

# Human SMAD4 is phosphorylated at Thr9 and Ser138 by interacting with NLK

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**Abstract** Smads are important intracellular effectors in signaling pathways of the transforming growth factor- $\beta$  (TGF- $\beta$ ). Receptor-activated Smads combine with a common Smad4 to translocate into the nucleus where they cooperate with other transcription factors to activate or repress transcription. *SMAD4* is an important tumor suppressor gene. Smad4 has been shown to be constitutively phosphorylated, but the kinase that performs this phosphorylation is unknown. In this study, Smad4 was identified to interact with Nemo-like kinase (NLK) by a yeast two-hybrid system, and this interaction was confirmed in vitro and in vivo. Furthermore, the linker sequence of Smad4 is sufficient for this specific interaction. NLK is a conserved Ser/Thr kinase. Using in vitro kinase assays, we identified that threonine 9 (Thr9) and Serine 138 (Ser138) within the N-terminal Mad homology1 (MH1) domain of Smad4 could be phosphorylated by NLK. Our research suggests that NLK may play a novel role in the regulatory of Smad4 through phosphorylation.

**Keywords** SMAD4 · NLK · Protein–Protein interaction · Phosphorylation

## Introduction

TGF- $\beta$  family can regulate diverse biological processes, including cell proliferation, differentiation, and apoptosis.

Smad family members, as the messenger of TGF- $\beta$  signaling pathway, play a key role in TGF- $\beta$  signal transduction. Smads have been classified into three subtypes according to their structures and functions: receptor-activated Smads (R-Smads), common mediator Smad (Co-Smad), Smad4, and inhibitory Smads (I-Smads) [1, 2]. Smad proteins have a conserved N-terminal MH1 domain, a conserved C-terminal MH2 domain, and a non-conserved linker region. The MH1 domain is highly conserved among R-Smads and Co-Smad, and functionally, is implicated in nuclear import and transcription by binding to DNA and interacting with nuclear proteins, such as vitamin D receptor (VDR), transcription factor Jun, transcription factor E3 (TFE3), and activating transcription factor 2 (ATF2) [1, 3]. MH2 domain is highly conserved among all Smads. It regulates Smad oligomerization, recognition of R-Smads by type I receptors, and interacts with several transcription factors [3–5]. The linker sequence is not conserved among Smads.

Smad4 is the core member of Smad family. It forms a complex with phosphorylated R-Smads and then translocates into the nucleus to active the transcription of downstream genes. Once Smad4 was mutated or deleted, the TGF- $\beta$  superfamily signal transduction network would not be able to activate, and the inhibition effect of TGF- $\beta$  superfamily for tumor development would disappear. Smad4 is modified by sumo-1 conjugation, phosphorylation, and ubiquitination. SUMO-1 modification serves to protect Smad4 from ubiquitin-dependent degradation and consequently enhances the growth inhibitory and transcriptional responses of Smad4 [6]. The major sumoylation sites in Smad4 are Lys-113 in the MH-1 domain and Lys-159 localized to its linker segment [7]. Besides SUMO-1 modification, mono- or oligo-ubiquitination also positively regulates Smad4, not linking it to degradation [7]. Poly-ubiquitination and proteasomal degradation of Smad4

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would be initiated when it forms ternary complex with I-Smads and Smurfs E3 ligase [8]. In addition, oncogenic Ras mutations cause a decrease in complex formation between Smad2/3 and Smad4 by degrading Smad4 through the ubiquitin-proteasome pathway, exhibiting a loss of TGF- $\beta$  anti-proliferative response [9].

Phosphorylation is another modification for Smad4. Smad4 can be phosphorylated in the linker region at Thr276 by extracellular-signal-regulated kinase (ERK). This phosphorylation is important for TGF- $\beta$ -induced nuclear accumulation and transcriptional activity of Smad4 [10]. Smad4 has been shown to be constitutively phosphorylated [11], but the site(s) of phosphorylation, the kinase(s) that performs this phosphorylation, and the significance of the phosphorylation of Smad4 are currently unknown [10]. In this report, we identified another kinase, NLK, which is also responsible for the phosphorylation of Smad4.

## Materials and methods

### Plasmids constructs

For protein–protein interaction assays in yeast, Smad4 was cloned into vector pDBLeu fused to Gal4 DNA binding domain (BD). NLK was cloned into pPC86 vector fused to Gal4 activation domain (AD). For in vitro binding analysis, Smad4 was cloned into vector pGEX-6p-1. For co-immunoprecipitation assays, Smad4 and its truncated mutants were cloned into pCDEF-Myc vector. For binding assays, NLK was cloned into pCDEF-Flag vector. For in vitro kinase assays, Smad4 and its point mutants were cloned into pGEX-6p-1 vector. Smad4 deletion mutants were cloned into vector pGEX-6p-1 or pET32a. Mutagenesis was performed using the Quik Change® Site-Directed Mutagenesis Kit (Stratagene).

### Yeast two-hybrid screening

The autoactivation of the *lacZ* reporter gene by pDBLeu-Smad4 was tested in the yeast strain Mav203. Then the stable BD-Smad4-transformed yeast cells were transformed with a human liver cDNA library (Invitrogen, California, USA) constructed in the pPC86 plasmid. More than  $2 \times 10^6$  cDNA colonies were screened on histidine-, leucine- and tryptophan-DropOut plates, containing 25 mM 3AT (Sigma, St. Louis, MO, USA) (SC-TLH + 25 mM 3AT). Positive clones were verified by  $\beta$ -galactosidase assays. Prey plasmids were isolated from His<sup>+</sup>/Leu<sup>+</sup>/LacZ<sup>+</sup> colonies and re-transformed into yeast along with either pDBLeu-Smad4 or pDBLeu to verify the specific interaction.

### In vitro GST pull-down assays

GST-Smad4 fusion protein was expressed in 0.1 mM IPTG-induced *E. coli* strain BL21 and purified by glutathione-Sepharose 4B beads (Invitrogen). Cell lysate from HEK293T cells expressing Myc-NLK was incubated with the Sepharose beads which had been pre-bound with GST-Smad4 proteins. Then the beads were washed thrice with cell lysis buffer (Cell Signaling, Beverly, MA). The pellets were subjected to SDS-PAGE analysis, and immunoblotted with mouse anti-c-Myc antibody (Sigma).

### Cell culture and co-immunoprecipitation assays

HEK293T cells were maintained in DMEM supplemented with 10% bovine calf serum (GIBCO), grown on 60 mm dishes at a concentration of  $6 \times 10^5$  cell/dish before the day of transfection. The relevant plasmids were transfected with Lipofectamine (Invitrogen). Forty-eight hours after transfection, cells were washed twice with ice-cold PBS and lysed with 400  $\mu$ l lysis buffer. Lysate was pretreated with protein A/G agarose (Santa Cruz, CA), and then immunoprecipitated with 1–2  $\mu$ g relevant antibody and protein A/G agarose at 4°C overnight. After washing thrice with lysis buffer, the precipitates were run on SDS-PAGE followed by Western blot detection.

### Protein purification

$6 \times$  His-tagged fusion protein was expressed in *E. coli* BL21 (DE3), purified with Ni<sup>2+</sup>-NTA agarose (Invitrogen) and eluted with elution buffer (50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole pH 8.0). GST-tagged fusion protein was expressed in *E. coli* BL21, purified with Sepharose 4B beads, and eluted with glutathione solution (50 mM Tris-HCl, 20 mM reduced glutathione, pH 8.0). Eluted solution was diluted to 10 ml by using  $1 \times$  PBS, added into the Microncon (Milipore, Boston, USA), and dialysed to 500  $\mu$ l or less. Lastly, purified protein was run on SDS-PAGE to determine the concentration, and then stored at  $-80^\circ\text{C}$  after adding 20% glycerol until use.

### In vitro kinase assays

Flag-NLK was expressed in HEK293T cells. Cell lysate was immunoprecipitated with anti-Flag antibody and protein A/G agarose. The beads were washed thrice with kinase buffer and then resuspended in kinase buffer with 1–2  $\mu$ g of recombinant Smad4 proteins and 1 mM ATP. The total volume of the reaction is 20  $\mu$ l. The reaction was carried out at 30°C for 30 min. Flag-NLK expressed in cells collected from one 60-mm dish is enough for one kinase reaction. For dephosphorylation assays, after

incubating the reaction at 30°C for 30 min, protein A/G agarose immunoprecipitated with Flag-NLK in the reaction mixture was first removed. Then 0.5  $\mu$ l of  $\lambda$ Pase (Biolabs, Hercules, CA) was added to the remaining system, and the reaction was carried out at 30°C for 30 min. All the reactions were then subjected to the Pro-Q Diamond Phosphoprotein Gel Stain (Sigma) analysis.

## Results

### Identification of Smad4/NLK interaction in a yeast two-hybrid system

To explore its potential interacting proteins, Smad4 was used as a bait to screen a human liver cDNA library by a yeast two-hybrid method. More than  $2 \times 10^6$  clones were screened and 19 positive clones were obtained. Database searches revealed that 4 out of the 19 clones encoded full-length NLK. To confirm the interaction between these two proteins, pPC86–NLK was co-transformed into Mav203 with pDBLeu–Smad4 or pDBLeu. Co-expression of Smad4 and NLK showed an evident effect of activation of all reporter genes (Fig. 1a).

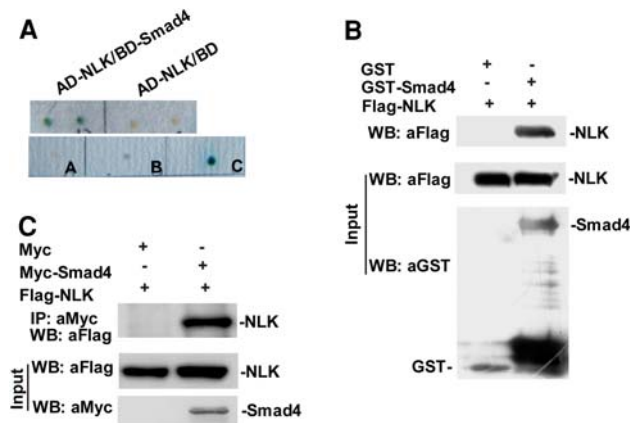
### Validation of Smad4/NLK interaction in vitro and in vivo

To further verify the interaction between Smad4 and NLK in vitro, GST pull-down assays were employed. Bacterially expressed GST–Smad4, being conjugated to glutathione–Sepharose beads, efficiently and specifically pulled down Flag–NLK. Conversely, GST alone did not (Fig. 1b).

To determine whether Smad4 interacts with NLK in vivo, Myc-tagged Smad4 and Flag-tagged NLK were transiently co-transfected into HEK293 cells. We found that NLK was present in the Myc–Smad4 immunoprecipitates (Fig. 1c right). This result indicated that Smad4 could interact with NLK in mammalian cells. Consistent with the yeast two-hybrid screening result, the in vitro GST pull-down assay and the in vivo co-immunoprecipitation assay also confirmed that Smad4 could bind to NLK specifically.

### Domain involved in the Smad4/NLK interaction

To identify the regions responsible for the interaction between Smad4 and NLK, three deletion mutants of Smad4 were constructed into Myc-tagged vectors according to its conserved domain sequence (Fig. 2a). These mutants, Myc–Smad4 $\Delta$ 1 (amino acids 1–140), -Smad4 $\Delta$ 2 (amino acids 141–320), and -Smad4 $\Delta$ 3 (amino acids 321–552), were co-transfected with Flag–NLK into HEK293T cells, respectively. Flag–NLK was detected in Myc–Smad4 $\Delta$ 2



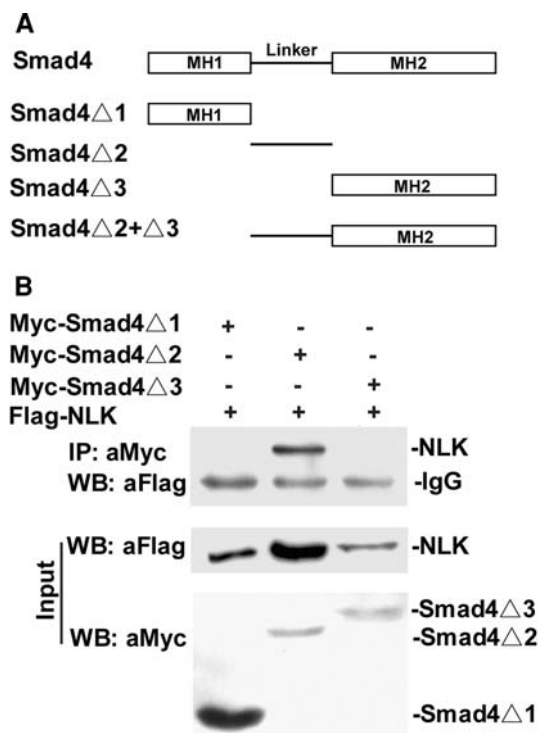
**Fig. 1** Smad4 interacts with NLK in vitro and in vivo. **a** Yeast two-hybrid interactions between Smad4 and NLK. Yeast strain Mav203 was co-transformed with pPC86–NLK/pDBLeu, or pPC86–NLK/pDBLeu–Smad4. Yellow color indicates no evident interaction. A, B, C are yeast controls with varying degrees of protein-protein interaction. **b** Smad4 can interact with NLK in vitro. Cell lysate expressing Flag–NLK was incubated with glutathione beads purified with GST or GST–Smad4 proteins expressed in *E. coli* BL21. After 4 h incubation, the bound proteins were eluted and detected by Western blot analysis with anti-Flag antibody. **c** Smad4 can interact with NLK in vivo. Myc–Smad4 and Flag–NLK were co-transfected into 293T cells; Myc-tagged vector and Flag–NLK were co-transfected as control. Lysate was immunoprecipitated with anti-Myc antibody and analyzed by immunoblotting with anti-Flag antibody (Color figure online)

co-precipitated complex, but not in Myc–Smad4 $\Delta$ 1 and Myc–Smad4 $\Delta$ 3 co-precipitated complex. The result indicates that the linker sequence of Smad4 is necessary for the specific interaction with NLK (Fig. 2b).

### NLK specifically phosphorylates Smad4 in vitro

NLK is a conserved Mitogen-activated protein kinase (MAPK)-type kinase, so we investigated whether Smad4 could be a substrate. Recombinant GST–Smad4 protein was used as the substrate in the phosphorylation assays. As shown in Fig. 3a, Smad4 can be evidently phosphorylated in the presence of NLK, but in the absence of NLK, GST–Smad4 showed no phosphorylation signal. This indicated that Smad4 can be phosphorylated by NLK in vitro.

To further identify the phosphorylation sequence in Smad4, two bacterially expressed recombinant proteins GST–Smad4- $\Delta$ 2 +  $\Delta$ 3 (amino acids 141–552) and pET32a–Smad4 $\Delta$ 3 (amino acids 321–552) were tested as the substrate of NLK. GST–Smad4 and GST–Smad4- $\Delta$ 2 +  $\Delta$ 3 both can be phosphorylated by NLK, while pET32a–Smad4- $\Delta$ 3 cannot. This indicated that the phosphorylation sequence of Smad4 was located in MH1 domain and the linker sequence of Smad4 (Fig. 3b). In addition, phosphatase  $\lambda$ Pase can dephosphorylate the NLK phosphorylated Smad4 (Fig. 3b). This result shows that NLK is the kinase responsible for the phosphorylation of Smad4.

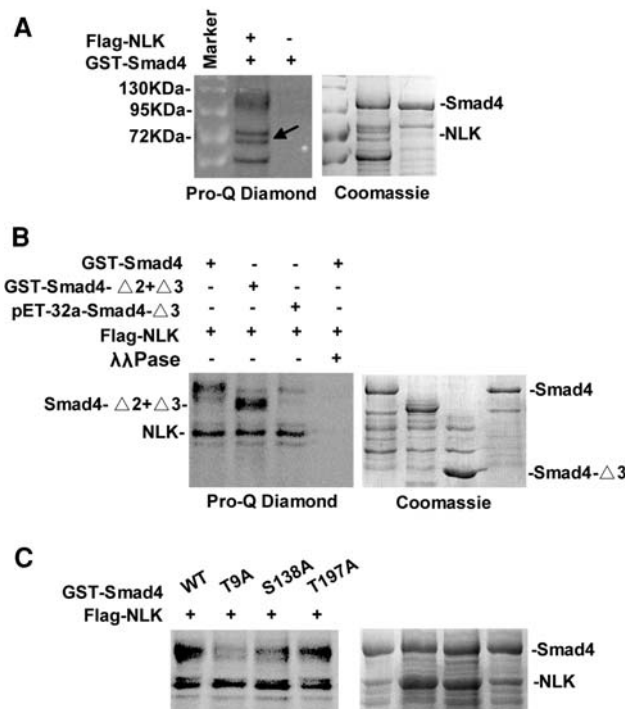


**Fig. 2** Identification of domain required for the interaction between Smad4 and NLK. **a** Schematic diagram of the structure of truncated mutant forms of Smad4. Smad4 contains an N-terminal MH1 domain, a C-terminal MH2 domain, and a connected variant linker region. **b** The linker domain of Smad4 is essential for the interaction with NLK. HEK293T cells were co-transfected with Myc-Smad4 derivatives and Flag-NLK. Cell lysate was immunoprecipitated with anti-Myc antibody and analyzed by immunoblotting with anti-Flag antibody

S/TP motifs are consensus sites for MAPK phosphorylation [12]. Examination of the primary sequences of the MH1 domain and the linker sequence of Smad4 revealed three potential NLK phosphorylation sites. To test whether these residues are indeed the sites for NLK, we altered them by site-directed mutagenesis. Smad4 mutants with its Thr9, Ser138, and Thr197 being replaced with alanine, respectively, were examined for their phosphorylation by NLK in vitro. Thr9A and Ser138A mutant proteins showed weaker phosphorylation compared to the wild-type protein, but the phosphorylation signal of Thr197A mutant was unchanged (Fig. 3c). This indicates that Thr9 and Ser138 are the NLK phosphorylation sites for Smad4 in vitro.

## Discussion

*Drosophila* Nemo is the founding member of the NLK family of serine/threonine protein kinases. Nemo can bind and phosphorylate Mad (homologous protein of Smad4) [13]. In this study, we identified the interaction between human NLK and Smad4, and identified NLK could



**Fig. 3** NLK can phosphorylate Smad4 in vitro. **a** GST-Smad4 fusion protein was incubated with or without cell-expressed Flag-NLK, together with 1mM ATP and kinase buffer at 30°C for 30 min. Reactions were run on SDS-PAGE and visualized with Pro-Q Diamond Phosphoprotein Gel Stain to see phosphorylated protein, and with coomassie blue stain. **b** Mapping of the Smad4 region that can be phosphorylated by NLK. GST-Smad4, GST-Smad4- $\Delta 2 + \Delta 3$ , and pET32a-Smad4- $\Delta 3$  were subjected to kinase assays with cell-expressed Flag-NLK as kinase. After incubation of these reactions at 30°C for 30 min, protein A/G agarose immunoprecipitated with Flag-NLK in the mixture was first removed from GST-Smad4 kinase assay reaction. With additional 0.5  $\mu$ l of  $\lambda$ ase to the remaining reaction system, the dephosphorylation reaction was carried out at 30°C for 30 min. Then all the reactions were subjected to Pro-Q Diamond Phosphoprotein Gel Stain analysis. **c** Thr9 and Ser138 are the phosphorylation sites for NLK. GST-Smad4-Thr9A, -Ser138A, and -Thr197A mutant fusion proteins were adjusted to the same concentration. These three mutant proteins and wild-type Smad4 were used as the substrates of Flag-NLK. GST-Smad4-Thr9A and -Ser138A showed weaker phosphorylation compared to the wild-type protein. The phosphorylation signal of GST-Smad4-Thr197A changed little

phosphorylate Smad4 in vitro. The interaction between Smad4 and NLK was discovered by yeast two-hybrid screen, and was further confirmed by the in vitro GST pull-down assay and the in vivo co-immunoprecipitation assay. Considering that NLK is a conserved MAPK-type kinase and Smad4 has been shown to be constitutively phosphorylated irrespective of the TGF- $\beta$  signal [10, 11], in vitro kinase assays were carried out to search the phosphorylation sites on Smad4 for NLK. Two novel phosphorylation sites in Smad4, Thr9, and Ser138 were identified for NLK. These two sites are located at the MH1 domain of Smad4, and are conserved in *Sus scrofa*,

<b>H.sapiens</b>	:	MDNMSITNTPTSNDACLIVHSLMCHRQGGSETFAKRAIESLVKKLKEKKDELDSLITAITTNGAHPKCVTIQRTLDD	:	80
<b>S.scrofa</b>	:	MDNMSITNTPTSNDACLIVHSLMCHRQGGSETFAKRAIESLVKKLKEKKDELDSLITAITTNGAHPKCVTIQRTLDD	:	80
<b>R.norvegicus</b>	:	MDNMSITNTPTSNDACLIVHSLMCHRQGGSETFAKRAIESLVKKLKEKKDELDSLITAITTNGAHPKCVTIQRTLDD	:	80
<b>M.musculus</b>	:	MDNMSITNTPTSNDACLIVHSLMCHRQGGSETFAKRAIESLVKKLKEKKDELDSLITAITTNGAHPKCVTIQRTLDD	:	80
<b>B.taurus</b>	:	MDNMSITNTPTSNDACLIVHSLMCHRQGGSETFAKRAIESLVKKLKEKKDELDSLITAITTNGAHPKCVTIQRTLDD	:	80
<b>X.laevis</b>	:	---MSITNTPTSNDACLIVHSLMCHRQGGSETFAKRAIESLVKKLKEKKDELDSLITAITTNGAHPKCVTIQRTLDD	:	77

<b>H.sapiens</b>	:	RLQVAGRKGFPFHVYARLWRWPDHLKHNELKHVKYCYAFDLKCDSCVCPYHYERVVSPGIDLSGLTLQSNAPSSMMVKL	:	160
<b>S.scrofa</b>	:	RLQVAGRKGFPFHVYARLWRWPDHLKHNELKHVKYCYAFDLKCDSCVCPYHYERVVSPGIDLSGLTLQSNAPSSMLVKD	:	160
<b>R.norvegicus</b>	:	RLQVAGRKGFPFHVYARLWRWPDHLKHNELKHVKYCYAFDLKCDSCVCPYHYERVVSPGIDLSGLTLQSNAPSSMLVKD	:	160
<b>M.musculus</b>	:	RLQVAGRKGFPFHVYARLWRWPDHLKHNELKHVKYCYAFDLKCDSCVCPYHYERVVSPGIDLSGLTLQSNAPSSMLVKD	:	159
<b>B.taurus</b>	:	RLQVAGRKGFPFHVYARLWRWPDHLKHNELKHVKYCYAFDLKCDSCVCPYHYERVVSPGIDLSGLTLQSNAPSSMLVKD	:	160
<b>X.laevis</b>	:	RLQVAGRKGFPFHVYARLWRWPDHLKHNELKHVKYCYAFDLKCDSCVCPYHYERVVSPGIDLSGLTLQSSAPSSGLLVKD	:	157

<b>H.sapiens</b>	:	EYVHDFE-GQPSLSI-EGHSIQTIQHPPSNRASTETYSTPALLAPSESNAATSTNFPNIPVASTSQPASILAGSHSEGLL	:	238
<b>S.scrofa</b>	:	EYVHDFE-GQPSLAT-EGHSIQTIQHPPSNRASTETYSTPALLAPSESNAATSTNFPNIPVASTSQPASILAGSHSEGLL	:	238
<b>R.norvegicus</b>	:	EYVHDFE-GQPSLPT-EGHSIQTIQHPPSNRASTETYSTPALLAPSESNAATSTNFPNIPVASTSQPASILAGSHSEGLL	:	238
<b>M.musculus</b>	:	EYVHDFE-GQPSLPT-EGHSIQTIQHPPSNRASTETYSTPALLAPSESNAATSTNFPNIPVASTSQPASILAGSHSEGLL	:	237
<b>B.taurus</b>	:	EYVHDFE-GQPSLSI-EGHSIQTIQHPPSNRASTETYSTPALLAPSESNAATSTNFPNIPVASTSQPASILAGSHSEGLL	:	238
<b>X.laevis</b>	:	EYGHDIYAEGQCSISAGDGLSIQTIQHPPSSRSSTETYSTNALLVPESEPSTPNPISFSSLPVASTSQPASILLATHNDGLL	:	237

**Fig. 4** The N-terminal sequences of human Smad4, and of *Sus scrofa*, *Rattus norvegicus*, *Mus musculus*, *Bos taurus*, *Xenopus laevis* Smad4 are aligned. Phosphorylation sites in human Smad4 are indicated by

asterisks. Thr9 and Ser138 in Smad4 are conserved in all these species, while Thr197 is replaced with an alanine in *Rattus norvegicus* and *Mus musculus*, and with an asparagine in *Xenopus laevis*

*Rattus norvegicus*, *Mus musculus*, *Bos taurus*, and *Xenopus laevis* (Fig. 4). These two phosphorylation sites may contribute to the constitutive phosphorylation of Smad4. Conversely, Thr197, which could not be phosphorylated by NLK, is found to be replaced by an alanine in *Rattus norvegicus* and *Mus musculus*, and by an asparagine acid residue in *Xenopus laevis* (Fig. 4).

NLK is a MAPK-type kinase which often functions as a negative regulator for Wnt signal and TGF- $\beta$  signal pathway through phosphorylation of interacting proteins. In *Xenopus* embryos, non-canonical Wnt-5a/Ca(2+) pathway activates the activities of endogenous TGF- $\beta$ -activated kinase (TAK1) and NLK, then inhibits  $\beta$ -catenin-induced transcriptional activation [14, 15]. NLK augmented NARF (NLK-associated RING finger protein, an E3 ubiquitin-ligase) binding and ubiquitylation of T-cell factor and lymphoid enhancer factor (TCF/LEF) complex in *Xenopus* embryos. The ubiquitylated TCF/LEF was subsequently degraded by the proteasome [16]. In human Wnt signaling pathway, activated NLK can directly bind and phosphorylate LEF-1/TCF, contributing to the down-regulation of LEF-1/TCF transcriptional activity [17]. In addition, Wnt-1 signal could activate NLK to phosphorylate c-Myb at multiple sites, subjecting c-Myb to ubiquitination and proteasome-dependent degradation [18]. The Wnt-NLK pathway also inhibits A-Myb activity by blocking A-Myb-induced transcriptional activation [19]. Taken together, we proposed that NLK might negatively regulate Smad4 through phosphorylation of it, inhibiting transcription activity of Smad4 or inducing the ubiquitination and proteasome-dependent degradation of Smad4.

Most importantly, we have identified the interaction between Smad4 and NLK, and identified two phosphorylation sites on Smad4 for NLK, which located at the MH1

domain of Smad4. Whether this phosphorylation is responsible for the constitutive phosphorylation of Smad4 and whether this phosphorylation negatively regulates Smad4 still need further investigation. Nevertheless, our results revealed a new mechanism for phosphorylation modification of Smad4, a pivotal protein involved in TGF- $\beta$  signaling.

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