

The RING domain of PIASy is involved in the suppression of bone morphogenetic protein-signaling pathway[☆]

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Abstract

Bone morphogenetic proteins (BMPs) play central roles in differentiation, development, and physiologic tissue remodeling. Recently, we have demonstrated that a protein inhibitor of activated STAT, PIASy, suppresses TGF- β signaling by interacting with Sma and MAD-related protein 3 (Smad3). In this study, we examined a PIASy-dependent inhibitory effect on BMP signaling. PIASy expression was induced by BMP-2 stimulation and suppressed BMP-2-dependent Smad activity in hepatoma cells. Furthermore, BMP-2-regulated Smads directly bound to PIASy. We also demonstrated that the RING domain of PIASy played an important role in PIASy-mediated suppression of Smad activity. We here provide evidence that the inhibitory action of PIASy on BMP-regulated Smad activity was due to direct physical interactions between Smads and PIASy through its RING domain.

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Bone morphogenetic proteins (BMPs) are members of the transforming growth factor β (TGF- β) superfamily that have been implicated in tissue growth and remodeling [1–3]. BMPs were initially identified by the ability of bone extracts to induce bone formation at extraskelatal sites [2]. BMPs bind to two types of transmembrane receptors, denoted type I and type II BMP receptors, which have serine/threonine kinase activity [3]. Upon ligand binding, type II receptors phosphorylate the type I receptors. The activated type I receptors then phosphorylate downstream Smads, Smad1, Smad5 or Smad8, which are transcription factors that regulate gene expression in response to BMPs [4–6].

BMP signaling has been also implicated in the metastatic potential of several human cancers [7–11]. BMP stimulates cell adhesion to type I and IV collagens, fibronectin, and vitronectin as well as down-regulated

expression of integrins [1,2,12], following by an increased cell spreading. BMP signaling also induces the expression of several distinct target genes, including members of the Id gene family through Smad proteins [13–15].

In attempt to identify novel Smad partners, we screened a mouse embryo cDNA library with yeast two-hybrid system using the MH2 domain of Smad7 as bait. We recently identified PIASy, which is one of the protein inhibitors of activated STAT family protein, as a protein that interacts specifically with Smad7 [16]. They have been originally identified as a cofactor that inhibits the transcriptional activation potential of STAT (signal transducers and activators of transcription) and in mammals five PIAS proteins (PIAS1, 3, α , β , and γ) have been reported [17]. PIAS1 and PIAS3 were initially cloned as transcriptional repressors of the Jak-STAT signaling pathway [18,19]. PIAS3 was originally identified as a specific co-repressor of signal transducer and activator of transcription 3 (STAT3) [20]. PIAS3 binds to STAT3 and inhibits its DNA-binding activity and thereby interferes with STAT3-mediated gene activation. PIAS1, another member of PIAS family, was

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originally identified as a co-repressor of STAT1 [18]. PIAS family proteins also function as a transcriptional cofactor for nuclear receptors [20–22]. Recently, PIAS family proteins have been proposed to function as a small ubiquitin-related modifier (SUMO)-E3 ligase [23]. PIAS1 and PIASy were shown to catalyze sumoylation of p53, LEF-1, and Tcf-4, respectively [24–27]. PIAS family proteins have the RING domain, and their SUMO-E3 ligase activities are dependent on this domain [23]. PIASy was demonstrated to interact with Smad3 and antagonizes Smad3-dependent transcriptional activation [16,28]. We also showed that expression of PIASy is induced by TGF- β [16].

In this study, we demonstrate a novel molecular mechanism for the inhibitory actions of PIASy on BMP-2 function: there are direct physical and functional interactions between BMP-regulated Smads and PIASy. We also demonstrate that the RING domain of PIASy plays an important role in PIASy-mediated suppression of Smad activity and the sequestration of Smad in nuclear bodies. These findings provide new insights into the regulation of BMP-2-signaling pathway that may have implications in reproductive physiology and the process of oncogenesis.

Materials and methods

Reagents and antibodies. Human recombinant BMP-2 was purchased from Strathmann Biotech GmbH (Germany). Expression vectors, FLAG-tagged Smad1, Smad8, Smad6, BMPR-IA (QD), 12xGCCG-LUC [29,30], T β R-I (T204D) [31], and p3TP-LUC [31], were kindly provided by Dr. K. Miyazono (Tokyo Univ., Tokyo, Japan) and Dr. J. Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY). Myc-tagged PIASy RING mutant (PIASy^{CA}) [27] were kindly provided by Dr. A. Kikuchi (Hiroshima Univ., Hiroshima, Japan). PIASy mutants were generated by PCR methods and sequenced (primer sequences are available upon request). Anti-Myc antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG antibody was purchased from Sigma (St. Louis, MO).

Cell culture, transfection, and luciferase assays. Human embryonic kidney carcinoma cell line, 293T, was maintained in DMEM containing 10% FCS and transfected by the standard calcium precipitation protocol [33]. Human hepatoma cell line Hep3B was maintained in DMEM containing 10% FCS [34]. Before stimulation, the cells were cultured for 12 h in DMEM containing 1% FCS followed by treatment with BMP-2 [31,32]. Hep3B cells ($2\text{--}2.5 \times 10^5$ in a 6-cm dish) were transfected by using FuGENE6 (Roche Diagnostics GmbH, Mannheim, Germany) following manufacturer's instructions. Luciferase assay was performed as described [33]. The cells were harvested 48 h after transfection, lysed in 100 μ l of PicaGene Reporter Lysis Buffer (Toyo Ink, Tokyo, Japan), and assayed for luciferase and β -galactosidase activities according to the manufacturer's instructions. Luciferase activities were normalized to the β -galactosidase activities. Three or more independent experiments were carried out for each assay.

Immunoprecipitation and immunoblotting. The immunoprecipitation and Western blotting assays were performed as described previously [33]. Cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, containing 1% NP-40, 1 μ M sodium orthovanadate, 1 μ M phenylmethylsulfonyl fluoride, and 10 μ g/ml each of

aprotinin, pepstatin, and leupeptin). The immunoprecipitates from cell lysates were resolved on 5–20% SDS-PAGE and transferred to Immobilon filter (Millipore; Bedford, MA). The filters were then immunoblotted with each antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham-Pharmacia Biotech).

Northern blot and RT-PCR analysis. After 12 h of incubation in 1% FCS, Hep3B cells were treated with BMP-2 (50 ng/ml) for the indicated time. Total RNAs were prepared by using Iso-Gen (Nippon Gene, Tokyo, Japan) and used in Northern blot analysis according to the established procedures [34]. A nylon membrane (Hybond N⁺, Amersham-Pharmacia Biotech) and radiolabeled cDNA probes were used, where indicated. RT-PCR was performed using RT-PCR high -Plus-Kit (TOYOBO, Tokyo, Japan). Human Id1 and G3PDH primers were used as described previously [35,36].

Results and discussion

PIASy expression is induced by BMP-2 and suppresses BMP-2-mediated transcription in Hep3B cells

In our previous report, we demonstrated that TGF- β -induced PIASy suppressed TGF- β -stimulated gene expression in a human hepatoma cell line, Hep3B cells [16]. We then examined whether PIASy expression is also regulated by BMP-2 in Hep3B cells. Hep3B cells were either left untreated or treated with BMP-2, and PIASy expression was monitored by Northern blot analysis. As shown in Fig. 1A, about 3-fold induction of PIASy expression was observed by treatment with BMP-2 in Hep3B cells. The level of PIASy mRNA expression increased about 3-fold at 6 h and did not alter until 24 h. We also examined BMP-2-induced Id1 expression through Smads [13–15]. Id proteins interact with a variety of basic helix-loop-helix (bHLH) and non-bHLH transcription factors that contribute to normal growth regulation. Therefore, dysregulated Id expression may contribute to oncogenesis [37–39], and their expression is increased in a variety of human cancers [40]. When we monitored the same RNA samples by RT-PCR, BMP-2 treatment strongly induced transient expression of Id1 at 3–6 h after stimulation in Hep3B cells.

To examine the effect of PIASy expression on BMP-2 signaling in Hep3B, we next performed the transient transfection assay. The BMP-2-mediated transcriptional responses were measured by using 12xGCCG-LUC, which is a reporter construct that directly detects Smad phosphorylation through BMP receptors [30]. Hep3B cells were transfected with 12xGCCG-LUC together with empty vector or PIASy, and treated with BMP-2 and LUC activities were determined. As shown in Fig. 1B, PIASy expression showed a significant decrease of BMP-2-stimulated 12xGCCG-LUC activation in Hep3B cells. These results demonstrated that expression of PIASy is induced by BMP-2 and inhibits BMP-2/Smad-mediated transcription activity in Hep3B cells.

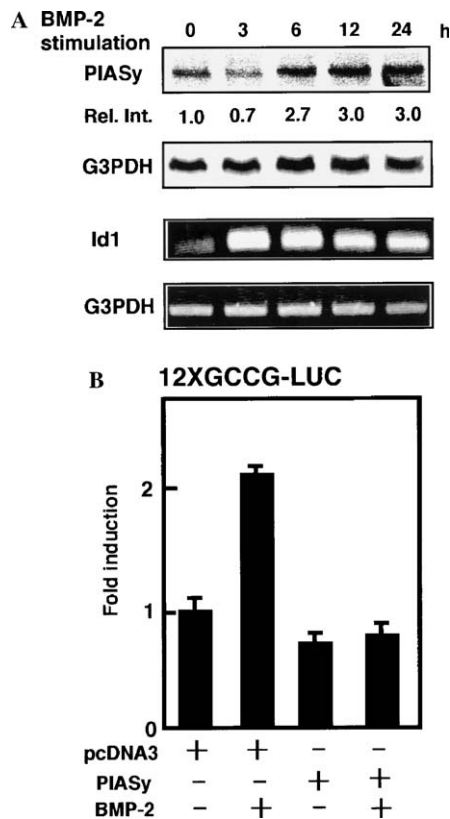


Fig. 1. PIASy expression is induced by BMP-2 and suppresses BMP-2-induced transcription in Hep3B cells. (A) BMP-2-induced PIASy expression in Hep3B cells. Twenty micrograms of total RNA isolated from cells treated or untreated with BMP-2 (50 ng/ml) for the indicated times were used in Northern blot analysis of PIASy expression. The same blot was probed with glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA as control (lower panel). Relative intensities (Rel. Int.) of the bands shown below the autoradiograms were determined by densitometric analysis. Same RNA samples were subjected to RT-PCR analysis using Id1 (upper panel) and G3PDH (lower panel) primers. RT-PCR products were separated on a 1% agarose gel. This figure is representative of three separate experiments. (B) Hep3B cells (6-well plate) were transfected with 12xGCCG-LUC (1 μ g) together with empty vector or PIASy (3 μ g). Forty eight hours after transfection, cells were stimulated with BMP-2 (50 ng/ml) for an additional 12 h. Cells were harvested and relative luciferase activities were measured. The results are presented as fold induction of luciferase activity from triplicate experiments, and the error bars represent the standard deviations.

PIASy suppresses BMP-signaling in 293T cells

To delineate the interactions between PIASy and BMP-signaling pathways further, we carried out transient transfection experiments in 293T cells using the respective receptors and the downstream activators for the BMP signaling, Smad1. In addition, in most of these experiments, a constitutively active form of BMP type IA receptor, BMPR-IA(QD), was used [30,41]. Expression of BMPR-IA(QD) in 293T cells resulted in an induction of 12xGCCG-LUC as previously described [34]. When 293T cells were transfected with 12xGCCG-LUC

together with BMPR-IA (QD), LUC expression was increased by 2- to 2.5-fold (Fig. 2A). Additional expression of Smad1 augmented 12xGCCG-LUC expression by 5- to 6-fold (Fig. 2A). We then examined the effect of expression of PIASy on BMP signaling in 293T cells. 293T cells were transfected with an expression vector for BMPR-IA(QD), Smad1 and 12xGCCG-LUC together with or without an inhibitory Smad, Smad6 or PIASy. As shown in Fig. 2A, PIASy expression but not PIAS1, PIAS3, and PIASX α suppressed BMPR-IA (QD)/Smad1-mediated 12xGCCG-LUC activation in a dose-dependent manner (Fig. 2B). These results suggest that BMP/Smad-mediated signal is suppressed by PIASy in a specific manner.

Physical interactions between PIASy and BMP-regulated Smads in vivo

One of the possible mechanisms that would be consistent with the data described above is that there are direct physical interactions between PIASy and BMP-regulated Smads. To investigate the association of PIASy with BMP-regulated Smads in vivo, 293T cells were transfected with either FLAG-tagged Smad1, Smad8 or Smad6 together with Myc-tagged PIASy. As shown in Fig. 3A, comparable amounts of Smads were expressed in each cell. Similarly, PIASy was expressed in samples containing Smads. Western blot analysis of associated proteins with an anti-FLAG antibody revealed that PIASy interacts with Smad1, Smad8, and Smad6 in 293T cells.

We next determined which domains of PIASy mediate interactions with Smad1, using a series of deletion mutants of PIASy as shown in Fig. 3B. Expression vectors encoding FLAG-tagged Smad1 and/or a series of Myc-tagged PIASy mutants were transiently transfected into 293T cells. Cells were lysed and subjected to immunoprecipitation with an anti-FLAG antibody. Immunoprecipitates were then used in Western blot analysis with an anti-Myc antibody. As shown in Fig. 3C, PIASy (1–100) lacking almost C-terminal domain was unable to bind Smad1. However, PIASy (1–202) and PIASy (100–510) showed a little lower binding affinity. These results indicate that PIASy–Smad1 interaction requires the N-terminal domain of PIASy. The data also suggest that the N-terminal domain of PIASy may contain a secondary binding site for Smad1.

The RING domain of PIASy is involved in the suppression of Smad-mediated transcription

PIASy has the RING domain, which has been identified as an important functional determinant of ubiquitin and SUMO E3 ligases [24–26]. Sumoylation proteins have been shown to localize to specific sub-nuclear structures, known as PML-containing nuclear

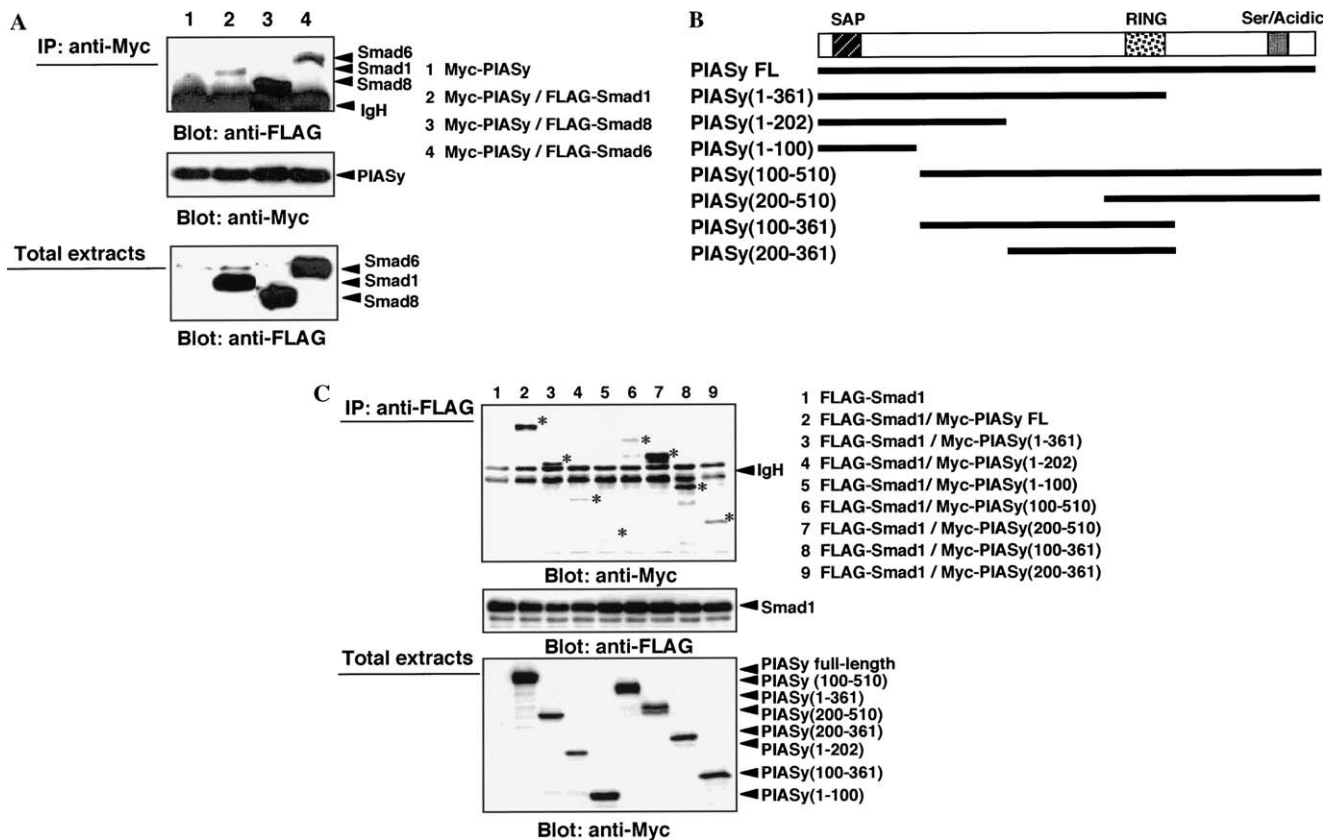


Fig. 3. Physical interactions between PIASy and BMP-regulated Smads. (A) 293T cells (1×10^7) were transfected with Myc-tagged PIASy (10 μ g) and/or FLAG-tagged Smad3, Smad1, Smad8, and Smad6 (10 μ g). Forty eight hours after transfection, cells were lysed and immunoprecipitated with an anti-Myc antibody, and immunoblotted with anti-FLAG antibody (upper panel) or anti-Myc antibody (middle panel). Total cell lysates (1%) were blotted with anti-FLAG antibody as indicated (lower panel). IgH, heavy chain of immunoglobulin. (B) Schematic line diagrams of PIASy. PIASy contains a putative chromatin-binding SAP domain, a C2HC3 RING domain (RING), and a C-terminal serine-rich and acidic domain (Ser/Acidic). (C) Mapping the Smad1 interaction domain of PIASy. 293T cells (1×10^7) were transfected with FLAG-tagged Smad1 (10 μ g) and/or full-length PIASy or PIASy mutants. Forty eight hours after transfection, cells were lysed and immunoprecipitated with an anti-ER α antibody, and immunoblotted with anti-Myc antibody (upper panel) or anti-FLAG antibody (middle panel). Total cell lysates (1%) were blotted with anti-Myc antibody (lower panel). The asterisks indicate the migration position of full-length PIASy or PIASy mutants. IgH, heavy chain of immunoglobulin.

to punctate structures in the nucleus such as a subset of PML nuclear bodies. Taken together, these observations show that the RING domain of PIASy is important not for the suppression of Smad activity but also for the sequestration of Smad1 in nuclear bodies.

Concluding remarks

Recent studies have documented the interaction of a large number of intracellular proteins with the effector molecules Smads to influence BMP signaling [43,44]. Whereas some of these proteins have been found to functionally cooperate together and activate Smads, others were found to repress Smad activity. Several molecular mechanisms have been proposed for the inactivation of BMP/Smad signaling pathway. In this study, we showed that BMP-2 induced PIASy expression suppressed BMP-2-mediated Smad activation in

hepatoma cells. We also demonstrated that a PIAS family protein, PIASy, interacted with BMP-regulated Smads and antagonized Smad-dependent transcriptional activation by BMPR. Furthermore, this inhibitory effect required the RING domain of PIASy, which mediates the sequestration of Smad1 in nuclear bodies.

Smad-dependent proteasome-mediated degradation of Smads themselves but also of cytoplasmic and nuclear Smad partners is an important aspect of TGF- β /BMP signaling as well. Smurf1 and Smurf2, which are HECT E3 ubiquitin ligases, selectively interact with BMP-regulated Smads to trigger their ubiquitination and subsequent degradation, and hence their inactivation [45,46]. Smurf1 and Smurf2 also bind to Smad7 and are recruited to the activated TGF- β receptor I by Smad7, leading to proteasomal degradation of the receptor [47,48]. Recently, four members of the mammalian PIAS (protein inhibitor of activated STAT) family, PIAS1, PIASx, PIASy, and PIASz, and a yeast

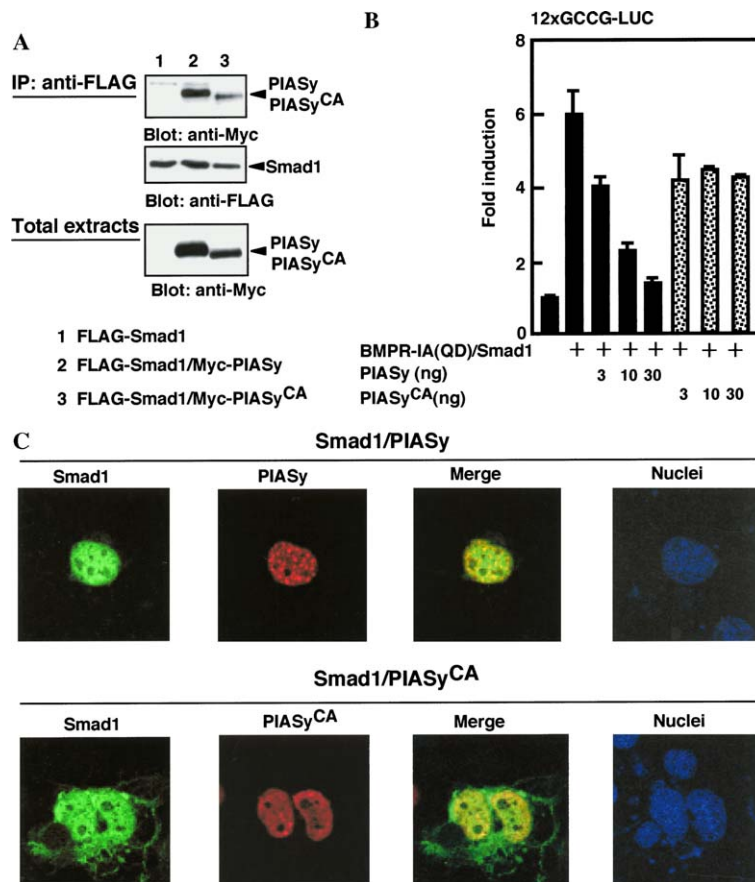


Fig. 4. Involvement of the RING domain in PIASy-mediated suppression of Smad activation. (A) 293T cells (1×10^7) were transfected with FLAG-tagged Smad1 (10 μ g) together with or without Myc-tagged PIASy (10 μ g) or Myc-tagged PIASy^{CA} (10 μ g). Forty eight hours after transfection, cells were lysed and immunoprecipitated with an anti-FLAG antibody, and immunoblotted with anti-Myc antibody (upper panel) or anti-FLAG antibody (middle panel). Total cell lysates (1%) were blotted with anti-Myc antibody as indicated (lower panel). (B) 293T cells (12-well plate) were transfected with 12xGCCG-LUC (0.3 μ g) and/or BMPR-IA(QD) (100 ng) and Smad1 (100 ng), together with or without an increasing amounts of PIASy or PIASy^{CA} as indicated. Forty eight hours after transfection, cells were harvested and relative luciferase activities were measured. The results are presented as fold induction of luciferase activity from triplicate experiments, and the error bars represent the standard deviations. (C) COS7 cells were cotransfected with FLAG-tagged Smad1 and Myc-tagged PIASy or Myc-tagged PIASy^{CA} by the calcium phosphate precipitation protocol. Forty eight hours after transfection, cells were fixed, reacted with an anti-FLAG polyclonal antibody and an anti-Myc monoclonal antibody, and visualized with an FITC-conjugated anti-rabbit antibody (Smad1) and a rhodamine-conjugated anti-mouse antibody (PIASy or PIASy^{CA}). These figures were merged. The same slide was also stained with DAPI.

PIAS homologue, Siz1, have been reported to have SUMO-E3 ligase activities toward various target proteins, including p53 and LEF1, and affect their transcriptional activity [24–26]. We and others demonstrated that PIASy stimulated the sumoylation of Smad3 *in vivo*, followed by suppression of TGF- β -mediated transactivation [16,28].

Our findings provide a novel mechanism of the negative regulation of BMP signaling by PIASy, which may be due to the sequestration of Smads in nuclear bodies by the RING domain of PIASy using the negative feedback loop. More detailed understanding of the interactions between activated Smads and PIASy will provide not only critical information on embryogenesis, organogenesis, and tissue homeostasis, but may also be instrumental in the development of new treatment strategies in related diseases such as cancer.

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