



# HOKKAIDO UNIVERSITY

|                  |                                                                                                                                                                             |
|------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Title            | Sumoylation of Smad3 stimulates its nuclear export during PIASy-mediated suppression of TGF- $\beta$ signaling                                                              |
| Author(s)        | Imoto, Seiyu; Ohbayashi, Norihiko; Ikeda, Osamu et al.                                                                                                                      |
| Citation         | Biochemical and Biophysical Research Communications, 370(2), 359-365<br><a href="https://doi.org/10.1016/j.bbrc.2008.03.116">https://doi.org/10.1016/j.bbrc.2008.03.116</a> |
| Issue Date       | 2008-05-30                                                                                                                                                                  |
| Doc URL          | <a href="https://hdl.handle.net/2115/33892">https://hdl.handle.net/2115/33892</a>                                                                                           |
| Type             | journal article                                                                                                                                                             |
| File Information | PIASy-Smad3.pdf                                                                                                                                                             |



***Title: Sumoylation of Smad3 stimulates its nuclear export during PIASy-mediated suppression of TGF- $\beta$  signaling***

*Authors:* Seiyu Imoto<sup>1\*</sup>, Norihiko Ohbayashi<sup>1\*</sup>, Osamu Ikeda<sup>1</sup>, Shinya Kamitani<sup>1</sup>, Ryuta Muromoto<sup>1</sup>, Yuichi Sekine<sup>1</sup> and Tadashi Matsuda<sup>1,\*</sup>

*Affiliation:* <sup>1</sup>Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-Ku Kita 12 Nishi 6, Sapporo 060-0812, Japan

\*These authors contributed equally to this work.

\**Address correspondence to:* Dr. Tadashi Matsuda, Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-Ku Kita 12 Nishi 6, Sapporo 060-0812, Japan TEL: 81-11-706-3243, FAX: 81-11-706-4990, E-mail:

tmatsuda@pharm.hokudai.ac.jp

*Running title:* Smad3 sumoylation regulates nuclear retention

## **Abstract**

Smad- and MAD-related protein 3 (Smad3) plays crucial roles in the transforming growth factor- $\beta$  (TGF- $\beta$ )-mediated signaling pathway, which produce a variety of cellular responses, including cell proliferation and differentiation. In our previous study, we demonstrated that protein inhibitor of activated STATy (PIASy) suppresses TGF- $\beta$  signaling by interacting with and sumoylating Smad3. In the present study, we examined the molecular mechanisms of Smad3 sumoylation during PIASy-mediated suppression of TGF- $\beta$  signaling. We found that small-interfering RNA-mediated reduction of endogenous PIASy expression enhanced TGF- $\beta$ -induced gene expression. Importantly, coexpression of Smad3 with PIASy and SUMO1 affected the DNA-binding activity of Smad3. Furthermore, coexpression of Smad3 with PIASy and SUMO1 stimulated the nuclear export of Smad3. Finally, fluorescence resonance energy transfer analyses revealed that Smad3 interacted with SUMO1 in the cytoplasm. These results suggest that PIASy regulates TGF- $\beta$ /Smad3-mediated signaling by stimulating sumoylation and nuclear export of Smad3.

*Keywords:* Smad3; TGF- $\beta$ ; PIASy; sumoylation; nuclear export

## Introduction

Transforming growth factor- $\beta$  (TGF- $\beta$ ) signaling is mediated through cell membrane transmembrane receptors (T $\beta$ Rs) located at the cell surface. These receptors are serine/threonine kinases, which in turn use the highly conserved members of the Sma- and MAD-related (Smad) family of transcription factors to transduce their signals to the nucleus. Two of the receptor-regulated Smads (R-Smads), Smad2 and Smad3, transduce signals for TGF- $\beta$ . On the other hand, Smad4, a co-Smad, acts as a heterodimeric partner for Smad2 and Smad3 for efficient DNA binding and transcriptional activation. When T $\beta$ Rs are activated by binding of their cognate ligands, Smad2 and Smad3 are phosphorylated by the T $\beta$ R-I serine/threonine kinase. Phosphorylated Smad2 and Smad3 then form stable hetero-complexes with Smad4 that translocate into the nucleus and activate transcription [1, 2].

Sumoylation occurs by the covalent attachment of SUMO, a ubiquitin-related polypeptide, to lysine residues. Sumoylation regulates the functions of the target proteins by changing their subcellular localization, protein-protein interactions and/or stability [3]. In a previous study, we demonstrated direct physical and functional interactions between Smad3 and protein inhibitor of activated STATy (PIASy) [4, 5]. PIAS proteins were first identified as specific cofactors that inhibit DNA binding of and transcriptional activation by the STAT family of transcription factors [6]. They have since been shown to also function as E3 SUMO ligases.

To date, five mammalian PIAS proteins have been identified, namely PIAS1, PIAS3, PIASx $\beta$ , PIASx $\gamma$  and PIASy, and they all function as SUMO E3 ligases with relative substrate specificities [7]. The PIAS family E3 ligases also induce sumoylation of Smad4 and modify TGF- $\beta$  family signaling pathways [8,9].

In the present report, we investigated the roles of PIASy-mediated sumoylation of Smad3 in TGF- $\beta$  signaling. First, we found that small-interfering RNA (siRNA)-mediated reduction of PIASy expression enhanced TGF- $\beta$ -induced gene expression in Hep3B cells. Importantly, coexpression of Smad3 with PIASy and SUMO1 reduced the DNA-binding activity of Smad3. Furthermore, coexpression of Smad3 with PIASy and SUMO1 stimulated the nuclear export of Smad3 as evaluated by confocal microscopy. Finally, fluorescence resonance energy transfer (FRET) analyses revealed that Smad3 interacted with SUMO1 in the cytoplasm. These results suggest that PIASy regulates TGF- $\beta$ /Smad3-mediated signaling by stimulating sumoylation and nuclear export of Smad3.

## Materials and Methods

*Reagents and antibodies.* Human recombinant TGF- $\beta$ 1 was purchased from Strathmann Biotech GmbH (Hamburg, Germany). Expression vectors, Smad3, T $\beta$ R-I (T204D), SUMO1, PIASy wild-type (WT), PIASy CA and p3TP-LUC were described previously [4, 5, 10]. Yellow-emitting (YFP)-SUMO1, cyan-emitting (CFP)-Smad3 and SUMO1-Smad3 constructs were generated by PCR methods and sequenced as described previously [11, 12]. Anti-HA, anti-Smad3, and anti-phospho-Smad3 (Ser 433/435) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG M2 and anti-HA antibody was purchased from Sigma (St Louis, MO). Anti-SUMO1 antibody was purchased from Medical & Biological Laboratories (Nagoya, Japan).

*Cell culture, transfection, siRNA, RT-PCR and quantitative real-time PCR.* The human hepatoma cell line Hep3B, human skin keratinocyte cell line HaCaT and human embryonic kidney carcinoma cell line 293T were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) [4]. Hep3B and HaCaT cells were transfected using jetPEI (PolyPlus-transfection, Strasbourg, France) according to the manufacturer's instructions. 293T was transfected by the standard calcium precipitation protocol [13]. A stable Hep3B transfectant expressing Myc-tagged PIASy or PIASy CA was established as described previously [4]. Hep3B cells were treated with an siRNA-

Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as previously described [14]. siRNAs targeting human PIASy used in this study were as follows: siPIASy, 5'-UUCUACCUGCAGAUGAACGAGAAAG-3'. At 24h after transfection, the cells were treated with TGF- $\beta$  (100 pM) for the indicated periods. The cells were harvested and total RNAs were prepared, and RT-PCR was as described previously [4]. Quantitative real-time PCR analyses of PAI-1 as well as the control G3PDH mRNA transcripts were carried out using the assay-on-demand<sup>TM</sup> gene-specific fluorescently labeled TaqMan MGB probe in an ABI Prism 7000 sequence detection system (Applied Biosystems).

*Immunoprecipitation and immunoblotting.* The immunoprecipitation and western blotting assays were performed as described previously [13].

*DNA-binding assay for Smad3.* The transfected 293T cells were then treated with or without TGF- $\beta$  (100 pM) for the indicated period and cell extracts were prepared as described [14]. To measure Smad3 DNA-binding, pull-down assays were performed using the immobilized Smad3 Consensus oligonucleotide-Sepharose (Santa Cruz Biotechnology) -Sepharose as described previously [14].

*Confocal imaging and FRET measurements.* Hep3B cells transfected with CFP-Smad3, YFP-SUMO1 and/or HA-PIASy by using jetPEI [11, 15]. At 24h after transfection, the cells were stimulated by TGF- $\beta$  (100 pM) for the indicated periods. The cells were fixed with a solution containing 4% paraformaldehyde and reacted with an anti-HA monoclonal antibody

(Sigma). The cells were then reacted with Alexa Fluor 647 anti-mouse IgG antibody (Invitrogen) and observed under a confocal laser fluorescent microscope. Images were obtained by using a Zeiss LSM 510 laser scanning microscope with an Apochromat x63/1.4 oil immersion objective and x4 zoom. CFP and YFP excitation was at 458 and 514 nm, respectively, and emitted fluorescence was measured at  $485 \pm 30$  nm (for CFP) and  $545 \pm 40$  nm (for YFP). Acceptor photobleaching was performed by excitation at 514 nm. Acceptor (YFP) emission at donor (CFP) excitation was corrected in some cases for direct activation of the acceptor. FRET measurements were performed as described previously [11, 16, 17]. The ratio image of YFP/CFP was created with the Scion Image (Scion Corporation) and used to represent FRET efficiency.

## Results and Discussion

### *PIASy regulates TGF- $\beta$ -induced gene expression and sumoylation of Smad3*

In a previous study, we showed that ectopic expression of PIASy negatively regulates TGF- $\beta$ -induced transcriptional activation [4]. To further clarify the physiological significance of this effect of PIASy on TGF- $\beta$ -induced transcriptional activation, we used a siRNA to reduce endogenous PIASy expression in Hep3B cells. Hep3B cells were transfected with a PIASy-specific siRNA or control siRNA, and their total RNAs were analyzed by RT-PCR. Reduced expression of PIASy was confirmed (Fig. 1A). As shown in Fig. 1B, TGF- $\beta$ -induced PAI-1 mRNA expression was significantly enhanced in PIASy siRNA-treated Hep3B cells, although PAI-1 mRNA expression was up-regulated by PIASy siRNA treatment even in the absence of TGF- $\beta$  stimulation, suggesting that PIASy may also influences the basal level of TGF- $\beta$ -induced transcriptional activation. Thus, endogenous PIASy regulates TGF- $\beta$ -induced transcriptional activation in Hep3B cells.

PIASy has the RING domain, which has been identified as an important functional determinant of ubiquitin and SUMO E3 ligases [18, 19, 20]. We previously demonstrated that a PIASy RING mutant (PIASy CA) failed to repress bone morphogenetic protein-mediated signaling [5]. We then examined the effect of PIASy CA expression on TGF- $\beta$ -induced

transcriptional activation using p3TP-LUC, which is one of the standard reporters for assessing TGF- $\beta$  activity [4]. As shown in Fig. 1B, PIASy WT but not CA showed an effective suppression of p3TP-LUC activation by an active form of T $\beta$ R-I (T $\beta$ R-I T204D) in 293T cells. To examine the effect of PIASy CA on TGF- $\beta$ -mediated signaling under more physiological conditions, we transiently expressed PIASy WT or CA together with p3TP-LUC in HaCaT cells, and cells were treated with or without TGF- $\beta$ . As shown in Fig. 1C, TGF- $\beta$ -induced p3TP-LUC expression was significantly reduced in PIASy WT but not CA expressed HaCaT cells. We next established a stable Hep3B transfectant expressing PIASy WT or CA. As shown in Fig. 1D, TGF- $\beta$ -mediated endogenous PAI-1 mRNA expression was markedly decreased in PIASy WT but not CA expressed Hep3B cells, suggesting that the RING domain plays an important role in PIASy-mediated repression of TGF- $\beta$ -induced transactivation.

We also examined the involvement of the RING domain on PIASy-mediated sumoylation of Smad3. Expression vectors encoding FLAG-tagged Smad3 and T $\beta$ R-I T204D together with or without SUMO1 and HA-tagged PIASy WT or CA were transiently transfected into 293T cells. The transfected 293T cells were lysed and immunoprecipitated with an anti-FLAG antibody. The immunoprecipitates obtained were analyzed by western blotting with an anti-FLAG or anti-SUMO1 antibody. As shown in Fig. 1E, PIASy WT but not CA induced sumoylation of Smad3, suggesting that the RING domain on PIASy is critical for sumylation

of Smad3. However, a recent study demonstrated that PIASy stimulates protein sumoylation independent of the RING domain [21]. Therefore, the involvement of the RING domain of PIASy in the PIASy-mediated sumoylation would need further and more detailed investigations.

#### *Sumoylation of Smad3 influences its DNA-binding activity*

To identify the molecular mechanisms underlying the effect of sumoylation during PIASy-mediated suppression of TGF- $\beta$ /Smad3-mediated transcriptional activation, we performed DNA pull-down assays using an immobilized Smad3 consensus oligonucleotide. Expression vectors encoding FLAG-tagged Smad3 with or without T $\beta$ R-I T204D, SUMO1 or HA-tagged PIASy were transiently transfected into 293T cells. The transfected 293T cells were lysed and subjected to DNA pull-down assays with Smad3 consensus oligonucleotide-conjugated Sepharose. The precipitates were subjected to western blot analysis with an anti-FLAG antibody. As shown in Fig. 2, Smad3-DNA complex formation was observed in T $\beta$ R-I T204D-transfected 293T cells. This complex formation was competed by an excessive amount of the Smad3 consensus oligonucleotide but not a mutant oligonucleotide. Furthermore, Smad3 was phosphorylated at Ser-433/435 by coexpression of T $\beta$ R-I T204D and the Smad3-DNA complex formation was not affected by additional expression of

SUMO1 or PIASy. Importantly, expression of both SUMO1 and PIASy together with Smad3 and T $\beta$ R-I T204D resulted in a marked reduction of the DNA-bound Smad3 content. These results indicate that sumoylation of Smad3 may influence its DNA-binding activity, but had no effect on its phosphorylation.

*Sumoylation of Smad3 by PIASy stimulates its nuclear export after TGF- $\beta$  stimulation*

Next, we investigated whether sumoylation of Smad3 affected its subcellular localization by examining the localization of Smad3 when coexpressed with PIASy or SUMO1 in Hep3B cells with or without TGF- $\beta$  treatment. As shown in Fig. 3A, CFP-Smad3 was localized in the cytoplasm in Hep3B cells in the absence of TGF- $\beta$ . In contrast, CFP-Smad3 translocated into the nucleus in the presence of TGF- $\beta$ . YFP-SUMO1 was localized throughout the cytoplasm and nucleus, although its localization was mainly in the nucleus. PIASy was localized in the nucleus as described previously. Even when CFP-Smad3 was coexpressed with both PIASy and YFP-SUMO1, CFP-Smad3 was detected in the cytoplasm in the absence of TGF- $\beta$  (Fig. 3B). However, its pattern was a little more diffuse. Similarly, YFP-SUMO1 seemed to be a little more diffusely localized in the nucleus in the presence of PIASy. These localizations appeared to be influenced by SUMO peptides added onto cellular proteins, including Smad3, by PIASy. Importantly, CFP-Smad3 coexpressed with PIASy and YFP-SUMO1 was still

localized in the cytoplasm after 30 min of TGF- $\beta$  treatment, although CFP-Smad3 coexpressed with PIASy and YFP-SUMO1 was localized in the nucleus after 10 min of TGF- $\beta$  treatment (Fig. 3B). These results show that sumoylation of Smad3 stimulates its nuclear export after TGF- $\beta$  stimulation. Furthermore, accumulation of PIASy was observed at the peripheral rim of the nucleus after 10 min of TGF- $\beta$  treatment. Differences in subnuclear localizations are known to be involved in changes of gene localizations during transcriptional activation and/or repression. Silenced genes appear to colocalize with heterochromatic chromatin in foci located near the nuclear periphery. The immunoglobulin gene has been shown to localize at the nuclear periphery in primary T cells that do not express this gene, whereas it is localized away from the nuclear periphery in pre-B cells in which the gene is activated [22]. Furthermore, the nuclear localizations of genes silenced by the transcription factor Ikaros are altered in comparison to cells that do not express Ikaros [23].

To further clarify the molecular interactions between Smad3, PIASy and SUMO1, we employed FRET analysis (Fig. 4). The occurrence of FRET was confirmed by a photobleaching experiment. The intensities of both CFP-Smad3 and YFP-SUMO1 were monitored before and after photobleaching. As expected, photobleaching of YFP-SUMO1 resulted in a marked increase in CFP-Smad3 intensity, suggesting that the intramolecular binding of Smad3 to SUMO1 by PIASy brings CFP close to YFP, thereby increasing FRET from CFP to YFP (Fig. 4A and B). Importantly, FRET between YFP-SUMO1 and CFP-Smad3

was preferentially observed in the cytoplasm when coexpressed with PIASy and treated with TGF- $\beta$  for 30 min. No significant FRET was observed in the cytoplasm without ectopic expression of PIASy, even when cells were treated with TGF- $\beta$  for 30 min. Therefore, sumoylation of Smad3 by PIASy stimulates its immediate export from the nucleus.

To further clarify the effect of sumoylation of Smad3 on its subcellular localization, we used a SUMO1-Smad3 fusion protein, which tagged Smad3 with SUMO1 at the N-terminus. It has been recently shown that SUMO-fusion proteins behave similarly to that of physiologically sumoylated proteins [12, 24]. Expression vectors for FLAG-tagged Smad3 or SUMO1-Smad3 were transfected into Hep3B cells and treated with or without TGF- $\beta$ . SUMO1-Smad3 fusion protein was well observed in cells as well as Smad3 by immunostaining, although its protein detection by immunoblotting was lower than that of Smad3 (Fig. 4C). As shown in Fig. 4C (right panels), both proteins were localized in the cytoplasm in Hep3B cells in the absence of TGF- $\beta$ . After TGF- $\beta$  stimulation, Smad3 translocated into the nucleus, however, SUMO1-Smad3 was still localized in the cytoplasm. These data strongly suggest that sumoylation of Smad3 may affect nuclear anchoring of Smad3.

#### *Concluding remarks*

In the present study, we have demonstrated that endogenous PIASy acts as a negative

regulator of TGF- $\beta$ -mediated transcription in Hep3B cells. Smad3 sumoylated by PIASy failed to bind to its specific consensus DNA oligonucleotide. Furthermore, overexpression of Smad3 together with PIASy and SUMO1 stimulated immediate export of Smad3 from the nucleus and the amount of SUMO1-bound Smad3 in the cytoplasm increased. Thus, the results of the present study indicate that sumoylation of Smad3 may play a regulatory role in TGF- $\beta$  signaling pathways by controlling its subcellular localization. Indeed, SUMO1-Smad3 fusion protein was localized in the cytoplasm with or without TGF- $\beta$  stimulation (Fig. 4C). This fact also supports that SUMO1 modification of Smad3 regulates its subcellular localization.

Recent accumulated studies have demonstrated that many proteins involved in cell-cycle regulation, proliferation, apoptosis and DNA repair are targets for sumoylation, and indicated that alterations in sumoylation could ultimately have impacts on cell growth, cancer development and drug responsiveness [9, 25]. Therefore, regulation of sumoylation is tightly linked to homeostasis, and dysregulation of sumoylation may play significant roles in pathological disorders. For example, Ubc9, an E2-conjugating enzyme required for sumoylation, has been found to be upregulated in an increasing number of human malignancies, including ovarian carcinoma, melanoma and lung adenocarcinoma [26]. Thus, it is likely that perturbation of the SUMO modification/deconjugation system will manifest itself in disease processes and represent a potential target for clinical applications against

diseases such as cancer.

## **Acknowledgements**

This study was supported in part by Sankyo Foundation of Life Science, Industrial Technology Research Grant Program in 2005 from New Energy and Industrial Technology Development Organization (NEDO) of Japan and Grant-in-Aid for scientific research from Ministry of Education, Culture, Sports, Science and Technology of Japan.

## References

- [1] C.H. Heldin, K. Miyazono, P. ten Dijke, TGF-beta signalling from cell membrane to nucleus through SMAD proteins, *Nature* 390 (1997) 465-471.
- [2] Y. Shi, J. Massague, Mechanisms of TGF-beta signaling from cell membrane to the nucleus, *Cell* 113 (2003) 685-700.
- [3] R.T. Hay, SUMO: a history of modification, *Mol. Cell.* 18 (2005) 1-12.
- [4] S. Imoto, K. Sugiyama, R. Muromoto, N. Sato, T. YamamotoT. Matsuda, Regulation of transforming growth factor-beta signaling by protein inhibitor of activated STAT, PIASy through Smad3, *J. Biol. Chem.* 278 (2003) 34253-34258.
- [5] S. Imoto, K. Sugiyama, T. YamamotoT. Matsuda, The RING domain of PIASy is involved in the suppression of bone morphogenetic protein-signaling pathway, *Biochem. Biophys. Res. Commun.* 319 (2004) 275-282.
- [6] K. Shuai, B. Liu, Regulation of gene-activation pathways by PIAS proteins in the immune system, *Nat. Rev. Immunol.* 5 (2005) 593-605.
- [7] D. Schmidt, S. Muller, PIAS/SUMO: new partners in transcriptional regulation, *Cell. Mol. Life Sci.* 60 (2003) 2561-2574.
- [8] P.S. Lee, C. Chang, D. Liu, R. Derynck, Sumoylation of Smad4, the common Smad mediator of transforming growth factor-beta family signaling, *J. Biol. Chem.* 278 (2003)

27853-27863.

[9] X. Lin, M. Liang, Y.Y. Liang, F.C. Brunicardi, X.H. Feng, SUMO-1/Ubc9 promotes nuclear accumulation and metabolic stability of tumor suppressor Smad4, *J. Biol. Chem.* 278 (2003) 31043-31048.

[10] H. Yamamoto, M. Ihara, Y. Matsuura, A. Kikuchi, Sumoylation is involved in beta-catenin-dependent activation of Tcf-4, *EMBO J.* 22 (2003) 2047-2059.

[11] C. Miyagi, S. Yamashita, Y. Ohba, H. Yoshizaki, M. Matsuda, T. Hirano, STAT3 noncell-autonomously controls planar cell polarity during zebrafish convergence and extension, *J. Cell. Biol.* 166 (2004) 975-981.

[12] R. Muromoto, M. Ishida, K. Sugiyama, Y. Sekine, K. Oritani, K. Shimoda, T. Matsuda T, Sumoylation of Daxx regulates IFN-induced growth suppression of B lymphocytes and the hormone receptor-mediated transactivation, *J. Immunol.* 177 (2006) 1160-1170.

[13] T. Matsuda, T. Yamamoto, A. Muraguchi, F. Saatcioglu, Cross-talk between transforming growth factor-beta and estrogen receptor signaling through Smad3, *J. Biol. Chem.* 276 (2001) 42908-42914.

[14] Y. Sekine, O. Ikeda, Y. Hayakawa, S. Tsuji, S. Imoto, N. Aoki, K. Sugiyama T. Matsuda, DUSP22/LMW-DSP2 regulates estrogen receptor-alpha-mediated signaling through dephosphorylation of Ser-118, *Oncogene* 26 (2007) 6038-6049.

- [15] R. Muromoto, Y. Sekine, S. Imoto, O. Ikeda, T. Okayama, N. Sato, T. Matsuda, BART is essential for nuclear retention of STAT3, *Int. Immunol.* 20 (2008) 395-403.
- [16] A. Miyawaki, R.Y. Tsien, Monitoring protein conformations and interactions by fluorescence resonance energy transfer between mutants of green fluorescent protein, *Methods Enzymol.* 327 (2000) 472-500.
- [17] R.E. Itoh, K. Kurokawa, Y. Ohba, H. Yoshizaki, N. Mochizuki, M. Matsuda, Activation of rac and cdc42 video imaged by fluorescent resonance energy transfer-based single-molecule probes in the membrane of living cells, *Mol. Cell. Biol.* 22 (2002) 6582-6591.
- [18] T. Kahyo, T. Nishida, H. Yasuda, Involvement of PIAS1 in the sumoylation of tumor suppressor p53, *Mol Cell* 8 (2001) 713-718.
- [19] V. Nelson, G.E. Davis, S.A. Maxwell, A putative protein inhibitor of activated STAT PIASy interacts with p53 and inhibits p53-mediated transactivation but not apoptosis, *Apoptosis* 6 (2001) 221-234.
- [20] S. Sachdev, L. Bruhn, H. Sieber, A. Pichler, F. Melchior, R. Grosschedl, PIASy, a nuclear matrix-associated SUMO E3 ligase, represses LEF1 activity by sequestration into nuclear bodies, *Genes Dev* 15 (2001) 3088-3103.
- [21] Z. Deng, M. Wan, G. Sui, PIASy-mediated sumoylation of Yin Yang 1 depends on their interaction but not the RING finger, *Mol. Cell. Biol.* 27 (2007) 3780-3792.
- [22] S.T. Kosak, J.A. Skok, K.L. Medina, R. Riblet, M.M. Le Beau, A.G. Fisher, H. Singh,

Subnuclear compartmentalization of immunoglobulin loci during lymphocyte development, Science 296 (2002) 158-162.

[23] B.S. Cobb, S. Morales-Alcelay, G. Kleiger, K.E. Brown, A.G. Fisher, S.T. Smale, Targeting of Ikaros to pericentromeric heterochromatin by direct DNA binding, Genes Dev. 14 (2000) 2146-2160.

[24] J.S. Steffan, N. Agrawal, J. Pallos, E. Rockabrand, L.C. Trotman, N. Slepko, K. Illes, T. Lukacsovich, Y.Z. Zhu, E. Cattaneo, P.P. Pandolfi, L.M. Thompson, J.L. Marsh. 2004. SUMO modification of Huntingtin and Huntington's disease pathology. Science 304 (2004) 100-104.

[25] J.S. Seeler, A. Dejean, Nuclear and unclear functions of SUMO, Nat. Rev. Mol. Cell. Biol. 4 (2003) 690-699.

[26] Y.Y. Mo, S.J. Moschos, Targeting Ubc9 for cancer therapy. Expert Opin. Ther. Targets. 9 (2005) 1203-1216.

## Figure legends

Fig. 1. PIASy regulates TGF- $\beta$ -induced gene expression and sumoylation of Smad3.

(A) Hep3B cells in a 6-well plate were transfected with control or PIASy siRNA using Lipofectamine2000. RT-PCR analysis was performed using PIASy or G3PDH primers. Hep3B cells in a 6-well plate were transfected with control or PIASy siRNA, and cells were stimulated with TGF- $\beta$  (100 pM) for the indicated periods. PAI-1 expression level was quantified by reverse transcription and quantitative real-time PCR analysis as described in Materials and Methods. Data represent the levels of PAI-1 mRNA normalized to that of a G3PDH internal control and are expressed relative to the value at time zero. Results are representative of three independent experiments, with standard deviations. \*,  $P < 0.03$  \*\*,  $P < 0.01$ .

(B) 293T cells in a 12-well plate were transfected with T $\beta$ R-I (T204D)(1  $\mu$ g) and p3TP-LUC (0.5  $\mu$ g) together with PIASy WT or CA (0.1, 0.3, 1.0  $\mu$ g). At 24h after transfection, the cells were harvested, and luciferase activities were measured. Results are representative of three independent experiments, with standard deviations.

(C) HeCaT cells in a 6-well plate were transfected with p3TP-LUC (0.5  $\mu$ g) together with PIASy WT or CA (1.0  $\mu$ g). At 36 h after transfection, the cells were stimulated with TGF- $\beta$  (100 pM) for additional 12 h. The stimulated cells were harvested, and luciferase activities

were measured. Results are representative of three independent experiments, with standard deviations.

(D) Hep3B/vector, Hep3B/PIASy WT or Hep3B/PIASy CA cells in a 12-well plate were stimulated with TGF- $\beta$  (100 pM) for 6 h. RT-PCR analysis was performed using the respective primers. An aliquot of total cell lysates (TCL) was analyzed by immunoblotting using anti-Myc antibody.

(E) 293T cells ( $1 \times 10^7$  cells) were transfected with FLAG-Smad3 (10  $\mu$ g) and T $\beta$ R-I (T204D)(3  $\mu$ g) together with or without SUMO1 (3  $\mu$ g) and HA-PIASy WT or CA (10  $\mu$ g). At 36 h after transfection, the cells were lysed, and immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-FLAG (upper) or anti-SUMO1 antibody (middle). TCL (1%) was blotted with anti-HA antibody (lower).

Fig. 2. Sumoylation of Smad3 influences its DNA binding activity.

293T cells ( $1 \times 10^7$  cells) were transfected with FLAG-Smad3 (10  $\mu$ g) and/or T $\beta$ R-I (T204D)(3  $\mu$ g), SUMO1 (3) and HA-PIASy (10  $\mu$ g). At 36 h after transfection, the cells were lysed, and pull-down assays were performed using the immobilized Smad3 Consensus oligonucleotide-Sepharose. Specifically bound proteins were analyzed by Western blot analysis using anti-FLAG antibody (upper). The cells were also immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-FLAG (middle) or anti-pSmad3 antibody (bottom).

Fig.3. Subcellular localization of Smad3 in the presence of PIASy and SUMO1

(A) Hep3B cells in a 6-well plate were transfected with CFP-Smad3 (cyan), YFP-SUMO1 (yellow), or HA-PIASy. The HA-PIASy-transfected cells were fixed and stained with anti-HA monoclonal antibody, then Alexa647-conjugated anti-mouse IgG antibody (red).

(B) Hep3B cells in a 6-well plate were transfected with CFP-Smad3 (cyan), YFP-SUMO1 (yellow), and HA-PIASy, and left untreated or treated with TGF- $\beta$  (100 pM) for the indicated periods. The cells were fixed and stained with anti-HA monoclonal antibody, then Alexa647-conjugated anti-mouse IgG antibody (red). Approximately 100 cells were classified according to cyan signals in the cytoplasm and nucleus. In 60 % of Smad3/SUMO1/PIASy-expressing cells, Smad3 was localized in the cytoplasm after TGF- $\beta$  stimulation (30min), whereas Smad3 was localized in the nucleus after TGF- $\beta$  stimulation (30 min) in more than 90 % of Smad3-expressing cells.

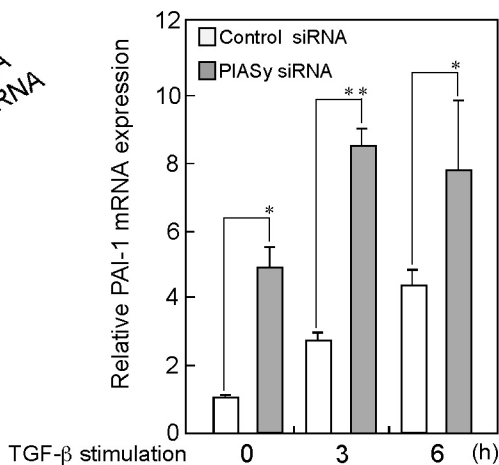
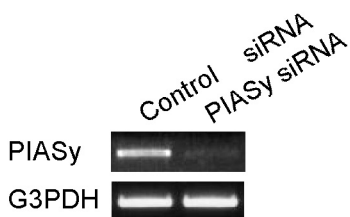
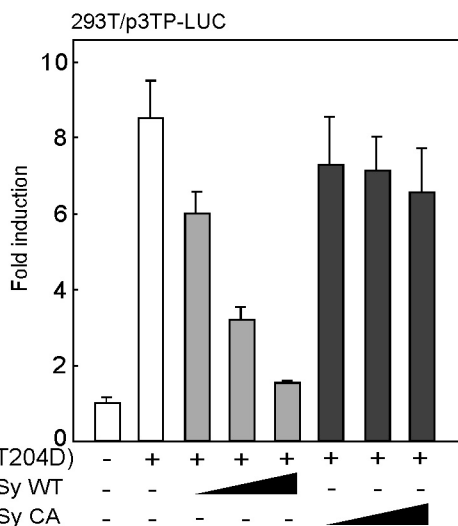
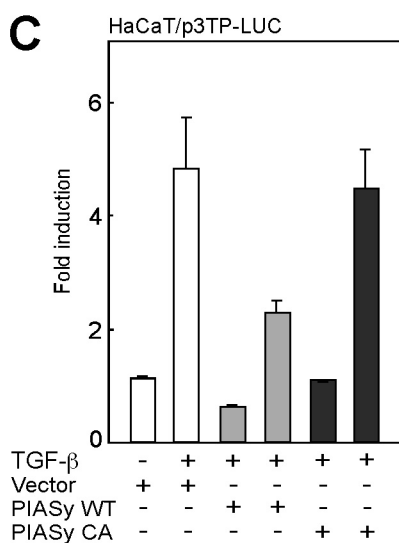
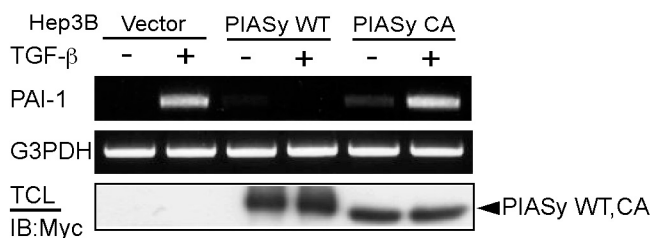
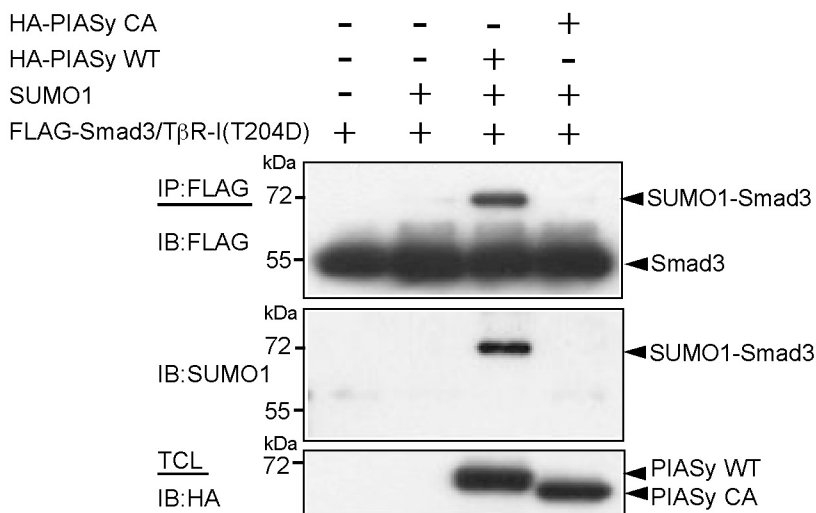
Fig.4. FRET occurs between CFP-Smad3 and YFP-SUMO1 in the presence of PIASy.

(A) Hep3B cells in a 6-well plate were transfected with CFP-Smad3 (cyan) and YFP-SUMO1 (yellow) together with HA-PIASy, and left untreated or treated with TGF- $\beta$  (100 pM) for 30 min. The cells were then exposed to maximal intensity laser light at 514 nm for 60 sec (acceptor photobleaching). The cells were also stained with anti-HA monoclonal antibody,

then Alexa647-conjugated anti-mouse IgG antibody (red). The representative cell images of pre- and postbleaching are shown.

(B) Hep3B cells in a 6-well plate were transfected with CFP-Smad3 (cyan) and YFP-SUMO1 (yellow) together with or without HA-PIASy, and left untreated or treated with TGF- $\beta$  (100 pM) for the indicated periods. The cells were then exposed to maximal intensity laser light at 514 nm for 60 sec (acceptor photobleaching). Average increase of donor (CFP) fluorescence after photobleaching indicated FRET occurring between CFP-Smad3 and YFP-SUMO1 in the cytoplasm or nucleus. \*,  $P < 0.03$  \*\*,  $P < 0.01$ .

(C) Domain structure of SUMO1-Smad3 is schematically shown. Hep3B cells in a 6-well plate were transfected with Smad3 or SUMO1-Smad3 and treated with or without TGF- $\beta$  (100 pM, 30 min). TCL (1%) of transfectants was analyzed by immunoblotting using anti-Smad3 antibody (left panels). (S), short exposure 1-2 min; (L), long exposure 5-10 min. The transfected cells were fixed and stained with anti-Smad3 antibody, and visualized with FITC-conjugated anti-rabbit antibody.

**A****B****C****D****E****Figure 1**

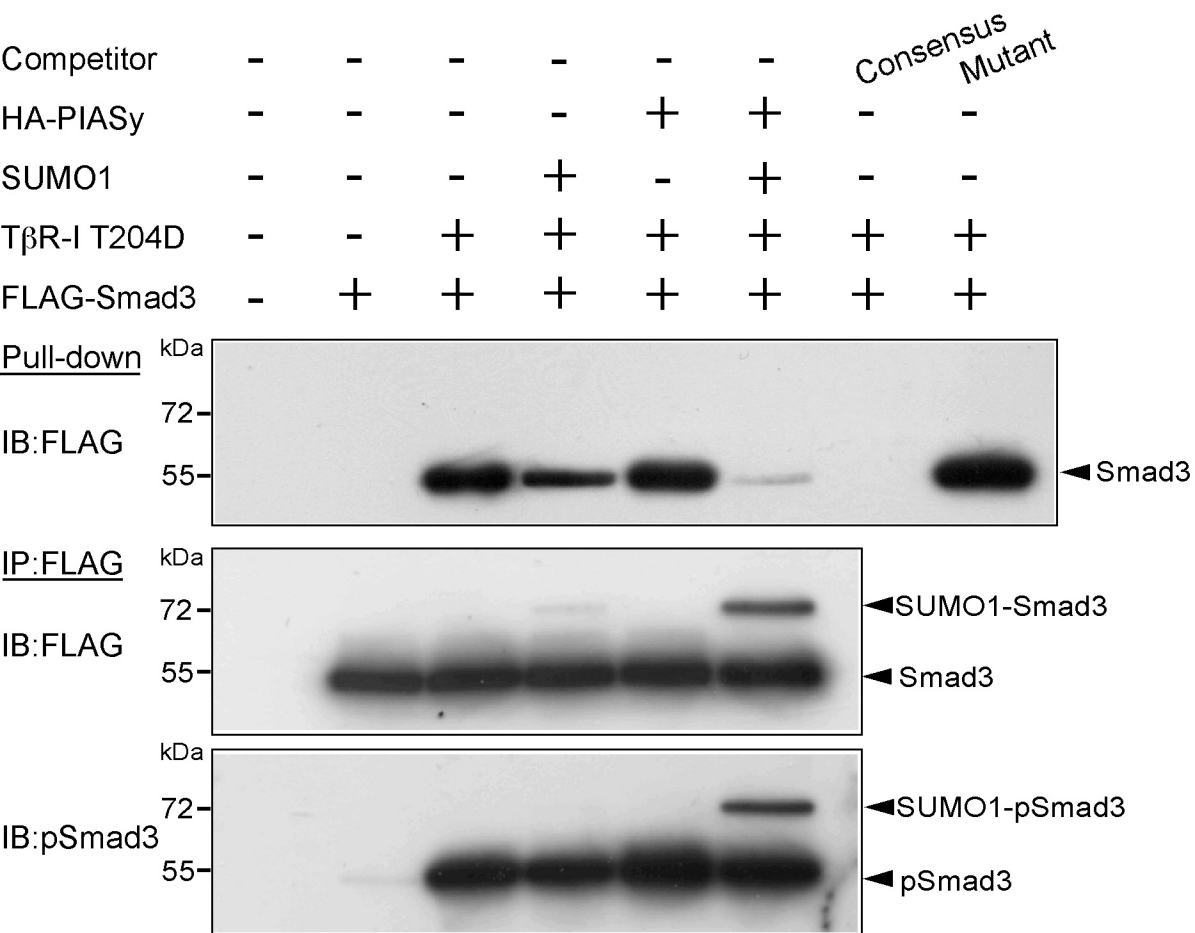


Figure 2

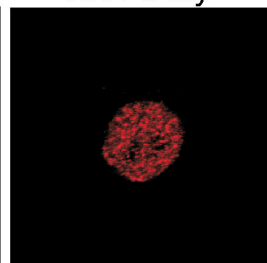
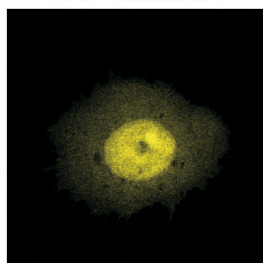
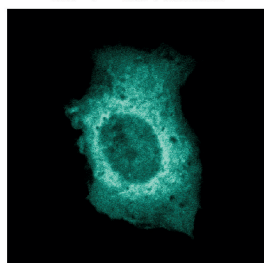
**A**TGF- $\beta$   
stimulation

CFP-Smad3

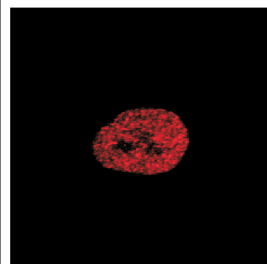
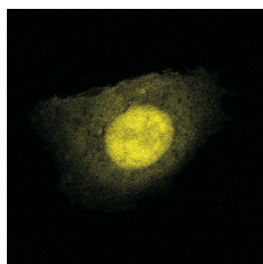
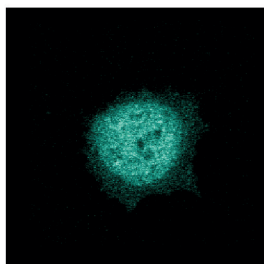
YFP-SUMO1

HA-PIASy

(-)

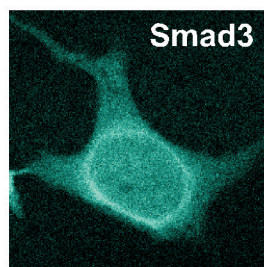


30 min

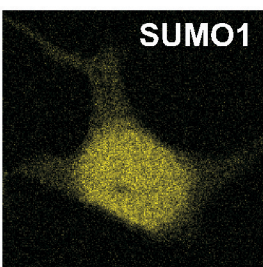
**B**TGF- $\beta$   
stimulation

CFP-Smad3/YFP-SUMO1/HA-PIASy

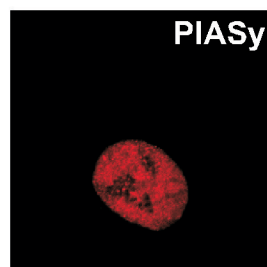
(-)



Smad3

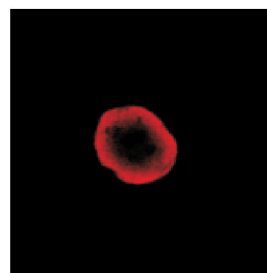
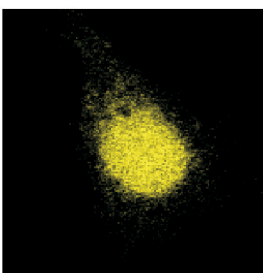
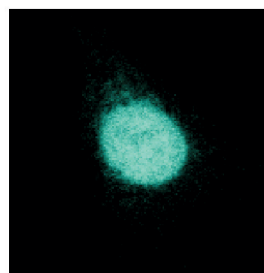


SUMO1



PIASy

10 min



30 min

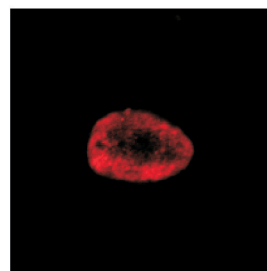
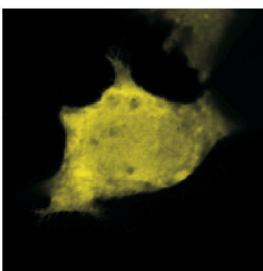
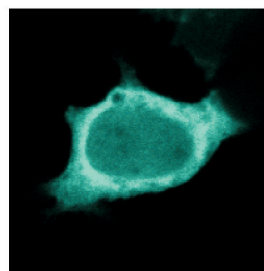


Figure 3

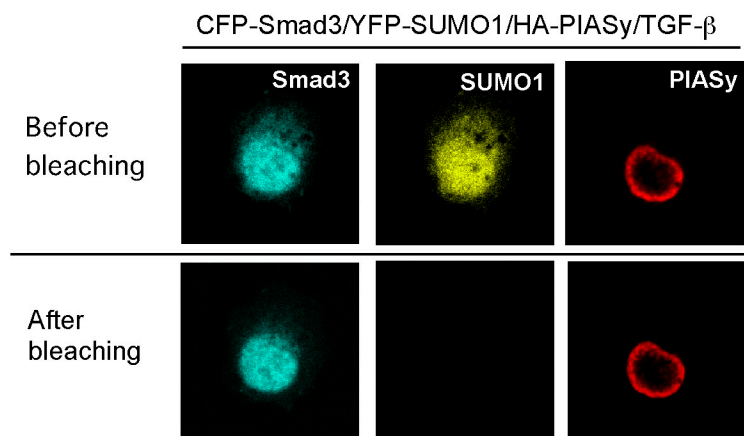
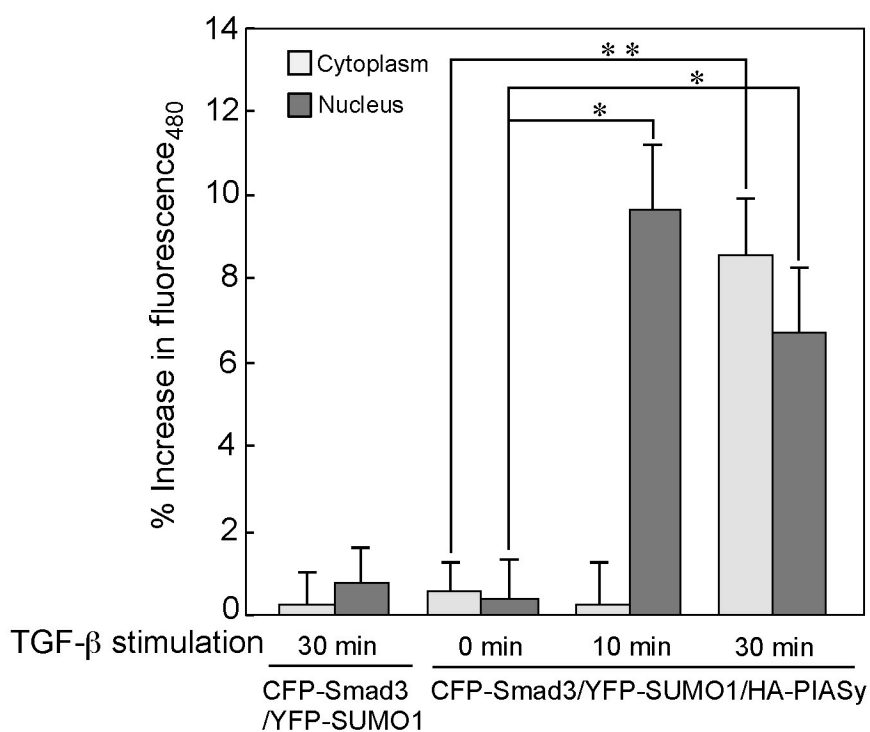
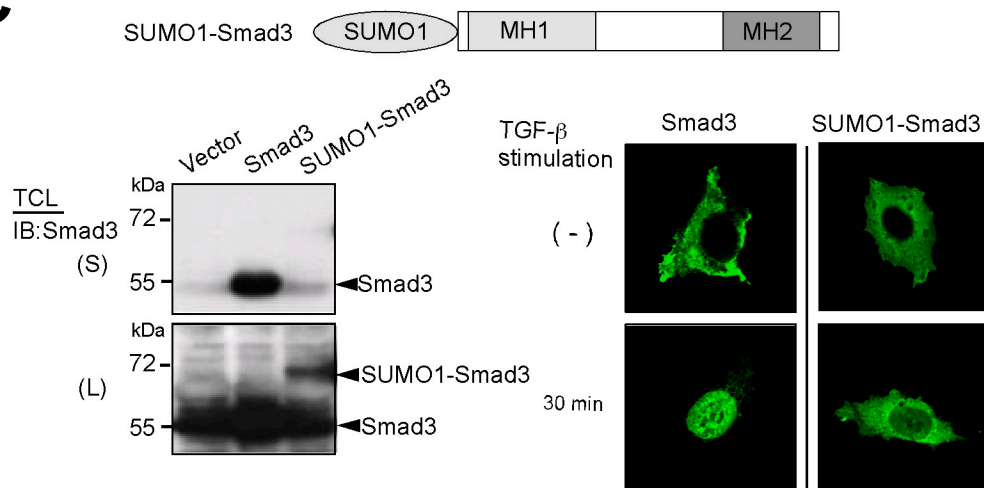
**A****B****C**

Figure 4