad1 and p300 (6). We are particularly interested in how SNIP1 activity is regulated. By sequence analysis, we noticed that SNIP1 contains several SUMOylation consensus motifs ϕkX(D/E). Thus, we sought to determine whether SNIP1 is SUMO-modified and what the biological function of this modification is. We first examined the SUMO modification of SNIP1 in cell co-transfection experiments. HEK293T cells were co-transfected with expression plasmids for HA-SNIP1 and FLAG-SUMO1 (Fig. 1A). Cell lysates were collected and subjected to immunoprecipitation of SUMO1 with anti-FLAG antibody, followed by anti-HA immunoblotting to detect the presence of SNIP1 in the precipitates. We specifically detected SNIP1 in SUMO1-immunoprecipitated products (Fig. 1A, top panel, lane 3), and this form of SNIP1 showed the molecular weight consistent with the addition of one SUMO moiety, when compared with SNIP1 IB with whole cell lysates (Fig. 1A, bottom panel).

The SUMOylation of SNIP1 was further confirmed under physiological conditions with endogenous levels of SNIP1 and SUMO. Lysates of HeLa cells were collected and subjected to immunoprecipitation of SUMO1 with anti-FLAG antibody, followed by anti-HA immunoblotting to detect the presence of SNIP1 in the precipitates. We specifically detected SNIP1 in SUMO1-immunoprecipitated products (Fig. 1A, bottom panel, lane 3), and this form of SNIP1 showed the molecular weight consistent with the addition of one SUMO moiety.
Identification of SNIP1 SUMOylation Sites—Having determined SNIP1 is SUMO modified, we next sought to determine the SUMO modification site(s) on SNIP1. SNIP1 can be roughly divided into two functional domains. The N-terminal domain of SNIP1 binds to transcription co-activator p300, Smad, and p65/RelA and is responsible for its repression function in TGF-β/BMP and NF-κB signaling (6, 7). The C-terminal region of SNIP1 contains a forkhead-associated domain that binds directly to c-Myc and activates the transcription of c-Myc target genes (11). Through sequence analysis, we found three perfectly matched consensus SUMOylation motifs in SNIP1 sequence (Fig. 2A). To determine the SUMO modification site(s), we introduced lysine to arginine mutation in each of these potential motifs and other lysine residues and tested the effect of each mutation on SNIP1 SUMOylation. Specifically, we generated expression plasmids for His-tagged SNIP1 and SNIP1 lysine mutants and co-transfected cells with FLAG-SUMO1 plasmid, respectively. Anti-FLAG immunoblotting revealed that wild-type SNIP1 is heavily SUMOylated, with three major bands migrating at 70, 100, and 120 kDa, respectively (Fig. 2B, lane 3). These bands were also present in the anti-SUMO1 immunoblot (data not shown). Mutation of Lys30 into Arg profoundly decreased SUMOylation of SNIP1 (Fig. 2B, lane 5), indicating that Lys30 is the major SUMOylation site on SNIP1. SUMOylation also occurred on Lys5 and Lys108 (Fig. 2B, lanes 4 and 6). On the other hand, mutations of Lys106, Lys245, Lys265, and Lys359 did not affect SNIP1 SUMOylation profile (Fig. 2B, lanes 7 and 9–11), indicating that these were not modified by SUMO. Double mutation at Lys30/Lys108 sites (Fig. 2C, lane 8) and triple mutation at Lys5/Lys30/Lys108 sites completely abolished SNIP1 SUMOylation (see Fig. 4B, lane 9). Together, we show that SNIP1 is SUMOylated at three lysine residues: Lys5, Lys30, and Lys108. Although the K5R and K108R mutants lost the 120 kDa and 100 kDa bands, respectively, the K30R mutant lost most of the bands. These results suggest that Lys30 is the major acceptor site for SUMOylation.

Regulations of SNIP1 SUMOylation by PIAS Family Protein and SENP Proteins—Protein SUMOylation can be remarkably enhanced by SUMO E3 ligases. Several families of E3 ligases including PIAS family proteins, SMC5/SMC6 complex, nucleoporin protein RanBP2, and polycomb protein Pc2 (13, 14) have been identified. To determine whether SNIP1 SUMOylation can be enhanced by E3 ligase, we tested the effect of E3 ligase families on SNIP1 SUMOylation. As shown in Fig. 3A, co-ex-
pression of PIAS family proteins including PIAS1, PIAS3, PIASxα, and PIASxβ significantly enhanced SNIP1 SUMOylation, although the expression of PIASxβ is weak (lanes 4–7). In contrast, either RanBP2 or Pc2 failed to promote SNIP1 SUMOylation (data not shown). These results suggest that PIAS family proteins serve as E3 ligases to promote SNIP1 SUMOylation.

To further confirm PIAS proteins can act as direct E3 ligases for SNIP1, we examined the ability of PIAS proteins to promote SNIP1 SUMOylation in vivo. HEK293T cells were transfected with combination of plasmids as indicated and tested for SUMOylation. Unmodified and SUMO-modified SNIP1 bands are indicated. B, PIAS proteins promote SNIP1 SUMOylation in vitro. Purified His-SNIP1 proteins (WT or triple mutant K5R/K30R/K108R) were incubated with purified proteins as indicated and tested for in vitro SUMOylation with anti-His immunoblotting. Unmodified and SUMO-modified SNIP1 bands are indicated. C, SENP proteins decrease SNIP1 SUMOylation in vivo. Whole cell lysates from HEK293T cells transfected with indicated plasmids were tested for SUMOylation with Ni-NTA precipitation (NTA ppt) and blotted with anti-FLAG and anti-SNIP1 antibodies (top panels). IB, immunoblot; WCL, whole cell lysate.

SUMOylation is a dynamic, reversible process. SENP family proteins are responsible for the specific removal of SUMO moiety from the conjugated substrates (13, 17). To explore whether SUMOylation of SNIP1 is subjected to regulation by SENP proteins, the effect of SENP on SNIP1 SUMOylation was determined in co-transfection experiments in HEK293T cells. Specifically, SNIP1 was co-transfected with or without SENP expression plasmids and immunoprecipitated from cell lysates. We found that co-expression of Myc-tagged SENP1 and SENP2, but not SENP3 plasmids, decreased the SUMOylation of SNIP1, indicated by the decreased intensity of SUMO-modified SNIP1 bands at ~70, 100, and 120 kDa (Fig. 3C, lanes 3–6), suggesting that SENP1 and SENP2 are the proteases for SNIP1 deSUMOylation. Our observation that SENP3 had no activity on removing the SUMO conjugation from SNIP1 is consistent with the reported function of SENP3 because it is more preferably responsible for the removal of SUMO2/SUMO3 than SUMO1 from substrates (17).
Ser35 Phosphorylation Can Enhance SNIP1 SUMOylation—
The major SNIP1 SUMOylation site, Lys30, fits into a consensus phosphorylation-dependent SUMOylation motif (PDSM, ψKX(D/E)XXSP) with a serine residue at amino acid 35 (Fig. 4A), where possible proline-directed serine phosphorylation regulates the SUMOylation on lysine residue (25, 26). We observed that SNIP1 is a phosphoprotein (data not shown), and Ser35 was recently identified as one of the phosphorylation sites by mass spectrometry proteomics analysis (27). To determine whether Ser35 phosphorylation regulates SNIP1 SUMOylation, we introduced a point mutation where Ser35 is either mutated into Ala35 (S35A) that cannot be phosphorylated or mutated into Asp35 (S35D) or Glu35 (S35E) to mimic phosphorylation confirmation and then compared the effect of these mutations on SNIP1 SUMOylation (Fig. 4B). S35D mutant exhibited a similar level of SUMOylation (lane 5 versus lane 3), indicating that Ser35 phosphorylation positively regulates SNIP1 SUMOylation. Further removal of SUMOylation at Lys5, Lys108, or both sites, did not completely abrogate SNIP1 SUMOylation (Fig. 4B, lanes 6–8 versus lane 4), indicating that Lys30 is still SUMOylated under these conditions. Thus, Ser35 phosphorylation can substantially enhance SNIP1 SUMOylation.

SNIP1 SUMOylation Regulates Its Transcriptional Repression Activities in TGF-β Signaling—After identifying SUMO modification on SNIP1, we next determined the functional significance of this SUMO modification, particularly its effect on SNIP1-regulated TGF-β activity. We first investigated the effect of SNIP1 SUMOylation on the transcriptional activation of Smad proteins. For this purpose, SNIP1 mutants harboring mutations that affect SNIP1 SUMOylation were generated. SUMO1-SNIP1 is the mutant in which SUMO1 was fused to the N terminus of SNIP1, mimicking a SUMO-modified form.
of SNIP. We reason that SUMO1-SNIP1 probably represents a truly SUMOylated SNIP1 because the N-terminal fusion is close to the receptor Lys. SNIP1 (K5R/K30R/K108R) is a SUMO-deficient mutant of SNIP1 in which all the SUMO acceptor lysine residues at positions 5, 30, and 108 were mutated into arginine. SUMO1-SNIP1 (K5R/K30R) is a mutant form of SUMO1-SNIP1 fusion construct in which lysine residues at 5 and 30 were mutated to arginine. These mutants or wild-type SNIP1 were then transfected into HaCaT cells individually, and the transcriptional activity of Smad protein was measured by using a luciferase reporter assay of 4xSBE-luc, a synthetic reporter gene driven by the promoter that contains four copies of SBE (Smad-binding element). As expected, wild-type SNIP1 inhibited the reporter gene transcription. We found SUMO deficiency mutant of SNIP1, SNIP1 (K5R/K30R/K108R), had a similar inhibitory effect as wild-type SNIP1. However, the SUMO-fused forms of SNIP, SUMO1-SNIP1 and SUMO1-SNIP1 (K5R/K30R), lost the inhibitory effect, suggesting that SUMO modification inhibited SNIP1 activity (Fig. 5A). Similar results were obtained with another Smad-regulated 3TP-luc reporter gene, which contains the AP1- and Smad-binding sites based on the sequence from both collagenase I and PAI-1 promoter (Fig. 5B). In addition to reporter assays, we further confirmed the inhibitory effect of SUMO modification on SNIP1 activity by examining the effect of SNIP1 SUMOylation on the transcript level of TGF-β target genes. Lung epithelial A549 stable cell lines expressing either GFP control, wild-type SNIP1, or SNIP1 (K5R/K30R/K108R) were generated. These stable cell lines expressed comparable levels of SNIP1 or its mutant (data not shown) and were treated with TGF-β for 48 h. As shown, TGF-β treatment resulted in a strong increase in the mRNA level of PAI-1 (Fig. 5C) and MMP2 (Fig. 5D) in GFP-expressing A549 cells as measured by quantitative PCR, and this TGF-β-induced PAI-1 and MMP2 transcription activation was attenuated in wild-type SNIP1 and SNIP1 (K5R/K30R/K108R) mutant-expressing cells, confirming that SNIP1 and non-SUMO modified form of SNIP1 inhibits TGF-β activity. However, SNIP1 inhibitory activity was lost after SUMO modification because PAI-1 and MMP2 transcription was enhanced in SUMO1-SNIP1-expressing cells. We also measured the protein level of PAI-1 and MMP2 by Western blotting, which was consistent with the quantitative PCR results (Fig. 5, E and F). Because Ser phosphorylation triggers SNIP1 SUMOylation, we investigated whether the SNIP1 with serine 35 mutation could affect TGF-β transcriptional responses. We first conducted SBE-luc reporter assay in HaCaT cells and found that SNIP1-S35A strongly suppressed TGF-β-induced SBE-luc reporter activity to the same extent as wild-type SNIP1 and SNIP1-K5/30/108R, whereas SUMO1-SNIP had no effect (Fig. 5G). The effect of SNIP1-S35A on transcription of endogenous genes was further assessed in A549 cell lines stably expressing SNIP1-S35A. Consistent with the reporter assay in Fig. 5G, SNIP1-S35A suppresses TGF-β-induced PAI-1 mRNA as effectively as SNIP1 and SNIP1 (K5R/K30R/K108R) (Fig. 5H), further suggesting that loss of SUMOylation (caused by loss of phosphorylation) retains the inhibitory effect of SNIP1 on TGF-β signaling. Therefore, we conclude that SUMO1-SNIP1 inhibits TGF-β pathway at the transcriptional level, and SUMO modification negatively regulates SNIP1 activity.

SUMOylation Rescues the Inhibitory Effect of SNIP1 in TGF-β-induced Cell Migration and Invasion—The TGF-β signaling pathway promotes the migration and invasion of established cancer cells (5). To further investigate the regulatory role of SUMO modification on SNIP1 physiological activity, we determined the effect of SNIP1 SUMO modification in TGF-β-induced cell migration and invasion. A549 stable cell lines expressing either GFP control, wild-type SNIP1 or SNIP1 mutants SUMO1-SNIP1 or triple mutant SNIP1-KR, i.e. SNIP1 (K5R/K30R/K108R), were used for this study. TGF-β-enhanced cell migration was determined by a typical in vitro scratch wound healing assay (28). After 7 h of TGF-β treatment, the wound gap in control GFP-expressing cells started to close, whereas the gap in SNIP1 and SNIP1 (K5R/K30R/K108R) cells healed more slowly than that in control cells. However, the wound in SUMO1-SNIP1 cells healed completely (Fig. 6A, bottom panel). These results show that SNIP1 attenuated TGF-β-induced cell migration, whereas SUMOylation inhibited SNIP1 activity. We also used Transwell assay to confirm that SNIP1 inhibited TGF-β-induced A549 cell migration, whereas SUMO modification on SNIP1 abolished SNIP1 activity (Fig. 6B) (29). After cells were cultured for 8 h in the Transwell chambers with TGF-β treatment, the number of SNIP1 or SNIP1-KR-expressing cells that had migrated through the membrane was approximately half of that of GFP-expressing cells, whereas the cell migration ability of SUMO1-SNIP1-expressing cells was similar to that of GFP-expressing cells, indicating that SUMO modification inactivated SNIP1.

To examine whether SUMO modification also affect the function of SNIP1 in TGF-β-related cell invasion, the in vitro invasion assay using Transwell system was conducted as described under “Experimental Procedures.” Similarly, SNIP1 attenuated TGF-β-induced cell invasion; SNIP1 and SUMO1-SNIP1 cells had the same invasion ability as GFP cells (Fig. 6C).

We next attempted to test the in vivo function of SNIP1 SUMOylation on tumorigenesis by injecting mouse tumor 4T1-luc cells in female Balb/c mice. SNIP1 promoted tumorigenesis likely through suppression of TGF-β signaling (Fig. 6D). In sharp contrast, the SUMO1-SNIP1 fusion failed to induce tumors as potently as SNIP1, which perfectly matched with its lost ability in suppressing TGF-β signaling (Fig. 6D). Surprising, the SNIP1-K5R/K30R/K108R mutant had moderate or little effect in inducing tumorigenesis (Fig. 6D). Because in the in vivo situation we did not “add” TGF-β as we could do in vitro, we could not exclude the effect of SNIP1 or its mutants in the regulation of other pathways such as c-Myc or NF-κB. Nonetheless, SUMOylation disables the tumor-promoting activity of SNIP1 (Fig. 6D).

The Mechanisms underlying SUMOylation-regulated SNIP1 Function in the TGF-β Pathway—As one of protein post-translational modifications, SUMOylation modulates the function of target proteins through regulating their stability, subcellular localization, protein-protein interaction, and transactivation activity (13, 14). Because SNIP1 is a nuclear transcription fac-
tor, we determined whether SUMO modification affect the transactivation activity of SNIP1 during TGF-β signal transduction. We used GAL4-Smad4 reporter system to test whether SNIP1 and SUMO-modified SNIP1 directly influence the TGF-β-induced, Smad-mediated transcriptional activity. As expected, SNIP1 repressed GAL4-Smad4-driven luciferase expression.
p300/CBP plays a crucial role in activating transcription by linking sequence specific transcription factors to transcription apparatus or providing a scaffold for the formation of complex transcriptional components (30). It has previously been reported that p300/CBP participates in TGF-β signaling pathway activation by interacting with Smads complex and facilitating the transcription process. By disrupting the formation of p300/Smads complex, SNIP1 directly blocks TGF-β signaling. To determine whether SUMOylation affects SNIP1-modulated the formation of p300/Smads complex, we compared the complex formation between p300 and Smad2 in the presence or absence of SNIP1 and SNIP1 SUMO mutants by co-immunoprecipitation assays. ALK5 (T202D), the constitutively active TGF-β type I receptor, was used to induce the TGF-β signaling and complex formation between p300 and Smad2. As shown, co-expression of ALK5 (T202D) significantly enhanced the interaction between Smad2 and p300 (Fig. 7B, lane 3 versus lane 4), and co-expression of SNIP1 strongly inhibited the formation of Smad2/p300 complex. SUMO-deficient SNIP1 (SNIP1 K5R/K30R/K108R) also showed a strong inhibition result (Fig. 7B, lane 5 versus lane 7). However, SUMO1-fused SNIP1 (SUMO1-SNIP1) lost SNIP1 inhibitory effect (Fig. 7B, lane 4 versus lanes 5 and 6). Similarly, SNIP1 but not SUMO1-SNIP1 also suppressed the interaction between Smad3 and p300 (Fig. 7C). These data suggest that SUMO modification may cause the conformational change of SNIP1 that modulates the inhibitory effect of SNIP1 on Smad recruitment of transcription co-activator.

Because SNIP1 can also directly bind to Smad2/4 to inhibit TGF-β signaling activity, we then asked whether SUMO modification can modulate the effect of SNIP1 on the Smad complex formation. We used co-immunoprecipitation to determine the interaction between Smad2 and Smad4. As shown, constitutively active TGF-β type I receptor ALK5 (T202D) promoted the interaction between Smad2 and Smad4 (Fig. 7D, lane 3 versus lane 4). Co-expression of SNIP1 and SNIP1 (K5R/K30R/K108R) mutant significantly attenuated the interaction between Smad2 and Smad4, but SUMO1-conjugated SNIP1 (SUMO1-SNIP1) lost this inhibitory ability (Fig. 7C, lane 4 versus lanes 5–7). The effect of SUMO modification on the SNIP1-regulated interaction between Smad3 and Smad4 was also observed in the same manner (Fig. 7E). Taken together, we have shown that SNIP1 inhibits TGF-β signaling by disrupting formation of the Smad complex and attenuating the recruitment of p300 to Smad proteins, whereas SUMO modification of SNIP1 inactivates SNIP1 and leads to the loss SNIP1-suppressed TGF-β signaling activity.

Discussion

As a suppressor for TGF-β/BMP and NF-κB signaling, SNIP1 has a crucial role in cellular growth and tumorigenesis, but the regulation of its activity is still largely unknown. Protein functions can be rapidly regulated by post-transcriptional modifications, which occur on the specific amino acid side chains and are often catalyzed by enzymes including kinases and ubiquitin/ubiquitin-like ligases. These enzymes regulate the function of different proteins and eventually maintain dynamic balance of intracellular environment. We report here that SNIP1 is subjected to SUMOylation both in vivo and in vitro. We have mapped three lysine residues, Lys5, Lys30, and Lys108, as the SUMOylation sites, because complete loss of SNIP1 SUMOylation requires mutations at all three lysine residues (Figs. 2C, 4B, and 8). Lys30 is the major conjugation site of SNIP1 SUMOylation (Figs. 2, B and C, and 4B). We also found SNIP1 is heavily SUMOylated with two major bands migrated at 100 and 120 kDa, respectively, indicating that SNIP1 could be SUMOylated at all three sites at the same time. Interestingly, the mutation of Lys30 totally abrogated the 100/120-kDa bands and dramatically decreased the level of the 70-kDa band, whereas the mutation of Lys108 and Lys5 abrogated only the 100- and 120-kDa bands, respectively. This finding indicates that the Lys30 SUMOylation may prime the SUMO modification of Lys108 and Lys5. Furthermore, the Lys108 SUMOylation may be indispensable to the SUMO modification of Lys5.

SNIP1 is a phosphoprotein (27, 31), where Ser35 was recently identified as one of the phosphorylation sites (27). The sequence around Lys30 and Ser35 resembles a consensus phosphorylation-dependent SUMOylation motif, where SNIP1 SUMOylation was predicted to be dependent on Ser35 phosphorylation (25, 26). Indeed, our phosphomimetic mutation study indicates that the Ser35 to Asp substitution does not change SNIP1 SUMOylation, whereas the Ser35 to Ala mutation substantially reduces SUMOylation (Fig. 4B). Therefore, there is a sequential event of modifications on SNIP1. That is,
FIGURE 6. SUMO modification reverses SNIP1-mediated inhibition in TGF-β-induced cell migration and invasion. A, stable overexpression of SNIP1 attenuates migratory responses in A549 cells. Cell migration was measured by wound healing assay. A549 cells harboring GFP, SNIP1, SUMO1-SNIP1, and SNIP1-KR (i.e. SNIP1 K5R/K30R/K108R mutant) were plated in 60-mm dishes and treated with TGF-β for 7 h post-wound. Representative photographs were taken at 0 h (top panel) and 7 h (bottom panel). B, Transwell migration assays of A549 cell lines stably overexpressing GFP, SNIP1, SUMO1-SNIP1, and SNIP1-KR. The number of cells in the whole Transwell chamber was counted for each group in three independent experiments. C, Transwell invasion assays of A549 stable cell lines was performed under the same conditions as migration assay, by using Matrigel-coated insert wells. D, SNIP1 promotes tumorigenesis in vivo. Luciferase-carrying 4T1 tumor cells (4T1-luc, 2 × 10^5 cells/mouse) expressing SNIP1, SUMO1-SNIP1, SNIP1-K5R/K30R/K108R, or empty GFP vector (served as negative control) were separately injected through tail vein into 4-week-old BALB/c female mice, yielding four groups (n = 10 mice/group). After injection, the mice were allowed for tumor development for 20 days and analyzed by bioluminescence using Xenogen IVIS imaging system.

FIGURE 7. SUMOylation of SNIP1 affects Smad interactions in the TGF-β signaling pathway. A, SUMOylation releases the inhibition of SNIP1 on TGF-β signaling. MDA-MB-468 cells were transfected with SNIP1, SUMO1-SNIP1, mutant, and SUMO1-SNIP1 mutant, together with GAL4-Smad4C and pFR-Luc reporter constructs. The cells were treated with 5 ng/ml of TGF-β for 8 h, and cell lysates were collected for luciferase assay. B and C, SUMOylation disables the inhibitory effect of SNIP1 on the TGF-β-induced interactions between Smad2 and p300 (B), as well as Smad3 and p300 (C). Expression plasmids for Myc-Smad2 or Smad3 and HA-p300 were co-transfected with indicated plasmids. Cell lysates were immunoprecipitated (IP) with HA antibody and immunoblotted with Myc antibody. D and E, SUMOylation dampens the inhibitory effect of SNIP1 on TGF-β-induced formation of the Smad2/4 (D) and Smad3/4 complexes (E). The experiment was carried out as described for B and C. IB, immunoblot; WCL, whole cell lysate.

SUMOylation of SNIP1

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Ser^{35} phosphorylation triggers SUMOylation on Lys^{30}, which is followed by Lys^{108} and Lys^{5}.

Unlike ubiquitination that often targets substrates for proteasomal or lysosomal degradation, protein SUMOylation has rather variable outcomes, which depends on the substrates themselves. Usually SUMOylation of transcription factors associates with transcription repression (13, 32–34). In this report, we have found that SNIP1 SUMOylation modestly affects the formation of Smad complex and the interaction between Smads and p300 (Fig. 7, B–E).

TGF-β signaling has two opposite functions in tumor progression, because it inhibits tumor initiation at the early stage but enhances tumor metastasis at the late stage. Because TGF-β signaling can stimulate cell invasion, stimulate metastasis, and enhance angiogenesis, targeting TGF-β has been reported as an efficient treatment for cancer therapy including breast cancer, pancreatic cancer, and melanoma (35–38). By suppressing TGF-β responses, SNIP1 plays an important role in inhibiting TGF-β-related tumor cell migration and invasion (Fig. 6, A–C). It is interesting that SUMO modification reverses the suppressing effect of SNIP1 on TGF-β signaling. It was previously reported that SUMOylation enables an increased stability of Smad4 to enhance TGF-β signaling (21). Thus, the effect of SNIP1 SUMOylation is in agreement with the overall positive effect of SUMOylation in TGF-β signaling. It is conceivable that targeting SNIP1 SUMOylation may be one way to manipulate TGF-β signaling during tumorigenesis.

Experimental Procedures

**Constructs**—Human SNIP1 cDNA was amplified by PCR from EST clone (IMAGE clones 4540112; Open Biosystems). His, HA, FLAG, and GAL4-tagged SNIP1 cDNA were constructed into modified pRK5 (Genentech), pcDNA3.1-HA, or pSG424. SUMO-SNIP1 chimera was constructed by in-frame fusion of SUMO1 (1–96) and SNIP1 (2–346) into CS2 vector. SNIP1 was subcloned into pTrcHisA (Invitrogen) for His-tagged protein expression in E. coli. GST fusion of N-terminal domain of p300 (2–670) was constructed by PCR. Different SNIP1 SUMOylation site(s) mutants were made by PCR-based mutagenesis and confirmed by sequencing. FLAG-SUMO1, Myc-SUMO1, FLAG-PIAS, and Myc-tagged SENP1–3 expression plasmids were described as previously (18).

**Lentivirus Production and Stable Cell Line Generation**—VSV-G pseudotyped high titers lentiviruses were generated by transient co-transfection of 293FT cells with a four-plasmid combination as follows: SNIP1 or SNIP1 mutant lentiviral vector, packaging plasmid RRE, REV, and envelope plasmid VSV-G. 48 h after transfection, the media containing lentiviruses were collected and used to infect host cells. Stable cells were selected in the presence of 1 ng/ml puromycin.

**Antibodies, Cell Lines, Transfection, and Immunoprecipitation**—Primary antibodies used in this study include the following: anti-SNIP1 (Bethyl Laboratories), anti-Myc (BD Bioscience), anti-FLAG (Sigma), anti-His (SeraTec), anti-HA (Cell Signaling Technology), anti-SUMO1 (Boston Biochem), anti-GAPDH (FL-335) (Santa Cruz), anti-MMP2 (D8N9Y) (Cell Signaling Technology), and anti-PAI-1 (C-9) (Santa Cruz).

HeLa, HEK293T, COS-1, A549, and MDA-MB-468 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS (Hyclone). HaCaT and 293FT cells were maintained in Eagle’s minimum essential medium (Hyclone) and 293FT Complete medium, respectively, supplemented with 10% FBS. Transient transfections were performed using Lipofectamine 2000 (Invitrogen). Immunoprecipitations were carried out as previously described (18) from cells lysed in TNET buffer (10 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA, 1.0% Nonidet P-40) plus protease inhibitors and phosphatase inhibitors.

**In Vivo SUMOylation Assay**—In vivo SUMOylation assays of transfected HA-SNIP1 and endogenous SNIP1 were carried out as previously described in modified radioimmune precipitation assay/SDS buffer, followed by dilution and immunoprecipitation with appropriate antibodies (18, 19). Alternatively, SUMOylation of transfected His-SNIP1 wild type and mutants,
were performed as previously (20) under denatured conditions with Ni-NTA beads (Qiagen).

**In Vitro SUMOylation Assay**—In vitro SUMOylation assay was performed as previously described using bacterially expressed and purified proteins (20, 21). Recombinant His-SNIP1 protein was purified from *E. coli* with Ni-NTA beads (Qiagen) following manufacturer’s protocol. Reaction was stopped with SDS sample buffer and analyzed by anti-SNIP1 immunoblot.

**Luciferase Reporter Gene Assay**—The cells seeded in 24-well plates were transfected by Lipofectamine 2000 (Invitrogen). 3TP-luc or 4xSBE-luc was used to detect repression of TGF-β signaling by SNIP1 in HaCaT cells as previously described (22). The data presented are means ± S.D. from at least three independent experiments.

**Quantitative Real Time PCR (qRT-PCR)**—The cells were harvested, and total RNAs were extracted with TRIzol reagent (Invitrogen) (23). 1 μg of total RNA was reverse-transcribed to cDNA by using the PrimeScript® RT reagent kit (TaKaRa). qRT-PCR was performed on an ABI PRISM 7500 sequence detector system (Applied Biosystems) using gene-specific primers for MMP2 with SYBR Green Master Mix (Applied Biosystems). TaqMan Assay System (Applied Biosystems) was used to detect the level of PAI-1. Primers used for each gene are listed as follows: 5'-TCTCCTGACATTGACCTTGGC-3' and 5'-CAAGGTGCTGGCTGAGTAGATC-3' for human MMP2, 5'-CGACCACTTTGTCAAGCTCA-3' and 5'-TTACTCTTGGAGGGCATGT-3' for human GAPDH. Samples were done in triplicate, and the data were analyzed using the 2^−ΔΔCT method.

**Cell Migration and Invasion Assay**—For monolayer wound healing assay, the cells were plated in a 60-mm dish. A scratch was made on cells with a sterile pipette tip after the cells reached 100% confluence, and cell debris were washed out with PBS. Wound size was measured at 0 and 7 h post-wound at the same area. For the Transwell migration assay, 5000 cells were used to detect the level of transfected β-galactosidase driven by pSV-β-gal (Promega). The data presented are means ± S.D. from at least three independent experiments.

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