*Title:* **Roles for Lysine residues of the MH2 Domain of Smad3 in Transforming Growth Factor-β Signaling**

*Running title:* Roles for lysine residues in Smad3 MH2 domain

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*Abbreviations:* TGF, transforming growth factor; Smad, Sma and MAD-related protein; MH, Mad homology; TβR, TGF-β receptor

**Abstract**

Sma and MAD-related protein 3 (Smad3) plays a key role in the intracellular signaling of the transforming growth factor-β (TGF-β) family of growth factors, which exhibits a diverse set of cellular responses, including cell proliferation and differentiation. Smad3 has the N-terminal Mad homology (MH) 1 and the C-terminal MH2 domains. The N-terminal MH2 domain is essential for the TGF-β-induced transcriptional activation, because the MH2 domain of Smad3 is involved in the interactions with several transcriptional cofactors as well as the type I TGF-β receptor (TβR-I). In this study, we examined the roles for four lysine residues (Lys-333, Lys-341, Lys-378, and Lys-409) in the Smad3 MH2 domain. Mutation of the lysine (K)-378 to arginine (R) (K378R) abolished the interaction with TβR-I, phosphorylation, transcriptional activation by an active TβR-I. The K341R mutant also failed to stimulate TGF-β-induced transcription by resting in the cytoplasm. However, the R409R mutant showed a higher transcriptional activity by stronger interactions with co-activators, such as p300/CBP. Furthermore, both the K341R and K378R mutants act as dominant-negative inhibitors in the TGF-β-induced target genes of endogenous TGF-β signal. Thus, the lysine residues of Smad3 MH2 domain play important roles in the transcriptional regulation of TGF-β signals through TβR-I.

## 1. Introduction

TGF-β family of growth factors regulates diverse biological processes. TGF-β inhibits proliferation of epithelial, endothelial and haematopoietic cells, regulates the differentiation of immune, neuronal, mesenchymal and epithelial cell types and modulates their apoptotic response [1-3]. TGF-β signaling is mediated through cell membrane transmembrane receptors located at the cell surface (TβRs) that are serine / threonine kinases, which in turn use the highly conserved members of the Smad family of transcription factors to transduce their signals to the nucleus [4, 5]. Two of the receptor-regulated Smads (R-Smads), Smad2 and Smad3, transduce signals for TGF-β. Co-Smad, Smad4, on the other hand, acts as a heterodimeric partner for Smad2 and Smad3 for efficient DNA binding and transcriptional activation [6, 7]. When TβRs are activated by the binding of their cognate ligands, Smad 2 and Smad3 are phosphorylated by the TβR-I serine-threonine kinase. Phosphorylated Smad2 and Smad3 then form stable hetero-complexes with Smad4 that translocate into the nucleus and activate transcription. Smad7, one of inhibitory Smads (I-Smads), stably interacts with activated TβR-I and inhibits TGF-β signal [8-10].

Whereas phosphorylation plays a central role in the activation of the Smad pathway, recent studies demonstrate that other types of post-transcriptional modifications of lysine residues on

Smads are also critical for TGF-β signals [11, 12]. Ubiquitylation occurs by attachment of one or several ubiquitin polypeptides to lysine residues of target proteins [13]. Proteasomal degradation of ubiquitinated R-Smads has been reported to be important for the maintenance of their steady state levels and for the shutdown of the activated Smad pathway after execution of its transcriptional roles [14]. Furthermore, I-Smads act as adaptor proteins that mediate ubiquitination and degradation of TβR-I [15]. Ubiquitination of Smad4 has been also reported to occur only in cancer cells and is thought to be associated with oncogenic mutations in the MH1 domain that lead to Smad4 inactivation [16, 17]. Recently, Lys-507 in Smad4-MH2 domain, which participates in the C-terminal phosphoserine recognition, has been also identified as a major target for ubiquitination [18].

Sumoylation occurs by the covalent attachment of SUMO, a ubiquitin-related polypeptide, to lysine residues [19]. Sumoylation also regulates the functions of a target protein by changing the subcellular localization, protein-protein interactions, and/or stability [20]. In the previous study, we demonstrated direct physical and functional interactions between Smad3 and protein inhibitor of activated STATy (PIASy) [5]. PIASy mediated sumolyation of Smad3 in the presence of Ubc9 and SUMO-1. We also demonstrated that PIASy the RING domain of PIASy plays an important role in PIASy-mediated suppression of Smad activity and the sequestration of Smad in nuclear bodies [21]. PIAS family E3 ligases also induced

sumoylation of lysine residues on Smad4 and modify TGF-β family signals [22-24].

Acetylation is also known to be a dynamic post-translational modification of lysine residues. Proteins with intrinsic histone acetyltransferase (HAT) activity act as transcriptional co-activators by acetylating histones and thereby induce an open chromatin conformation, which allows the transcriptional machinery access to promoters [25]. CBP/p300 and P/CAF proteins interact with a large number of transcription factors, such as the Smads [26-30], as well as components of the basic transcriptional machinery. Acetylation of Smad7 on lysine residues by p300 has been also reported [31]. Acetylation or mutation of these lysine residues stabilizes Smad7 and protects it from TGF-β-induced degradation.

In this report, we show the roles of four lysine residues in the Smad3-MH2 domain in TGF-β signaling. We introduced mutations to lysine residues within the Smad3-MH2 domain, which were predicted to play important roles in interactions with the receptor complexes and cofactors, and examined their capabilities of binding to TβR-I as well as of transcriptional activation of TGF-β signal. The mutation of Lys-341 and Lys-378 showed a marked decrease of transcriptional activity by an active TβR-I. The mutation of Lys-378 abolished the binding ability to TβR-I and phosphorylation by TβR-I. However, the mutation of Lys-341 showed the normal binding ability to TβR-I and phosphorylation by TβR-I, but failed to translocate into

nucleus by TGF-β signal, suggesting Lys-341 is essential for nuclear translocation of Smad3. Contrarily, the mutation of Lys-409 showed an enhanced transcriptional activation through a stronger interaction with co-activators, CBP/p300. These results suggest that the lysine residues of Smad3-MH2 domain play important roles in the TGF-β superfamily signals through interactions with receptor complexes and co-regulators.

## 2. Materials and Methods

### 2. 1. Reagents and antibodies

Human recombinant TGF-β1 was purchased from Strathmann Biotech GmbH (Hamburg, Germany). Expression vectors, FLAG-tagged murine Smad3, Smad4 [28], p300 and Ski [32] were kindly provided by Dr. K. Miyazono (Tokyo Univ., Tokyo, Japan). TβR-I (T204D), p3TP-LUC [33], CBP, SUMO-1, HA-tagged PIASy [34] and FLAG-SMRT [35] were kindly provided by Dr. J. Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY), Dr. H. Ariga, Dr. T. Taira (Hokkaido Univ., Sapporo, Japan) and Dr. H. Nakajima (Inst. Mol. Cell. Biosci., Tokyo Univ., Tokyo, Japan), respectively. Smad3 K333R, K341R, K378R and K409R mutant constructs were generated by PCR methods and sequenced (primer sequences are available upon request). To obtain mammalian expression vectors for glutathione S-transferase (GST)-fusion Smad3-MH2 (GST-Smad3-MH2) K333R, K341R, K378R and K409R mutants, Smad3-MH2 K333R, K341R, K378R or K409R mutant fragments were generated by PCR methods and sequenced (primer sequences are available upon request) and inserted into pEBgs vector [36]. Anti-HA, anti-Myc, anti-GST, anti-phospho-Smad3 (Ser 433/435) and anti-CBP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FLAG M2 antibody was purchased from Sigma (St Louis, MO). Anti-SUMO-1 antibody was purchased from Medical & Biological Laboratories (Nagoya, Japan).

Anti-acetylated lysine antibody was purchased from Cell Signaling TECHNOLOGY (Beverly, MA).

2. 2. Cell culture, transfection and luciferase assays

Human hepatoma cell line, Hep3B, was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Hep3B cells were transfected with the wild-type Smad3 (Smad3 WT), Smad3 K333R, K341R, K378R or K409R mutant and using jetPEI (PolyPlus-transfection, Strasbourg, France) according to the manufacturer's instructions. Stable Hep3B cell transformants were selected with 500 μg/ml G418 (Sigma). Hep3B cells were transfected with p3TP-LUC using jetPEI. Forty-eight hrs after transfection, the cells were treated with TGF-β (40 pM) for the indicated period. The cells were harvested and assayed for the luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions. At least three independent experiments were carried out for each assay.

2. 3. Immunoprecipitation and immunoblotting

The immunoprecipitation and Western blotting assays were performed as described previously [37]. Human embryonic kidney carcinoma cell line, 293T, was maintained in DMEM containing 10% FCS and transfected by the standard calcium precipitation protocol

[37]. The cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, containing 1% NP-40, 1 □M sodium orthovanadate, 1 □M phenylmethylsulfonyl fluoride and 10 □g/ml each of aprotinin, pepstatin and leupeptin). The immunoprecipitates from cell lysates were resolved on 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).

2. 4. Indirect Immunofluorescence

Hep3B cells were maintained in DMEM containing 10% FCS transfected with FLAG-Smad3 WT, K333R, K341R, K378R or K409R mutants by using jetPEI. Thirty hrs after transfection, the cells were stimulated by TGF-□ (40 pM). Thirty minutes after stimulation, the cells were fixed with a solution containing 4% paraformaldehyde and reacted with a rabbit anti-FLAG antibody (Sigma). The cells were then reacted with a FITC-conjugated anti-rabbit IgG (Chemicon, Temecula, CA, USA) and observed under a confocal laser fluorescent microscope. At the same time, the nuclei in the cells were stained with 4', 6-diamidino-2-phenylindole (DAPI) (Wako, Tokyo, Japan). Images were obtained by using a Zeiss LSM 510 laser scanning microscope with an Apochromat x63/1.4 oil immersion objective and x4 zoom.

2. 5. RT-PCR analysis

After 12 hrs of culture in 1% FCS, each stably Hep3B transformants was treated with TGF-β (40 pM) for 2 hrs. Total RNAs were prepared by using Iso-Gen (Nippon Gene, Tokyo, Japan) and used in RT-PCR analysis performed using RT-PCR high -Plus- Kit (TOYOBO, Tokyo, Japan). Human PAI-1 and G3PDH primers were used as described previously [5].

## 3. Results and Discussion

### 3. 1. Roles for the lysine residues in the Smad3-MH2 domain in the activation of TGF-β-mediated transcription

The Smad3-MH2 domain is known to bind to a diverse group of proteins, which do not contain a common sequence motif [38]. Our previous studies also demonstrated that PIASy interacted with Smad3 through its MH2 domain [5]. Furthermore, overexpression of Smad3-MH2 restored the inhibition by PIASy [5]. We also showed the RING domain of PIASy, which mediates sumoylation, is important for the suppression of Smad activity [21]. These findings led us to examine the roles for lysine residues in the Smad3-MH2 domain. As shown in Fig. 1A, there are four lysine residues in the Smad3-MH2 domain (Lys-333, Lys-341, Lys-378, and Lys-409). The Smad3-MH2 domain is well conserved with those of R-Smads and Co-Smad. Lys-333, Lys-341 and Lys-409 are located in the β strand B8, B9 and the α helix H5 of Smad3, respectively. Lys-378 in Smad3 is located on the L3 loop that is recognized by the L45 loop, which is located immediately adjacent to the Gly/Ser-rich (GS) region of TβR-I [39]. In Smad2, it is reported that a positively charged surface pocket formed by the L3 loop and the B8 strand, referred to as the 'loop-strand pocket', is required for interactions with the activated TβR-I [40]. Furthermore, B8 and B9 strands have relation with the interaction of Smad anchor for receptor activation (SARA) and Smad2 [39]. To elucidate whether the Smad3 K333R, K341R, K378R and K409R mutants affect on the TGF-β signaling pathway,

we created a series of the full-length Smad3 K333R, K341R, K378R and K409R mutants of four lysine residues to arginine residues. We also made GST-Smad3-MH2 K333R, K341R, K378R or K409R mutants. These mutant proteins expressed well in 293T cells comparable with wild-type proteins (Fig. 1B). To first examine whether these K333R, K341R, K378R and K409R mutants have any effect on TGF-β-mediated transcriptional activation through Smad3, we performed a transient transcription assay using Hep3B. The TGF-β-mediated Smad3 transcriptional responses were measured by p3TP-LUC, which is one of the standard reporters for assessing TGF-β activity [33]. As shown in Fig. 2, the WT, K333R and K409R mutants induced the transcriptional activation of p3TP-LUC in Hep3B cells by TGF-β. Importantly, the K409R mutant enhanced more potently than the WT or K333R mutant. In contrast, both the K378R and K341R mutants failed to induce the transcriptional activation of p3TP-LUC. These results suggest that Lys-341 and Lys-378, but not Lys-333 and Lys-409, in the Smad3-MH2 domain are essential for activation of TGF-β signals.

3. 2. Role for Lysine-378 in the interaction of Smad-MH2 domain with Type-I TGF-β receptor

Phosphorylation-triggered heteromeric assembly between Smad3 and Smad4 is mediated by the C-terminal MH2 domain. To investigate whether the Smad3-MH2 K333R, K341R, K378R and K409R mutants physically interact with TβR-I (T204D), 293T cells were

transfected with HA-tagged TβR-I (T204D) and the GST-Smad3-MH2 K333R, K341R, K378R or K409R mutants. Pull-down assay for the GST-Smad3-MH2 WT, K333R, K341R, K378R or K409R mutant was performed and followed by Western blot analysis with anti-HA antibody. As shown in Fig. 3A, the GST-Smad3-MH2 WT, K333R, K341R and K409R mutants strongly bound to TβR-I (Fig. 3A). In contrast, the GST-Smad3-MH2 K378R mutant showed a marked reduction of the interaction with TβR-I (T204D)(Fig. 3A). Furthermore, phosphorylation of Smad3 by TβR-I (T204D) was also affected in the GST-Smad3-MH2 K378R mutant (Fig. 3B). These results indicate that Lys-378 is necessary for the interaction with and phosphorylation by TβR-I (T204D). We next examined whether the GST-Smad3-MH2 K333R, K341R, K378R and K409R mutants physically interact with co-Smad, Smad4. As shown in Fig. 3C, the GST-Smad3-MH2 K333R, K341R, K378R and K409R mutants exhibited the interaction with Smad4 as well as Smad3-MH2 WT. This result suggests that the K333R, K341R, K378R and K409R mutations of Smad3-MH2 domain have no effect on their association with Smad4.

3. 3. Interactions of the Smad3 K333R, K341R, K378R or K409R mutant with transcriptional co-factors

Smad3 has also been shown to interact with several transcription factors including c-Jun, c-Fos, ATF-2, and steroid receptors [42, 43]. Notably, transcriptional activity of Smad proteins

depends on co-activators such as CBP/p300 and co-repressors such as TG-interacting factor (TGIF), Ski, or SnoN [44]. It has been proposed that these proteins control transcription by modifying the access to chromatin by acetylation (co-activator) or deacetylation (co-repressors) of core nucleosomal histones. The transcriptional co-activators p300/CBP interact in a ligand-dependent manner with Smad3 through the MH2 domain [29]. Thus, p300/CBP positively regulate Smad-mediated transcriptional activation. We first examined whether the K333R, K341R, K378R and K409R mutants normally interact with co-activators, p300/CBP in the GST-pull down or co-immunoprecipitation experiments. As shown in Fig.4A-D, the WT, K333R and K409R mutants interacted with p300/CBP, whereas the binding potential of the K409R mutant was stronger than that of the WT or the K333R mutant. Therefore, these results may support the enhanced transcriptional activity of the K409R mutant as shown in Fig. 2. Furthermore, as shown in Fig. 4B, D, both the K341R and K378R mutants failed to interacted with p300/CBP. Similarly, the GST-Smad3-MH2 K378R mutant could not interact with p300/CBP, although the GST-Smad3-MH2 K341R mutant exhibited a weak interaction with p300/CBP (Fig. 4A, C). These interactions may cause the lower transcriptional activation as shown in Fig. 2. We also tested whether these Smad3-p300 interactions is functional. To this end, we examined acetylation of Smad3 by p300 using anti-acetylated lysine specific antibody. As shown in Fig. 3E, the WT, K333R and K409R mutants were acetylated in the presence of p300 in 293T cells, whereas no acetylation of Smad3 was observed in the absence

of p300 (data not shown).

We previously demonstrated the physical and functional interactions between Smads and PIASy through the MH2 domain [5]. PIASy inhibited Smad-mediated transcriptional activation in a dose-dependent manner and mediated SUMO-1 modification of Smads [21]. We then tested whether PIASy interacts with the K333R, K341R, K378R and K409R mutants and mediates sumoylation of them. As shown in Fig. 5A, B, PIASy associated with the K333R, K341R, K378R or K409R mutants or GST-Smad3-MH2 mutants as well as the Smad3 WT. Moreover, co-expression of PIASy and SUMO-1 together with theses mutants resulted in comparable sumoylation of GST-Smad3-MH2 mutant proteins with the GST-Smad3-MH2 WT (Fig. 5C), whereas no sumoylation of Smad3-MH2 was observed in the absence of PIASy (data not shown). These results indicate that PIASy mediates sumoylation at least two lysine residues in the Smad3-MH2 domain. Furthermore, none of these mutants showed a resistance for the PIASy-mediated suppression of transcriptional activation (data not shown).

We also examined physical interactions with other co-repressors, such as Ski and SMRT. As shown in Fig. 5D, Ski interacted with all GST-Smad3-MH2 mutants as well as GST-Smad3 MH2 WT. Interestingly, we found that co-repressor, SMRT also interacts with Smad3 through the MH2 domain (Fig. 5E). Like Ski, SMRT interacted with all GST-Smad3-MH2 mutants as well as GST-Smad3-MH2 WT. We could not observe any stronger binding of GST-Smad3-

MH2 K341R and K378R mutants to these repressors, suggesting the lower transcriptional activation of the K341R and K378R mutants may depend on the interactions with TβR-I or co-activators, p300/CBP.

3. 4. Subcellular localization of the Smad3 K333R, K341R, K378R or K409R mutant

We next assessed the subcellular localization of the K333R, K341R, K378R and K409R mutants. Expression vectors for FLAG-tagged Smad3 WT, K333R, K341R, K378R or K409R mutant was transfected into Hep3B. Forty-eight hrs after transfection, the cells were fixed and reacted with anti-FLAG polyclonal antibody, and visualized with an fluorescein isothiocyanate-conjugated antibody. As shown in Fig. 6A, the WT and all mutants localized in the cytoplasm in the absence of TGF-β. Thirty minutes after stimulation with TGF-β, the WT, K333R and K409R mutants translocated into the nucleus. However, both the K341R and K378R mutants localized in the cytoplasm after TGF-β stimulation (Fig. 6A), and no accumulation in the nucleus was observed even after the prolonged TGF-β-stimulation up to 12 hrs (data not shown). The time course analysis of the subcellular localization also demonstrated that both the K341R and K378R mutants failed to translocate into nucleus (Fig. 6B). It is reasonable that the K378R failed to translocate into nucleus because this mutant was not activated by TβR-I (Fig. 3A, B), whereas the K341R mutant was normally activated by TβR-I. We next examined the effect of K341R and K378R mutants on the nuclear

translocation of Smad4 in response to TGF-β, although these mutants could associate with Smad4 (Fig. 3C). Surprisingly, co-expression of Smad4 with these mutants resulted in the failure of the nuclear translocation of Smad4 after TGF-β stimulation (Fig. 6C). These results indicate that Lys-341 and Lys-378, but not Lys-333 and Lys-409, in the Smad3-MH2 domain are necessary for the TGF-β-induced nuclear translocation of not only Smad3 but also Smad4 in Hep3B cells. Moreover, Lys-341 may be a critical residue for the nuclear import of Smad3, since Lys-341 is not involved in the interaction with TβR-I. Lys-333, Lys-341 and Lys-378 of Smad3 are highly conserved not only among all R-Smads but also for the Co-Smad (Smad4). In addition, Lys-375 and Lys-420 of Smad2 corresponding residues to Lys-333 and Lys-378 of Smad3, directly coordinate the phosphorylated C-terminus and play critical roles in hetero-oligomerization [40]. Lys-383 of Smad2 corresponding to Lys-341 of Smad3, is shown to form an intermolecular H bond with SARA in association with SARA [40], suggesting that the K341R mutation of Smad3 may also affect the interaction with SARA. A previous study demonstrated that a nuclear localization signal (NLS) of Smad3 exists in the N-terminal region of Smad3-MH1 domain [45]. An NLS-like motif was conserved among all Smads, not only is responsible for constitutive nuclear localization of the isolated Smad3-MH1 domain but also is crucial for Smad3 nuclear import in response to TGF-β. Mutations in this motif completely abolished TGF-β-induced Smad3 nuclear translocation. Furthermore, the Smad3 mutants with the NLS mutations are dominant-negative inhibitors of TGF-β-induced

transcriptional activation. The isolated Smad3-MH1 domain was also demonstrated to display the specific binding to importin-β, which is diminished or eliminated by mutations in the NLS [46]. Full-length Smad3 exhibited weak but specific binding to importin-β, which is enhanced after phosphorylation by TβR-I [46]. Moreover, it has been recently shown that the nuclear import of Smad3 is dependent on the interaction with FG-repeat-containing nucleoporins such as CAN/Nup214 [47]. A surface hydrophobic corridor within the MH2 domain of Smad3 is also shown to be critical for association with CAN/Nup214 and the nuclear import. These findings suggest that the K341R mutant may partly participate in a surface hydrophobic corridor. Further detailed analysis of this K341R mutant will be very useful to understand the nuclear import mechanisms for Smad3 and Smad4.

3. 5. Both the Smad3 K341R and K378R mutants act as dominant-negative inhibitors of TGF-β-induced transcriptional activation

We further examined whether the K333R, K341R, K378R and K409R mutants act as dominant-negative inhibitors of TGF-β-induced transcriptional activation. To examine the effect of the K333R, K341R, K378R and K409R mutants on the TGF-β-signaling pathway under more physiological conditions through endogenous proteins, we established the stable transformants expressing the WT, K333R, K341R, K378R or K409R mutant in Hep3B cells (Fig. 7A). In Hep3B cells, TGF-β treatment induced Smad3-mediated plasminogen activator

inhibitor-1 (PAI-1) expression [5]. We then tested endogenous PAI-1 mRNA expression in these transformants by TGF-β. As shown in Fig. 7B, when we monitored the RNA samples by RT-PCR, endogenous PAI-1 mRNA expression by TGF-β was the markedly decreased in transformants with the K341R or K378R mutants, but not in those with the WT, K333R or K409R. These results suggest that both the K341R and K378R mutants act as dominant-negative inhibitors of TGF-β-induced transcriptional activation in Hep3B cells.

Smad-MH2 domain is known to be sensitive to mutations. Gly-421 in Smad2-MH2 domain is a highly conserved glycine residue whose mutation to serine in Drosophila Mad [48] or to aspartic acid in C. elegans Sma-2 [49] causes null or severe developmental phenotypes. The corresponding mutation in Smad1 inhibits BMP-induced phosphorylation of Smad1 [50]. In Smad2, the G421S mutation inhibited Smad2 binding to the receptor and TGF-β-dependent phosphorylation [51]. As shown in Fig. 3A and B, Smad3 K378R mutant exhibited the similar defect with Smad2 G421S mutant, since Lys-378 of Smad3 is adjacent to Gly-379 corresponding to Smad2 Gly-421. A missense mutation of Asp-450 to Glu (D450E) in Smad2-MH2 domain was also reported in a case of colorectal cancer. This mutant was reported to be defective in phosphorylation by TβR-I [52]. Asp-537 of Smad4 corresponding to Asp-450 of Smad2 was located at an interface of oligomer formation, and the Smad4 D537E mutant, as well as Smad2 D450E, was defective in oligomerization [40, 53]. Similarly, Smad3 D407E

mutant was not phosphorylated by TβR-I, and inhibited the phosphorylation of wild-type Smad2 and Smad3 as a dominant negative protein [54].

Our present findings revealed that Lys-378 is necessary for interaction with and phosphorylation by TβR-I, suggesting that Lys-378 is a critical residue for Smad3 activation. Importantly, Smad3 K341R mutant failed in nuclear translocation, indicating that Lys-341 is required for nuclear import of Smad3. In contrast, the K409R mutant enhanced transcriptional activity through a stronger interaction with co-activators, p300/CBP, suggesting that Lys-409 is also involved in the regulation of TGF-β-mediated transcription. Thus, the present study indicates that lysine residues in the MH2 domain of Smad3 may play important roles in TGF-β signaling pathway.

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**Figure legends**

Fig. 1. **Lysine residues in the Smad3-MH2 domain and its mutants.**

A, The functional domains of Smad3 are schematically shown. Sequence in the Smad3-MH2 domain contains four lysine residues shown in bold. The L3 loop is boxed. B, 293T cells ($1x10^7$ cells) were transiently transfected with FLAG-tagged full-length Smad3 (5 μg) or GST-tagged Smad3-MH2 (10 μg) for the Smad3 WT, K333R, K341R, K378R or K409R mutants. Protein expression levels of the Smad3 WT, K333R, K341R, K378R and K409R mutants were examined from total lysates (1%) by Western blot with anti-FLAG or anti-GST antibodies.

Fig. 2. **Roles of the lysine residues in the Smad3-MH2 domain in the activation of TGF-β-mediated transcription.**

Hep3B cells in a 6-well plate were transfected with p3TP-LUC (0.5 μg) together with the Smad3 WT, K333R, K341R, K378R or K409R mutant (0.5 μg). Thirty-six hrs after transfection, the cells were stimulated with TGF-β (40 pM) for additional 6 hrs. The stimulated cells were harvested, and luciferase activities were measured. The results are indicated as fold induction of luciferase activity from triplicate experiments, and the error bars represent the S.D.

Fig.3. **Physical interactions of the Smad3 K333R, K341R, K378R or K409R mutant with type-I TGF-β receptor.** A, 293T cells ($1x10^7$ cells) were transfected with GST-Smad3-MH2 (10 μg) WT, K333R, K341R, K378R or K409R mutant together with HA-tagged TβR-I (T204D)(3 μg). Forty-eight hrs after transfection, the cells were lysed, and pull down with glutathione-Sepharose beads (GSH bound), followed by immunoblotting with anti-HA (upper) or anti-GST antibody (middle). Total cell lysates (1%) were blotted with anti-HA antibody (lower) to monitor the expression of TβR-I. B, 293T cells ($1x10^7$ cells) were transfected with GST-Smad3-MH2 (10 μg) WT, K333R, K341R, K378R or K409R mutant together with HA-tagged TβR-I (T204D)(3 μg). Forty-eight hrs after transfection, the cells were lysed. Total cell lysates (1%) were blotted with anti-phopsho-Smad3 (Ser 433/435)(pSmad3) (upper), anti-GST antibody (middle) or anti-HA antibody (lower). C, 293T cells ($1x10^7$ cells) were transfected with GST-Smad3-MH2 (10 μg) WT, K333R, K341R, K378R or K409R mutant together with FLAG-tagged Smad4 (5 μg). Forty-eight hrs after transfection, the cells were lysed, and pull down with glutathione-Sepharose beads (GSH bound), followed by immunoblotting with anti-FLAG (upper) or anti-GST antibody (middle). Total cell lysates (1%) were blotted with anti-FLAG antibody (lower) to monitor the expression of Smad4.

Fig.4. **Physical and functional interactions of the Smad3 K333R, K341R, K378R or K409R mutant with transcriptional co-activators.** A, 293T cells ($1x10^7$ cells) were transfected with GST-Smad3-MH2 (10 □g) WT, K333R, K341R, K378R or K409R mutant together with HA-tagged p300 (15 □g). Forty-eight hrs after transfection, the cells were lysed, and pull down with glutathione-Sepharose beads (GSH bound), followed by immunoblotting with anti-HA (upper) or anti-GST antibody (middle). Total cell lysates (1%) were blotted with anti-HA antibody (lower) to monitor the expression of p300. B, 293T cells ($1x10^7$ cells) were transfected with FLAG-tagged full-length Smad3 (□g) WT, K333R, K341R, K378R or K409R mutant together with FLAG-tagged p300 (15 □g). Forty-eight hrs after transfection, the cells were lysed and immunoprecipitated with anti-FLAG antibody and immnoblotted with anti-HA (upper) or anti-FLAG antibody (middle). Total cell lysates (1%) were blotted with anti-HA antibody (lower) to monitor the expression of p300. C, 293T cells ($1x10^7$ cells) were transfected with GST-Smad3-MH2 (10 □g) WT, K333R, K341R, K378R or K409R mutant together with CBP (15 □g). Forty-eight hrs after transfection, the cells were lysed, and pull down with glutathione-Sepharose beads (GSH bound), followed by immunoblotting with anti-CBP (upper) or anti-GST antibody (middle). Total cell lysates (1%) were blotted with anti-CBP antibody (lower) to monitor the expression of CBP. D, 293T cells ($1x10^7$ cells) were transfected with FLAG-tagged full-length Smad3 (□g) WT, K333R, K341R, K378R or K409R mutant together with CBP (15 □g). Forty-eight hrs after transfection, the cells were

lysed and immunoprecipitated with anti-FLAG antibody and immnoblotted with anti-CBP (upper) or anti-FLAG antibody (middle). Total cell lysates (1%) were blotted with anti-CBP antibody (lower) to monitor the expression of CBP. E, 293T cells ($1x10^7$ cells) were transfected with full-length Smad3 (□g) WT, K333R, K341R, K378R or K409R mutant together with FLAG-tagged p300 (15 □g). Forty-eight hrs after transfection, the cells were lysed and immunoprecipitated with anti-FLAG antibody and immnoblotted with anti-acetylated lysine (Ac-Lys) (upper) or anti-GST antibody (middle). Total cell lysates (1%) were blotted with anti-HA antibody (lower) to monitor the expression of p300.

Fig.5. **Physical and functional interactions of the Smad3 K333R, K341R, K378R and K409R mutants with PIASy or transcriptional co-repressors.** A, 293T cells ($1x10^7$ cells) were transfected with GST-Smad3-MH2 (10 □g) WT, K333R, K341R, K378R or K409R mutant together with HA-tagged PIASy (5 □g). Forty-eight hrs after transfection, the cells were lysed, and pull down with glutathione-Sepharose beads (GSH bound), followed by immunoblotting with anti-HA (upper) or anti-GST antibody (middle). Total cell lysates (1%) were blotted with anti-HA antibody (lower) to monitor the expression of PIASy. B, 293T cells ($1x10^7$ cells) were transfected with FLAG-tagged full-length Smad3 (□g) WT, K333R, K341R, K378R or K409R mutant together with HA-tagged PIASy (15 □g). Forty-eight hrs after transfection, the cells were lysed and immunoprecipitated with anti-FLAG antibody and

immnoblotted with anti-HA (upper) or anti-FLAG antibody (middle). Total cell lysates (1%) were blotted with anti-HA antibody (lower) to monitor the expression of PIASy. C, 293T cells ($1x10^7$ cells) were transfected with FLAG-tagged full-length Smad3 (□g) WT, K333R, K341R, K378R or K409R mutant together with HA-tagged PIASy (15 □g) together with SUMO-1 (5 □g). Forty-eight hrs after transfection, the cells were lysed and immunoprecipitated with anti-FLAG antibody and immnoblotted with anti-FLAG antibody (upper). Total cell lysates (1%) were blotted with anti-HA antibody (lower) to monitor the expression of PIASy. D, 293T cells ($1x10^7$ cells) were transfected with GST-Smad3-MH2 (10 □g) WT, K333R, K341R, K378R or K409R mutant together with Myc-tagged Ski (10 □g). Forty-eight hrs after transfection, the cells were lysed, and pull down with glutathione-Sepharose beads (GSH bound), followed by immunoblotting with anti-Myc (upper) or anti-GST antibody (middle). Total cell lysates (1%) were blotted with anti-Myc antibody (lower) to monitor the expression of Ski. E, 293T cells ($1x10^7$ cells) were transfected with GST-Smad3-MH2 (10 □g) WT, K333R, K341R, K378R or K409R mutant together with FLAG-tagged SMRT (5 □g). Forty-eight hrs after transfection, the cells were lysed, and pull down with glutathione-Sepharose beads (GSH bound), followed by immunoblotting with anti-FLAG (upper) or anti-GST antibody (middle). Total cell lysates (1%) were blotted with anti-FLAG antibody (lower) to monitor the expression of SMRT.

Fig. 6. **Subcellular localization of the Smad3 K333R, K341R, K378R or K409R mutant in TGF-β-stimulated Hep3B cells.** A, Hep3B cells were transfected with FLAG-tagged Smad3 WT, K333R, K341R, K378R or K409R mutant. Thirty hrs after transfection, cells were stimulated with TGF-β (40 pM) for 30 min, and then fixed and reacted with rabbit anti-FLAG polyclonal antibody and visualized with fluorescein isothiocyanate-conjugated anti-rabbit antibody. The same slide was also stained with DAPI for the nuclei staining. B, Hep3B cells were transfected with FLAG-tagged Smad3 WT, K333R, K341R, K378R or K409R mutant. Thirty hrs after transfection, cells were stimulated with TGF-β (40 pM) for the indicated periods. Quantitative analysis of the subcellular localization of Smad3. Approximately 100 cells were classified according fluorescein signals in the cytoplasm (yellow), cytoplasm and nucleus (red) and nucleus (blue). The results represent the means of three individual experiments, in which 100 cells were counted. C, Hep3B cells were transfected with FLAG-tagged Smad4 together with Myc-tagged Smad3 WT, K341R or K378R mutant. Thirty hrs after transfection, cells were stimulated with TGF-β (40 pM) for 30 min, and then fixed and reacted with anti-FLAG polyclonal antibody and anti-Myc monoclonal antibody, and visualized with fluorescein isothiocyanate-conjugated anti-rabbit antibody and rhodamine conjugated anti-mouse antibody. The same slide was also stained with DAPI for the nuclei staining.

Fig. 7. **Both the Smad3 K341R and K378R mutants act as dominant-negative inhibitors of TGF-β-induced transcriptional activation**

A, Stable Hep3B transformants expressing the Smad3 WT, K333R, K341R, K378R or K409R mutant were established. Total extracts (1%) of each transformant were examined with Western blot using anti-FLAG antibody to monitor the expression of the Smad3 WT, K333R, K341R, K378R or K409R mutant. B, Stable Hep3B cell transformants were treated or untreated with TGF-β (40 pM) for 2 hrs. Total RNA samples isolated from these cells were subjected to RT-PCR analysis using PAI-1 (upper panels) and glyceraldehydes-3-phosphate dehydrogenase (G3PDH) (lower panels) primers. RT-PCR products were separated on a 1% agarose gel. This figure is representative of three separate experiments.

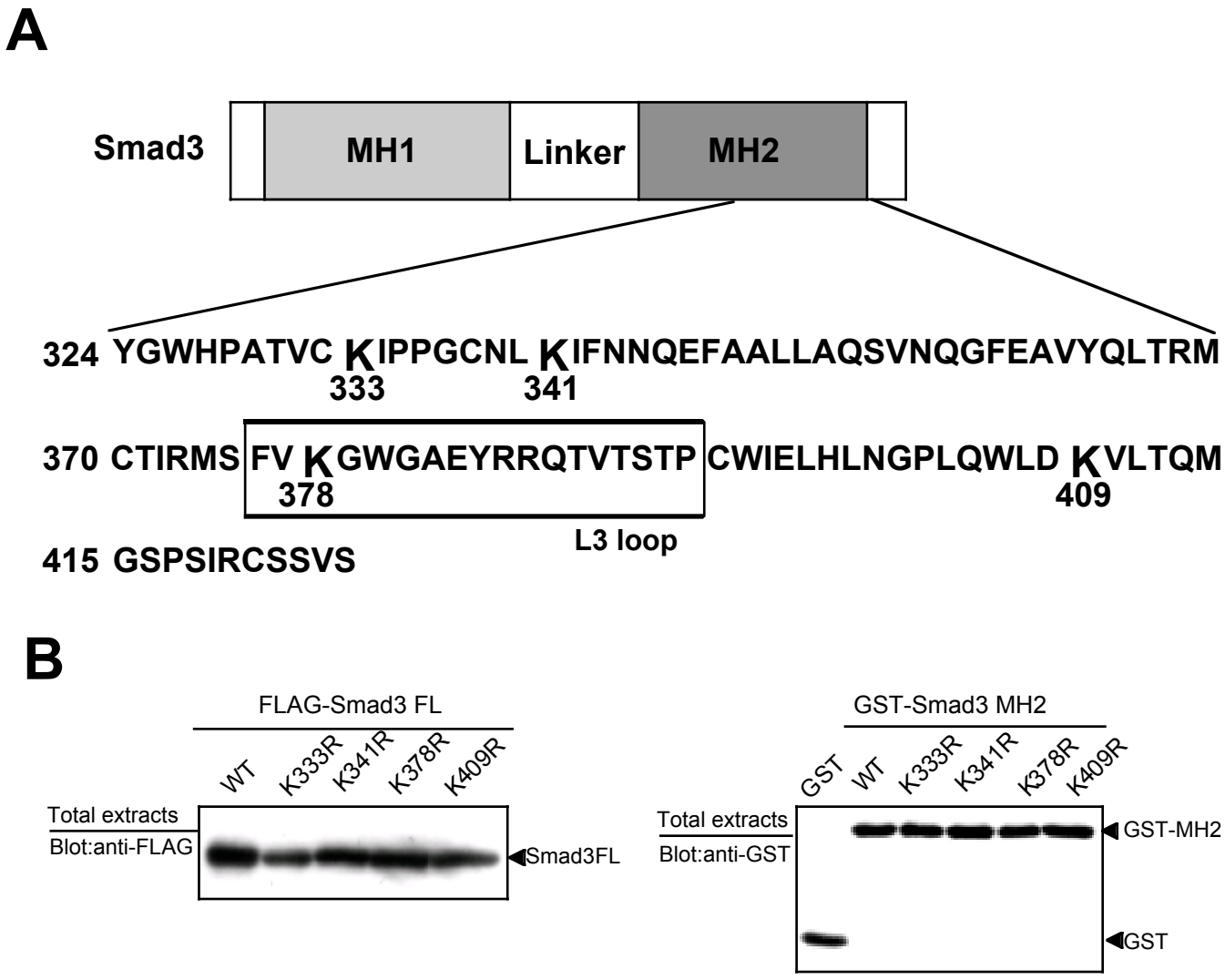
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Fig. 1

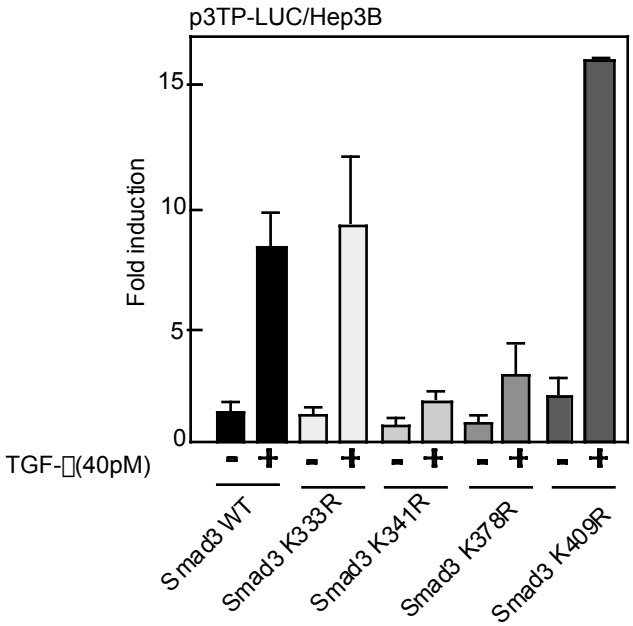
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**Fig. 2**

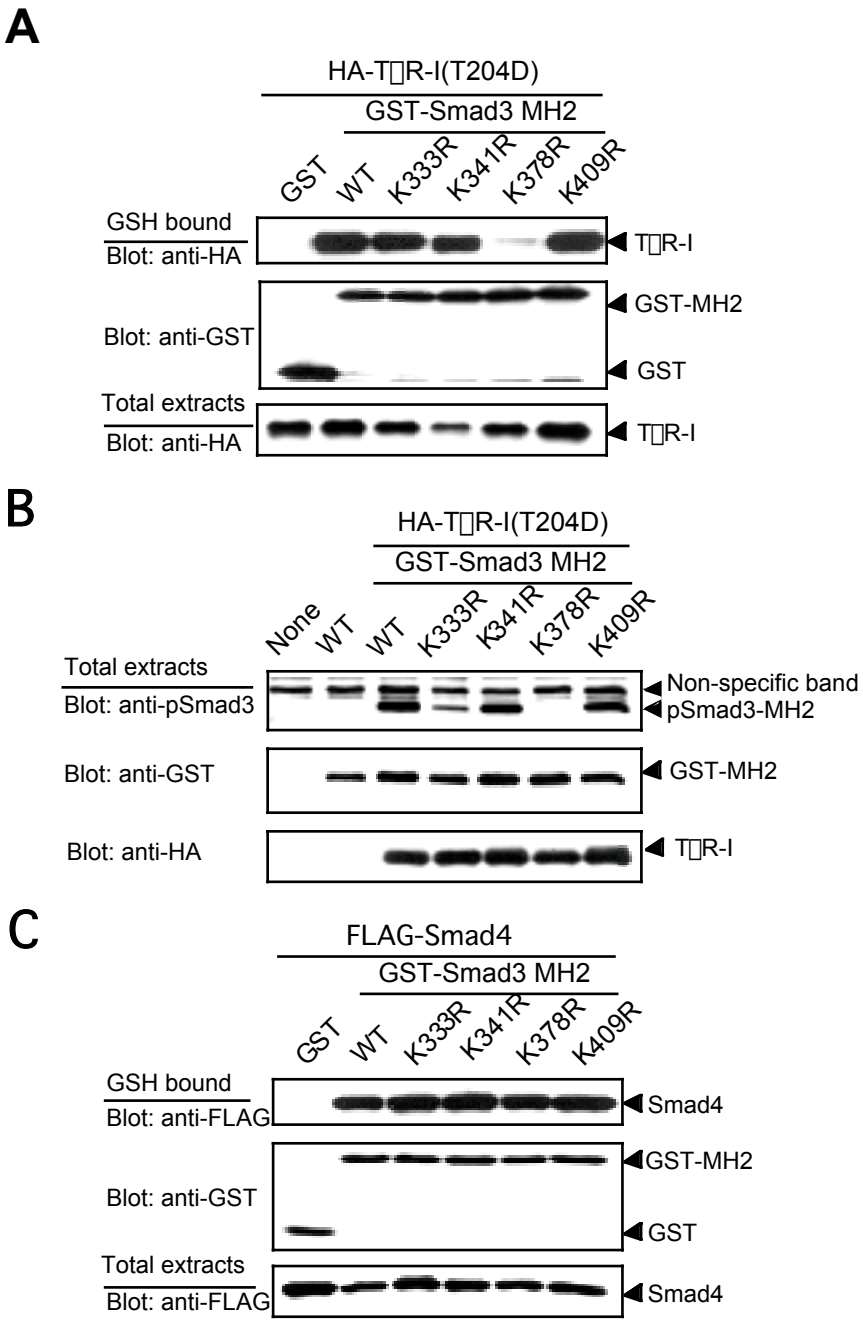
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**Fig. 3**

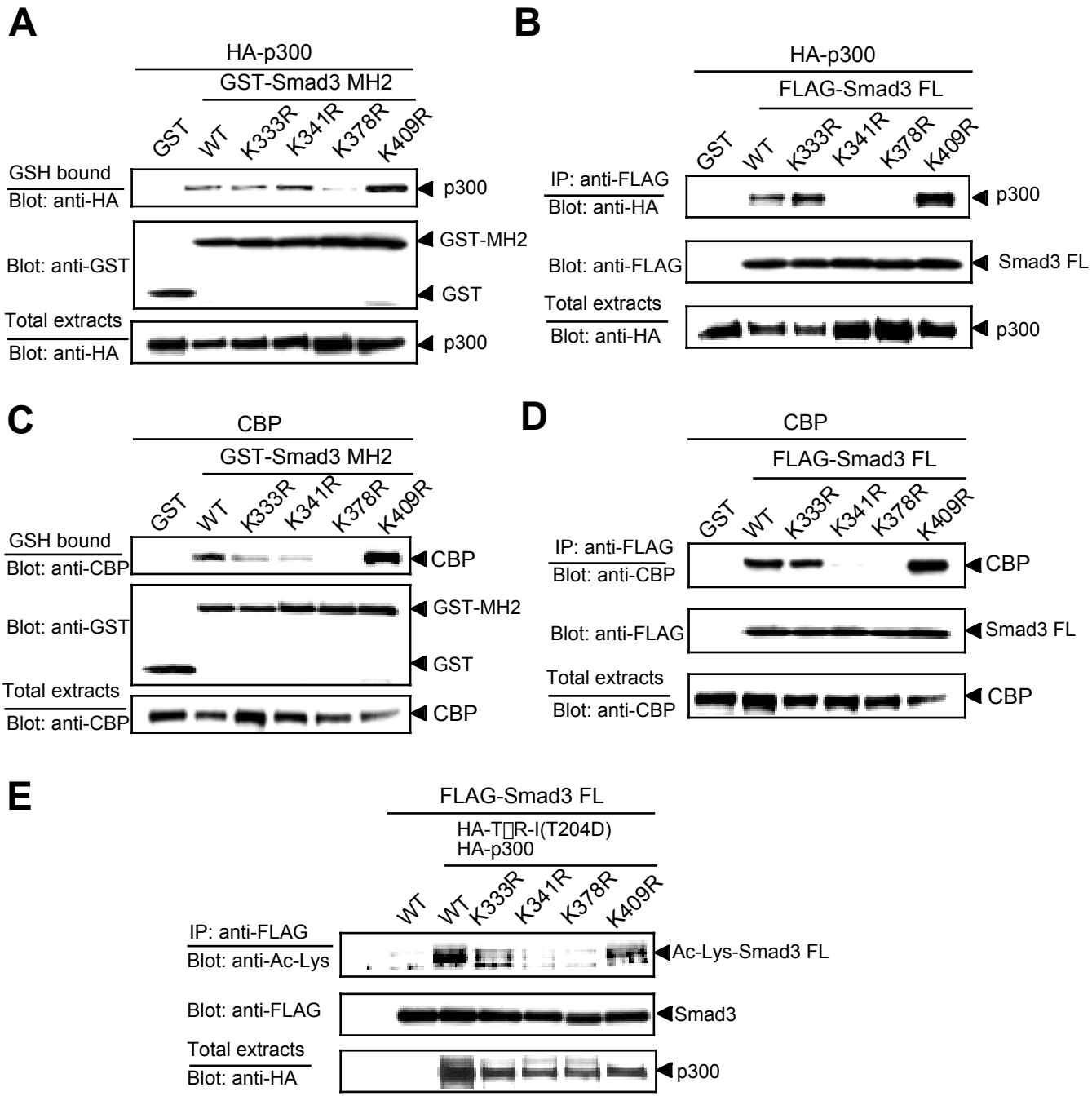
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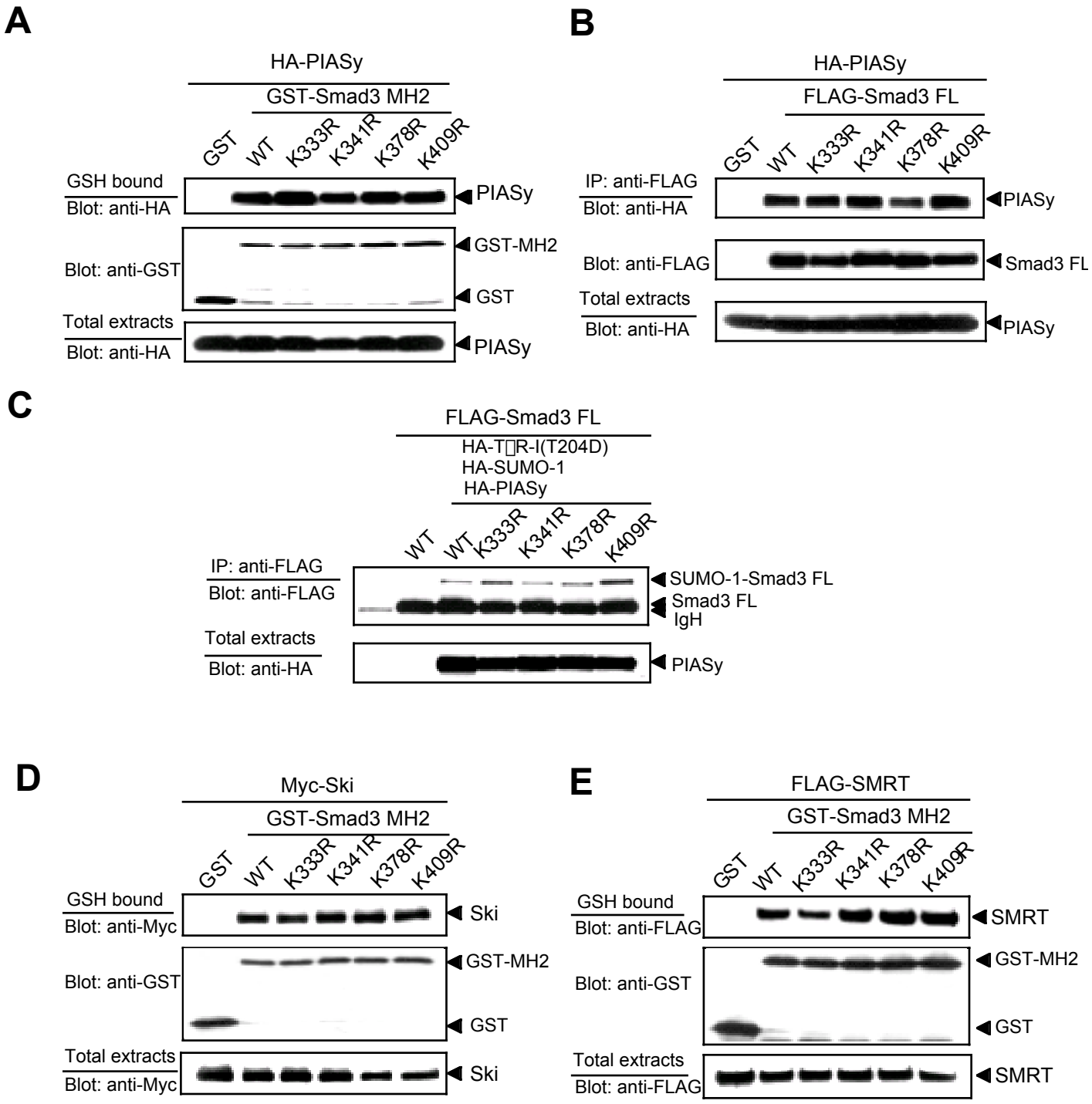
**Fig. 4**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SMRT

**Fig. 5**

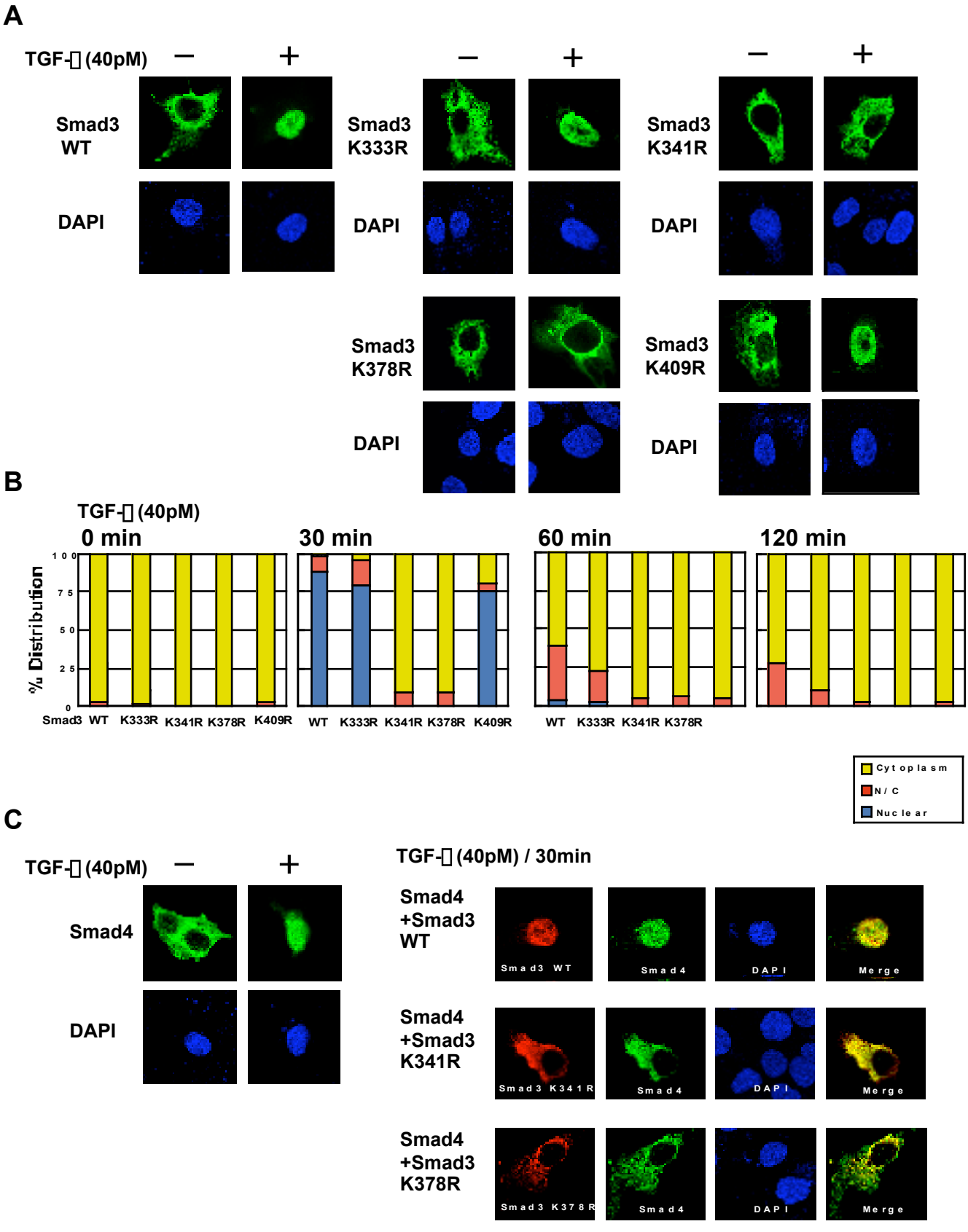
A
TGF-β (40pM)
−
+
Smad3 WT
DAPI
Smad3 K333R
Smad3 K341R
Smad3 K378R
Smad3 K409R
B
TGF-β (40pM)
0 min
30 min
60 min
120 min
% Distribution
100
75
50
25
0
Smad3 WT K333R K341R K378R K409R
WT K333R K341R K378R K409R
WT K333R K341R K378R
Cytoplasm
N/C
Nuclear
C
TGF-β (40pM)
Smad4
DAPI
TGF-β (40pM) / 30min
Smad4 +Smad3 WT
Smad4 +Smad3 K341R
Smad4 +Smad3 K378R
Smad3 WT
Smad4
DAPI
Merge
Smad3 K341R
Smad3 K378R

Fig. 6

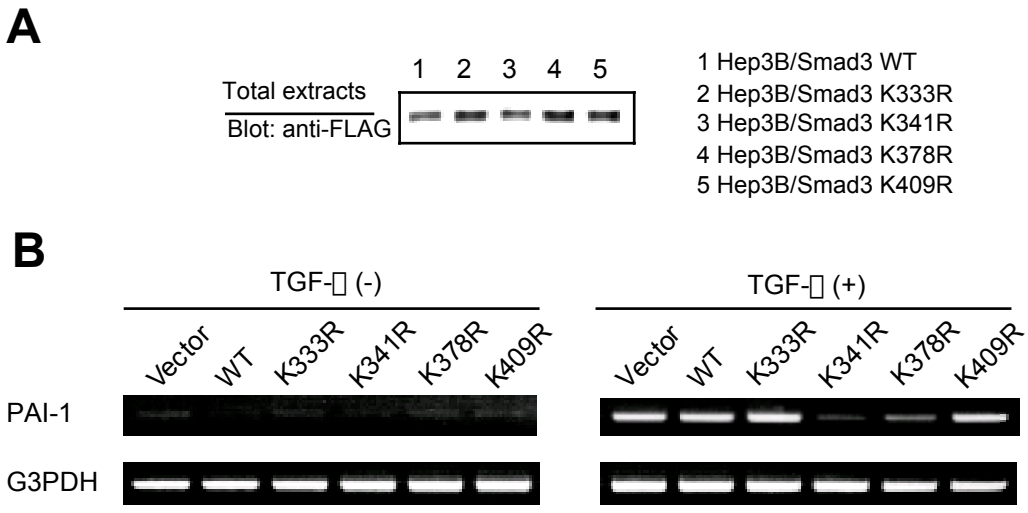
A
1 2 3 4 5
Total extracts
Blot: anti-FLAG
1 Hep3B/Smad3 WT
2 Hep3B/Smad3 K333R
3 Hep3B/Smad3 K341R
4 Hep3B/Smad3 K378R
5 Hep3B/Smad3 K409R
B
TGF-β (-)
TGF-β (+)
Vector
WT
K333R
K341R
K378R
K409R
PAI-1
G3PDH

**Fig. 7**