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Original Article

Protein tyrosine phosphatase PTPN2 regulates TGF-β signaling through Smad4 dephosphorylation

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Abstract: Transforming Growth Factor beta (TGF-β) is a multifunctional cytokine that regulates cell proliferation, differentiation, and apoptosis. Dysregulation of the TGF-β signaling is one of the major mechanisms underlying tumor progression. We have previously reported that anaplastic lymphoma kinase (ALK) phosphorylates Smad4 at Tyr95, which compromises the DNA-binding ability of Smad4 and thus renders ALK-positive cancer cells resistant to TGF-β tumor-suppressive action. In this study, we demonstrated that tyrosine phosphatase PTPN2 positively regulated TGF-β signaling through dephosphorylating Smad4 at the Tyr95 site. Both *in vitro* and cell-based assays revealed that PTPN2 bound to and dephosphorylated Smad4, thereby preserving the DNA-binding ability of Smad4. Furthermore, overexpression of PTPN2 restored TGF-β transcriptional and growth inhibitory responses in ALK-positive cancer cells. Consistently, Spermidine, an activator of PTPN2, also promoted TGF-β-induced gene expression, apoptosis, and anti-proliferation effect. Taken together, we revealed that PTPN2 functioned as a tumor suppressor to antagonize the inhibitory effect of tyrosine phosphorylation of Smad4 and to ensure the proper TGF-β growth inhibitory signaling in cancer cells.

Keywords: Smad, ALK, tyrosine phosphorylation, tumor suppressor, spermidine

Introduction

Members of the transforming growth factor-β superfamily (TGF-β) are multifunctional cytokines widely expressed in organisms. The TGF-β signaling pathway is involved in a variety of cellular processes, including cell proliferation, survival, differentiation, epithelial-mesenchymal transition, and stem-cell renewal [1-3]. Hence, dysregulation of the TGF-β signaling pathway attributes to tumor initiation and progression [4-6]. Given the complicated role of TGF-β in tumor development, it is critical to investigate the intricate regulation of TGF-β signaling in tumorigenesis.

Once the TGF-β receptors are activated, the TGF-β receptor complex recruits and activates Smads, the intracellular transducers of TGF-β signaling. The Smads form heteromeric complexes consisting of two phosphorylated receptor-regulated Smads (R-Smads) and one common Smad (co-Smad) in the cytoplasm, which are transported to the nucleus and regulate the transcription of target genes. Smad proteins are regulated by a variety of molecules and signaling pathways as well as posttranslational modifications. For example, Zhang *et al.* have reported that constitutively active anaplastic lymphoma kinase (caALK) phosphorylates Smad4 on tyrosine to attenuate its DNA-binding ability, and thus disables the TGF-β tumor suppressor functions [7]. This finding highlighted the importance of tyrosine phosphorylation in regulating TGF-β activity in tumor cells.

Protein tyrosine phosphatase non-receptor type 2 (PTPN2) is a member of the classical non-receptor protein tyrosine phosphatase family that is ubiquitously expressed, especially

Table 1. Information for antibodies utilized in the study

Antibody	Source and Catalog number
PTPN2	ABclonal A1808
PTPN2	Proteintech 11214-1-AP
PTPN2	Cell Signaling Technology 58935S
Smad2/3	Cell Signaling Technology 8685S
P-Smad3	Cell Signaling Technology 9520S
GAPDH	Sigma G8795
Actin	Sigma A5441
Smad4	Cell Signaling Technology 46535S
p21	Cell Signaling Technology 2947S
PAI-1	Abcam ab66705
cMyc	Cell Signaling Technology 5605S
PARP	Cell Signaling Technology 9532S
Cleaved PARP	Cell Signaling Technology 5625S
GST	Abcam ab9085
FLAG	GNI 4110-FG
HA	GNI 4110-HA
HA	Cell Signaling Technology 3724
GFP	Santa Cruz sc-9996

in lymphoid and hematopoietic cells. PTPN2 was originally discovered from human peripheral T cells, thus was first named TC-PTP [8]. Alternative splicing of PTPN2 mRNA generates two splice variants of 48 kD and 45 kD. The 48 kD splice variant (TC48) is localized to the endoplasmic reticulum (ER) by its C-terminal hydrophobic tail, while the 45 kD variant (TC45) targets to the nucleus [9, 10]. It is widely acknowledged that PTPN2 acts as a negative regulator in several oncogenic protein tyrosine kinase (PTK) signaling pathways by dephosphorylating and inactivating substrates, including epidermal growth factor receptor (EGFR) [11, 12] and Janus-activated kinases (JAKs) [13].

Protein phosphorylation and dephosphorylation are reversible dynamic processes that regulates many signaling events. Given that Tyr95 phosphorylation of Smad4 results in Smad4 inactivation, it is of great significance to identify tyrosine phosphatase(s) that can dephosphorylate Smad4, thereby restoring the TGF-β signaling in cells. Herein, we performed a functional phosphatase library screen and identified PTPN2 as the first tyrosine phosphatase of Smad4. We found that PTPN2 could dephosphorylate Smad4 at Tyr95 and restore the DNA binding capability of Smad4. Furthermore, the PTPN2 agonist Spermidine could enhance TGF-β responses in cells with Smad4 Tyr95 phosphorylation. These results illustrate the critical function of PTPN2 in regulating the TGF-β signaling and its role in tumorigenesis.

Materials and methods

Plasmids

Human PTPN2 cDNA was obtained by PCR, and the PTPN2-D182A mutant was generated by PCR-based mutagenesis and confirmed by sequencing. The N-terminal HA-tagged PTPN2 and PTPN2-D182A were constructed in expression vector pRK3H. Fusion of GFP-PTPN2 was generated by PCR and cloned into pBobi-puro vector. Lentiviral tet-on expression plasmids of PTPN2 were constructed by subcloning into pCMV-Tet-on vector. PTPN2 and PTPN2-D182A mutant were also subcloned into pGEX4T1 vector for *E. coli* expression as GST fusion protein. Expression plasmids of NPM-ALK, Smad4, Smad4-Y95E, Smad4-Y95F were described previously [7].

Antibodies and reagents

The antibodies used in this study are listed in **Table 1**. The following chemicals or growth factors were purchased: SB431542 (S4317), Spermidine (S0266) and Puromycin from Sigma-Aldrich; TGF-β1 (TGFB1) from Stem RD; G418 from Amresco.

Cell culture and transfection

SUDHL-1, EAP9, H2228, and Karpas 299 cells were maintained in 1640 medium supplemented with 10% FBS. HaCaT cells were cultured in MEM medium with 10% FBS, and HEK293T were cultured in DMEM medium with 10% FBS. All cells were free of mycoplasma contamination and were maintained in a 37°C humidified incubator with 5% CO_2. For cell transfection, HaCaT cells were transfected with X-tremeGENE (Roche Applied Science), while HEK293T cells were transfected with PEI (Polyscience).

Lentivirus production and generation of stable cell lines

Lentiviral plasmid pBobi-GFP-PTPN2 together with viral packaging vectors (pVSV-G, pMDLg/pRRE, and pRSV-Rev) were transfected into

HEK293T cells, while pCMV-Tet-on-PTPN2 was co-transfected with pLP1, pLP2, and pVSV-G into HEK293T cells. After 48 h of transfection, culture medium containing lentiviral particles was collected and used to infect target cells. Cells stably expressing target genes were selected by puromycin or G418 resistance.

RNA interference

For transient knockdown of PTPN2, siRNAs that specifically target human PTPN2 were synthesized (RiboBio) and transfected into cells using Lipofectamine RNAiMAX (Thermo Fisher Scientific). siRNA sequences were as follows: siPTPN2-1: GTACAGGACTTTCCTCTAA; siPTPN2-2: GGAACAGAATAGGTCTAGA; siPTPN2-3: GGTAGACACTTGTCTTGTT; negative control siRNA was provided by RiboBio.

qRT-PCR

Total RNAs were extracted by Trizol reagent (Invitrogen). The reverse transcription of RNAs was conducted using PrimeScript RT Master Mix Perfect Real Time (TaKaRa). qRT-PCR was performed on CFX Connect Real-Time PCR System (Bio-Rad) using the indicated primers and Power SYBR Green PCR Master Mix (ABI). The sequences of PCR primers were as follows: for human *GAPDH*: 5'-CGACCACTTTGTCAAGCTCA-3' and 5'-TTACTCCTTGGAGGCCATGT-3'; for human *PTPN2*: 5'-TCTTATTCAGACCCCAGATCAAC-3' and 5'-TGATCAAAGGCAGGAGATAAGTC-3'; for human *CDKN2B*: 5'-ACATGCGTAAACGACACTCTCTGG-3' and 5'-TTTAGCATCTGTCGTCGCTTGCA-3'; for human *CDKN1A*: 5'-ACCATGTGGACCTGTCACTGT-3' and 5'-TTAGGGCTTCCTCTTGGAGAA-3'; for human *SERPINE1*: 5'-TTTGCAGGATGGAACTACGG-3' and 5'-CAAGAGTGATGGCAATGTGAC-3'; for human *cMyc*: 5'-CTGGTGCTCCATGAGGAGA-3' and 5'-CTCTGACCTTTTGCCAGGAG-3'; for human *SMAD7*: 5'-CCAACTGCAGACTGTCCAGA-3' and 5'-CAGGCTCCAGAAGAAGTTGG-3'; for mouse *Serpine1*: 5'-CCTGACATGTTTAGTGCAACCC-3' and 5'-TTTTGCAGTGCCTGTGCTAC-3'; for mouse *Smad7*: 5'-CCAACTGCAGGCTGTCCAGA-3' and 5'-CAGGCTCCAGAAGAAGTTGG-3'.

Cell lysis and immunoprecipitation

Cells were treated with TGF-β (2 ng/ml) or other reagents for indicated time, washed with ice-cold PBS, and then lysed on ice with NEDT buffer (50 mM Tris-HCl [pH 8.0], 2 mM EDTA, 150 mM NaCl, 1% NP-40, and protease and phosphatase inhibitors added before use). The cell lysate was cleared by centrifugation, and the supernatant was subjected to immunoprecipitation with indicated primary antibody followed by protein agarose beads (G&E healthcare). After extensive washing, the immunoprecipitated proteins, as well as the whole cell lysates as input control, were separated by SDS-PAGE followed by western blot analysis.

Western blot analysis

Standard western blot protocol was followed. Briefly, whole cell lysate and IP samples were boiled with SDS loading buffer at 95°C for 10 min, separated by SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membrane (Milipore), incubated with primary and secondary antibodies, and detected with an enhanced chemiluminescence staining kit (Cyanagen).

In vitro phosphatase assay

The phosphatase PTPN2 protein was produced as GST fusion protein in *E. coli* and purified by glutathione-Sepharose 4B beads. The protocol for the production of GST-tagged fusion protein was previously described [14]. To obtain ALK-phosphorylated Smad4, Smad4 expression plasmid was transfected into HEK293T without or with NPM-ALK-expressing plasmid co-transfection, and Smad4 was purified by immunoprecipitation. For phosphatase assay, the recombinant GST-PTPN2 was incubated with the immunopurified phospho-Smad4 in dephosphorylation buffer (50 mM Tris, 50 mM Bis-Tris, 2 mM DTT, pH 7.5) at 25°C for 1 h. After reaction, the samples were analyzed by western blot.

In vitro binding assay

GST-PTPN2 fusion protein conjugated on Glutathione-Sepharose 4B beads was produced as described above. Smad4 and Smad4 mutants (Smad4-Y95E and Smad4-Y95F) were *in vitro* translated using Quick Coupled Transcription/Translation System (Promega). *In vitro* binding between GST-PTPN2-beads and Smad4 as well as Smad4 mutants was carried out by co-incubation in GST-binding buffer (50 mM Tris, 150 mM NaCl, 0.5% NP-40) at 4°C for 4 h. After extensive washing, samples co-

precipitated with Glutathione-Sepharose 4B beads were analyzed by western blot.

DNA-pulldown assay

DNA pulldown assay using biotinylated SBE oligonucleotides was carried out as previously described [15]. Briefly, HEK293T cells were transfected with indicated plasmids, and the cells were lysed with DNA pulldown buffer (10 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 0.1% NP-40 and 10% glycerol). The cell lysates were then incubated with 50 pmol of biotinylated SBE oligonucleotides at RT for 30 minutes followed by incubation with Streptavidin beads (GE healthcare) at RT for 15 minutes. After extensive washing, the Streptavidin beads-conjugated SBE-protein complexes were precipitated and subjected to SDS-PAGE and western blot analysis.

Cell proliferation assay

Cells were treated with TGF-β as indicated. Cell proliferation was examined by using Cell Counting Kit-8 (MCE) and EDU cell proliferation Apollo®488 kit (Ribobio) according to the manufacturer's instructions.

Chromatin immunoprecipitation (ChIP) assay

Cells were treated with spermidine and/or TGF-β as indicated and crosslinked by 1% of formaldehyde at room temperature for 10 min. ChIP assay was performed by using ChIP Assay Kit (Pierce) according to the manufacturer's instructions. Antibodies used in ChIP assay were indicated in the text. The amount of co-precipitated DNA was detected by qPCR. The sequences of qPCR primers used were as follows: for human *CDKB2B*: 5'-CTGCCTGGGGATGAATTTAAC-3' and 5'-GGTTTCACTGTGGAGACGTTG-3'; for human *CDKB1C*: 5'-TCTCGCTGTCCTCTCCTCTC-3' and 5'-GCACTAGTACTGGGAAGGTC-3'; for human *SERPINE1*: 5'-GCAGGACATCCGGGAGAGA-3' and 5'-CCAATAGCCTTGGCCTGAGA-3'.

Mouse xenograft tumor model

Female Balb/c nude mice (6 weeks old) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and provided with standard chow and tap water ad libitum. All animal experiments were performed according to the guidelines of the Zhejiang University Committee for Experimental Animal Studies and Ethics. To generate xenograft tumor, a total of 1×10^7 EAP9 cells stably overexpressing GFP or GFP-PTPN2 were mixed with Matrigel Matrix (Corning, 1:1) and then injected subcutaneously into the flank of mice (n=9). Tumor growth was monitored every 3 days using calipers, and mice were euthanized at day 23 after implant. Tumors were resected, weighed, and processed for western blot analysis.

Statistical analysis

Data were presented as mean ± SEM from three biologically independent experiments. Statistical analysis was performed using One Way ANOVA. *P* values < 0.05 were considered statistically significant.

Results

PTPN2 dephosphorylated Smad4

To identify protein tyrosine phosphatases that regulate Smad4 by posttranscriptional modification, a library of protein tyrosine phosphatases was constructed and screened using Smad4 p-Tyr95 specific antibody (**Figure 1A**). The screening results showed that PTPN2 dephosphorylated Smad4 at Tyr95 when it was co-expressed with Smad4 in HEK293T cells. Given the important role of Tyr95 phosphorylation in Smad4-mediated TGF-β signaling, we tested whether PTPN2 could affect TGF-β response by using a luciferase reporter gene under the control of Smad binding element (SBE) to monitor the TGF-β/Smad activity, and our data showed that the nucleus isoform (TC45) of PTPN2, but not the TC48 isoform, rescued the Tyr95 Smad4-suppressed TGF-β response (**Figure 1B**). Since TC48 localizes in the ER, it is reasonable that this isoform could not regulate TGF-β signaling. Therefore, our study focused on the function of the TC45 isoform of PTPN2.

To explore whether the phosphatase activity of PTPN2 was essential for its effect, we generated a PTPN2 mutant of D182A, which resulted in the loss of PTPN2 phosphatase activity. As shown in **Figure 1C**, in a co-transfection experiment, the expression of PTPN2, but not the PTPN2-D182A mutant, decreased

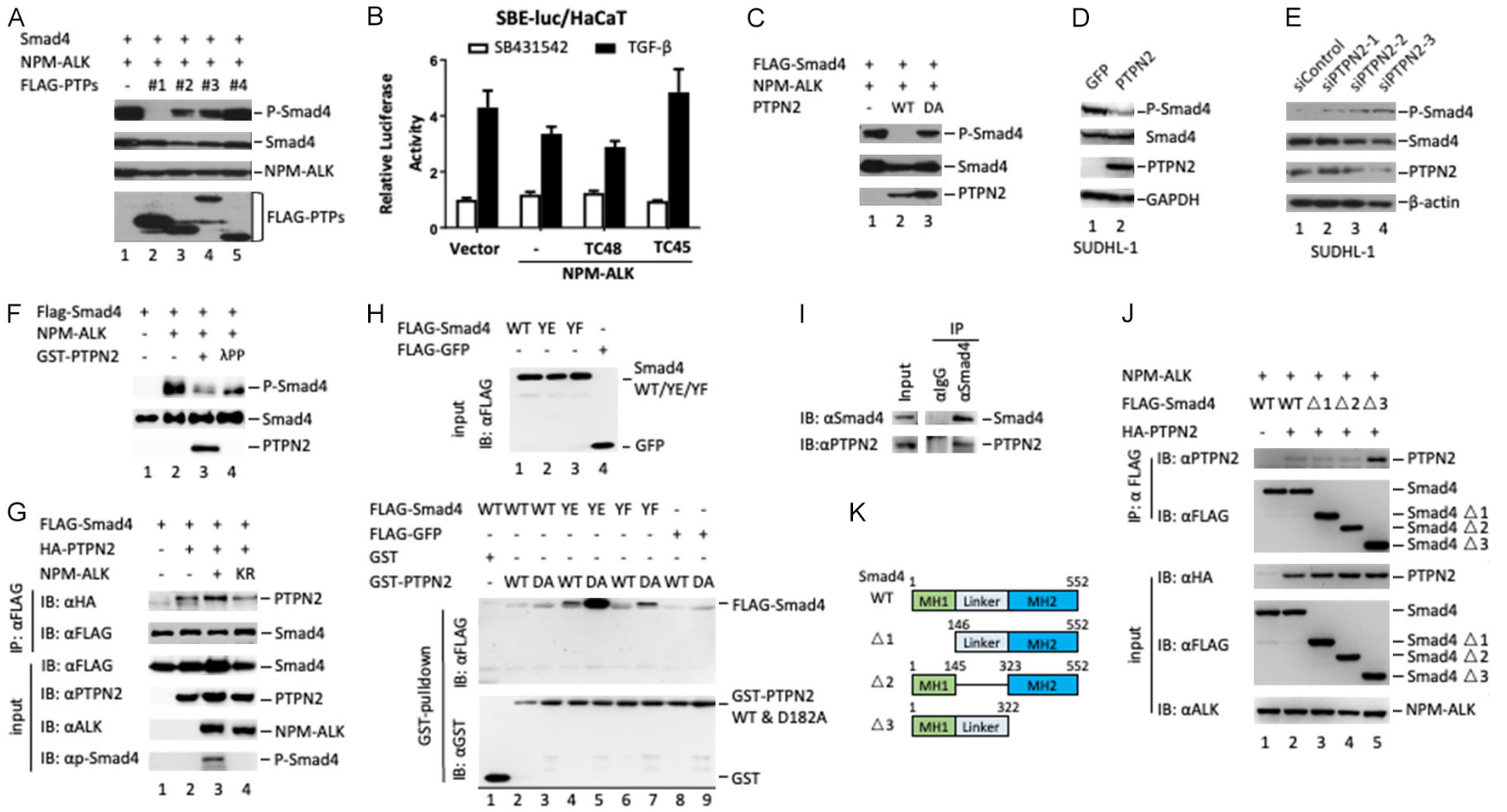

Figure 1. PTPN2 interacts with and dephosphorylates Smad4. (A) Functional screening of tyrosine phosphatases that dephosphorylate Smad4. Smad4 and NPM-ALK were transiently expressed in HEK293T with protein tyrosine phosphatases, and the phosphorylation of Smad4 was detected by P-Smad4 (Y95) antibody. (B) The nuclear isoform of PTPN2 (TC45), but not the 48kD isoform, rescues the TGF-β response. SBE-luc reporter activity was examined. The cells were treated with 5 μM SB431542 or 2 ng/ml TGF-β for 18 h. (C) Wildtype PTPN2, but not PTPN2-D182A mutant, dephosphorylates Smad4. Smad4 and NPM-ALK were transiently expressed in HEK293T with PTPN2 or PTPN2-D182A mutant co-expression. After transfection for 24 h, cells were collected for western blot analysis. (D) Stable overexpression of PTPN2 reduces the Tyr-95 phosphorylation of Smad4 in SUDHL-1 cells. SUDHL-1 cells stably overexpressing GFP or PTPN2 were collected for western blot analysis. (E) Knockdown of PTPN2 by siRNA increases the Tyr-95 phosphorylation of Smad4. SUDHL-1 cells transfected with siControl, siPTPN2-1, siPTPN2-2, and siPTPN2-3 were collected for western blot analysis. (F) PTPN2 dephosphorylates Smad4 *in vitro*. FLAG-Smad4 was transfected in HEK293T with or without NPM-ALK and immunoprecipitated as the substrate for *in vitro* assay. GST-PTPN2 was purified by Glutathione-Sepharose 4B beads. GST-PTPN2 and Smad4 were mixed and incubated at 25°C for *in vitro* dephosphorylation. The phosphorylation level of Smad4 were detected by western blot analysis. λPP, lambda protein phosphatase was used as a positive control. (G) Smad4 interacts with PTPN2, which is enhanced by NPM-ALK but not by kinase-dead mutant of NPM-ALK. HEK293T cells were transfected with HA-PTPN2 and FLAG-Smad4, as well as NPM-ALK or its kinase-dead mutant. Co-IP and western blot were carried out. (H) PTPN2 directly binds to the phospho-mimic Smad4. GST-PTPN2 was mixed with *in vitro*-synthesized FLAG-tagged Smad4 for *in vitro* binding assay. GST-bound proteins and input were detected by western blot. (I) Endogenous interaction of Smad4 and PTPN2 in SUDHL-1 cells. Cell lysates were collected and immunoprecipitated with the indicated antibodies. Smad4 and PTPN2 were detected by western blot. (J) PTPN2 binds to the MH1-linker region of Smad4. HEK293T cells were transfected with FLAG-Smad4 or its deletion mutants as well as HA-PTPN2. Co-IP and western blot were carried out. (K) Schematic diagram of the deletion constructs of Smad4.

the phosphorylation level of Smad4 at Tyr95, confirming the dephosphorylation activity of PTPN2 and the loss of catalytic activity in the DA mutant. We further examined the activity of PTPN2 towards endogenously phosphorylated Smad4. SUDHL-1 is a lymphoma cell line harboring the gene fusion *NPM-ALK*, which leads to the constitutive activation of NPM-ALK [16, 17] and the Tyr95 phosphorylation of Smad4 [7]. We found that stably overexpression of PTPN2 in SUDHL-1 cells resulted in a decreased Smad4 Tyr95 phosphorylation (**Figure 1D**). Conversely, knockdown of PTPN2 increased the Smad4 Tyr95 phosphorylation level in SUDHL-1 cells (**Figure 1E**). Therefore, PTPN2 regulated Smad4 Tyr95 phosphorylation, and this function required its phosphatase activity.

To distinguish whether the effect of PTPN2 on Smad4 phosphorylation was direct or indirect mediated by other phosphatase(s), we performed an *in vitro* dephosphorylation assay. The purified phospho-Smad4 (P-Smad4) was obtained from HEK293T cells by co-transfection with NPM-ALK and subsequent immunoprecipitation, while PTPN2 was produced as GST-PTPN2 fusion protein. After co-incubation of phospho-Smad4 and PTPN2 in the dephosphorylation buffer, the Smad4 Tyr95 level was significantly decreased (**Figure 1F**), indicating a direct dephosphorylation of Smad4 by PTPN2.

PTPN2 interacts with Smad4

Since a physical interaction is normally required for the enzyme and its substrates, a Co-IP assay was carried out to determine the interaction between PTPN2 and Smad4. In a co-transfection experiment, we could detect the presence of PTPN2 in the Smad4-immunoprecipitated complex (**Figure 1G**, lane 2), indicating the association of these two proteins. Interestingly, co-expression of NPM-ALK enhanced the interaction between PTPN2 and Smad4. This effect required the kinase activity, as the kinase-dead mutant NPM-ALK-K210R didn't exert this effect (**Figure 1G**, lanes 3 and 4). Importantly, the physiological interaction between PTPN2 and Smad4 was confirmed by endogenous co-immunoprecipitation in SUDHL-1 (**Figure 1I**). In addition, in vitro GST-pulldown experiment was performed to determine the direct interaction between Smad4 and PTPN2. GST, GST-PTPN2, and GST-PTPN2-D182A protein absorbed on Glutathione-Sepharose 4B beads were used for this purpose. We were particularly interested in the binding activity of PTPN2-D182A as it has been previously reported that D182A mutant of PTPN2 is not only inactive but also capable of forming a stable complex with substrates, thus making it a "substrate trapping" mutant [18]. Pure Smad4 proteins, including wildtype Smad4, phospho-mimic mutant of Smad4 (Smad4-Y95E), and phosphorylation-defective mutant (Smad4-Y95F) were obtained by *in vitro* translation. The binding reaction of purified GST fusion proteins with Smad4 was illustrated in **Figure 1H**. The GST-pulldown result showed that the Smad4-Y95E directly interacted with PTPN2, while Smad4-Y95F displayed a weaker interaction with PTPN2. As predicted, the "substrate trapping" mutant of PTPN2-D182A exhibited stronger interaction with Smad4 than wildtype PTPN2. Together, these results indicated that there was a direct interaction between PTPN2 and Smad4, and Tyr95 phosphorylation of Smad4 might play an important role in the process.

To further understand the structural requirement for the Smad4-PTPN2 interaction, we generated a series of deletion mutants of Smad4, which were tested for binding with PTPN2, as shown in **Figure 1K**. The Co-IP experiment showed that all Smad4 truncations except the deletion of MH2 domain could bind to PTPN2 similarly as wildtype did; however, deletion of the MH2 domain dramatically enhanced the interaction between Smad4 and PTPN2 (**Figure 1J**). According to the report by Hata et al., there is an intramolecular interaction between the MH1 and MH2 domain, which inhibits Smad4 activity [19]. Hence, we speculated that deletion of the MH2 domain released the autoinhibition of Smad4, leading to an open confirmation of Smad4 for its interaction with PTPN2.

Overexpression of PTPN2 enhances the TGF-β-induced transcriptional response

Smad4 is the intracellular effector of the TGF-β signaling pathway. It has been reported that oncogenic ALK abrogates the TGF-β-induced response by phosphorylating Smad4 [7]. Since we discovered that PTPN2 could dephosphorylate Smad4 on Tyr95, we were curious whether PTPN2 could rescue the abrogated TGF-β response in ALK-positive cells. To test this, we used SBE-luc reporter assay to investigate the functional consequence of ALK and PTPN2 on

TGF-β signaling. Our data showed that ectopic expression of PTPN2, but not PTPN2-D182A, markedly rescued the TGF-β-mediated transcriptional activation reduced by NPM-ALK in HaCaT cells (**Figure 2A** and **2B**). Similar results were also obtained in H2228 cells, an EML4-ALK-positive non-small cell lung carcinoma (NSCLC) cell line [20]. Ectopic expression of PTPN2 in H2228 strongly enhanced TGF-β-mediated transcriptional activation, as measured by synthetic promoter-driven reporters, SBE-luc and CAGA-luc (**Figure 2C** and **2D**) and natural promoter-driver reporters, PAI-1-luc and SMAD7-luc (**Figure 2E** and **2F**).

To further explore the TGF-β response and PTPN2 function in ALK-positive cells, we generated a SUDHL-1 cell line stably overexpressing PTPN2 (**Figure 2G**) and found that overexpression of PTPN2 markedly upregulated the TGF-β-induced transcription of *CDKN2B*, *CDKN1A*, and *SMAD7* (**Figure 2H-J**). In addition, we generated a PTPN2 tet-on cell line in EAP9 cells, a mouse lung tumor cell line harboring the *EML4-ALK* fusion gene [7]. Consistently, inducible overexpression of PTPN2 increased the transcription of *Serpine1* and *Smad7* (**Figure 2K-M**), indicating that PTPN2 could sensitize ALK-positive cells to TGF-β signal.

PTPN2 restores the anti-proliferative activity of Smad4

Since we observed that PTPN2 rescued the TGF-β-induced transcriptional response in cells, we reasoned that PTPN2 could consequently restore the anti-proliferative activity of Smad4. Therefore, we examined the effect of PTPN2 on the proliferation of ALK-positive cells. CCK-8 assay showed that inducible overexpression of PTPN2 attenuated the proliferation of both EAP9 and H2228 cells (**Figure 3A** and **3B**). The inducible overexpression of PTPN2 in these cells was confirmed (**Figures 2K** and **3C**). In agreement, the inducible expression of PTPN2 also restrained the colony formations of H2228 in the presence of TGF-β, as determined by clonogenic assay (**Figure 3D**). Furthermore, overexpression of PTPN2 in SUDHL-1 cells caused a basal reduction of cell proliferation and induced a more profound decrease in cell proliferation when treated by TGF-β (**Figure 3E**). To further understand the anti-proliferation function of PTPN2, we examined the cell death marker (cleaved-PARP) in SUDHL-1 cells and found that overexpression of PTPN2 promoted the TGF-β-induced apoptosis of SUDHL-1 cells (**Figure 3F**). Together, these results suggested that PTPN2 restored the anti-proliferative activity of Smad4 in ALK positives cells.

To support the findings from cell-based assays, we further investigated the function of PTPN2 *in vivo* by using xenograft tumor model. EAP9 cells stably overexpressing GFP or GFP-PTPN2 were subcutaneously injected into nude mice, and the effect of PTPN2 on tumor growth was evaluated. In consistent with the growth inhibitory effect observed *in vitro*, overexpression of GFP-PTPN2 repressed the tumor growth, resulting in a smaller tumor in mice (**Figure 3G**, **3H**).

PTPN2 reverses the effect of tyrosine phosphorylation on the DNA-binding activity of Smad4

Our previous study has reported that oncogenic ALK impairs the DNA-binding activity of Smad4 by phosphorylating Smad4 at the Tyr95 site [7]. If PTPN2 dephosphorylated Smad4, we speculated that PTPN2 would reverse the effect of ALK. We then performed a DNA-pulldown assay to investigate the effect of PTPN2 on Smad4 DNA binding. As expected, PTPN2 expression could rescue the attenuated binding of Smad4 on biotin-labeled SBE (**Figure 4A**). However, PTPN2 was unable to affect the binding of Smad4-Y94F, the phosphorylation-defective mutant of Smad4 (**Figure 4B**). Similarly, PTPN2 could rescue the DNA binding of wildtype Smad4, but not its phospho-mimic mutant, Smad4-Y95E (**Figure 4C**). These results indicated that PTPN2 rescued the Smad4 DNA-binding ability through dephosphorylating Smad4 at Tyr95.

In addition to Smad4 phosphorylation, we also exploited whether PTPN2 influenced other step(s) during TGF-β signal transduction. First, the phosphorylation of Smad2 and Smad3 were analyzed in H2228 cells, and overexpression of PTPN2 showed no influence on Smad2 nor Smad3 phosphorylation upon TGF-β stimulation (**Figure 4D**). Since Smad2/3/4 complex formation is a critical step in TGF-β signal transduction, we examined the effect of PTPN2 on Smads complex formation by co-immunoprecipitation experiments. In both HEK293T and H2228 cells, the inducible overexpression of PTPN2 had no effect on the endogenous Smads complex formation upon TGF-β treatment (**Figure 4E**, **4F**).

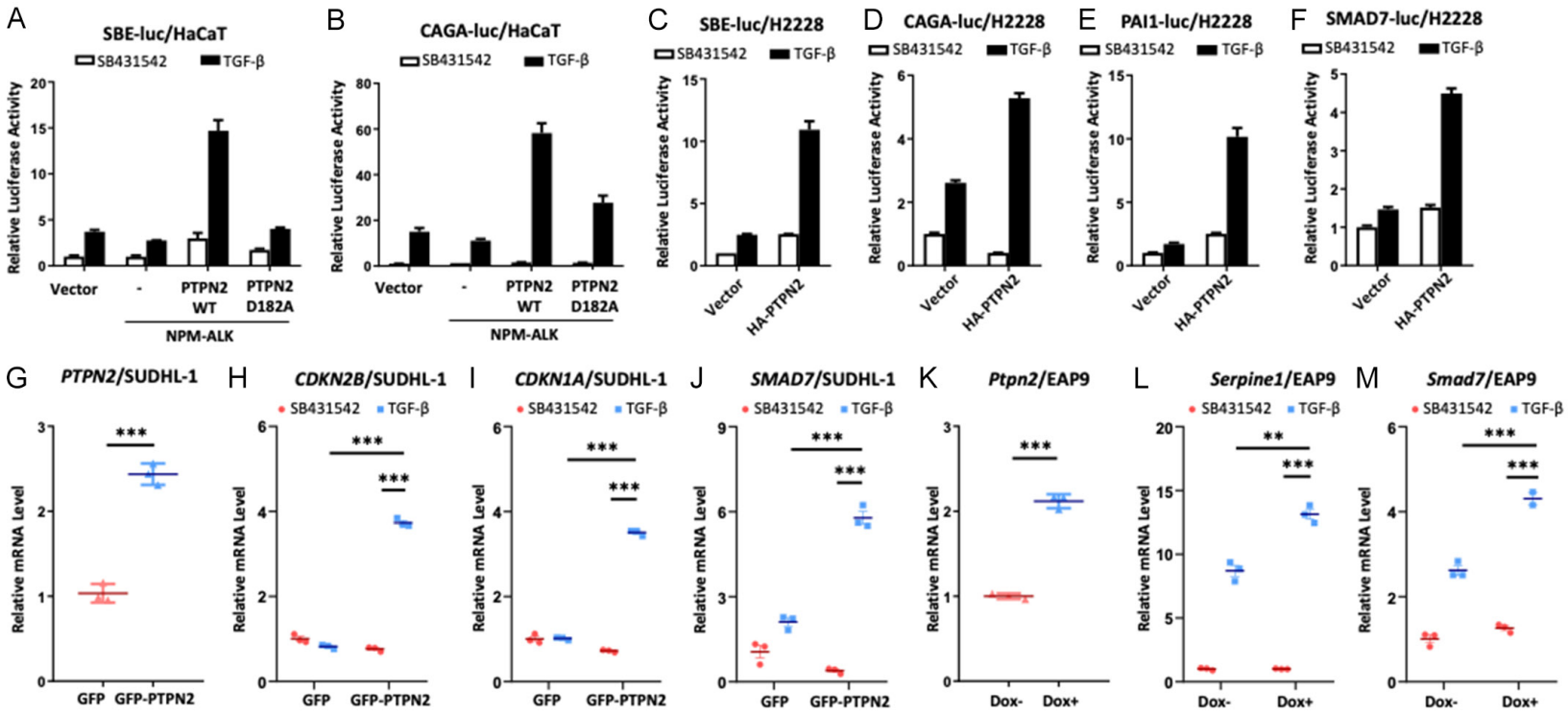

Figure 2. Overexpression of PTPN2 enhances TGF-β-induced transcriptional response. (A, B) PTPN2 restores TGF-β-induced transcriptional responses dampened by NPM-ALK in HaCaT cells. The luciferase activity of SBE-Luc (A) and CAGA-Luc (B) reporter genes was measured. The cells were treated with 5 μM SB431542 or 2 ng/ml TGF-β for 18 h. (C-F) PTPN2 enhances TGF-β-induced transcriptional responses in H2228 cells. The luciferase activity of reporter genes was measured: SBE-Luc (C), CAGA-Luc (D), PAI1-Luc (E), and SMAD7-Luc (F). The cells were treated with 5 μM SB431542 or 2 ng/ml TGF-β for 18 h. (G) PTPN2 was stably overexpressed in SUDHL-1 cells. The expression of PTPN2 in stable cells was confirmed by qRT-PCR. (H-J) Stable overexpression of PTPN2 restores the TGF-β-induced transcription of *CDKN2B* (H), *CDKN1A* (I), and *SMAD7* (J). SUDHL-1 stable cells were treated with 5 μM SB431542 or 2 ng/ml TGF-β for 8 h and subjected to qRT-PCR. (K) The expression of PTPN2 was induced by Doxycycline (Dox) in EAP9 tet-on stable cells. EAP9 stable cells were treated with or without 1000 ng/ml Dox for 24 h and subjected to qRT-PCR. (L, M) Inducible overexpression of PTPN2 enhances TGF-β-induced transcription of *Serpine* (L) and *Smad7* (M) in EAP9 cells. EAP9 tet-on stable cells expressing PTPN2 by Dox induction were treated with 5 μM SB431542 or 2 ng/ml TGF-β for 2 h and subjected to qRT-PCR.

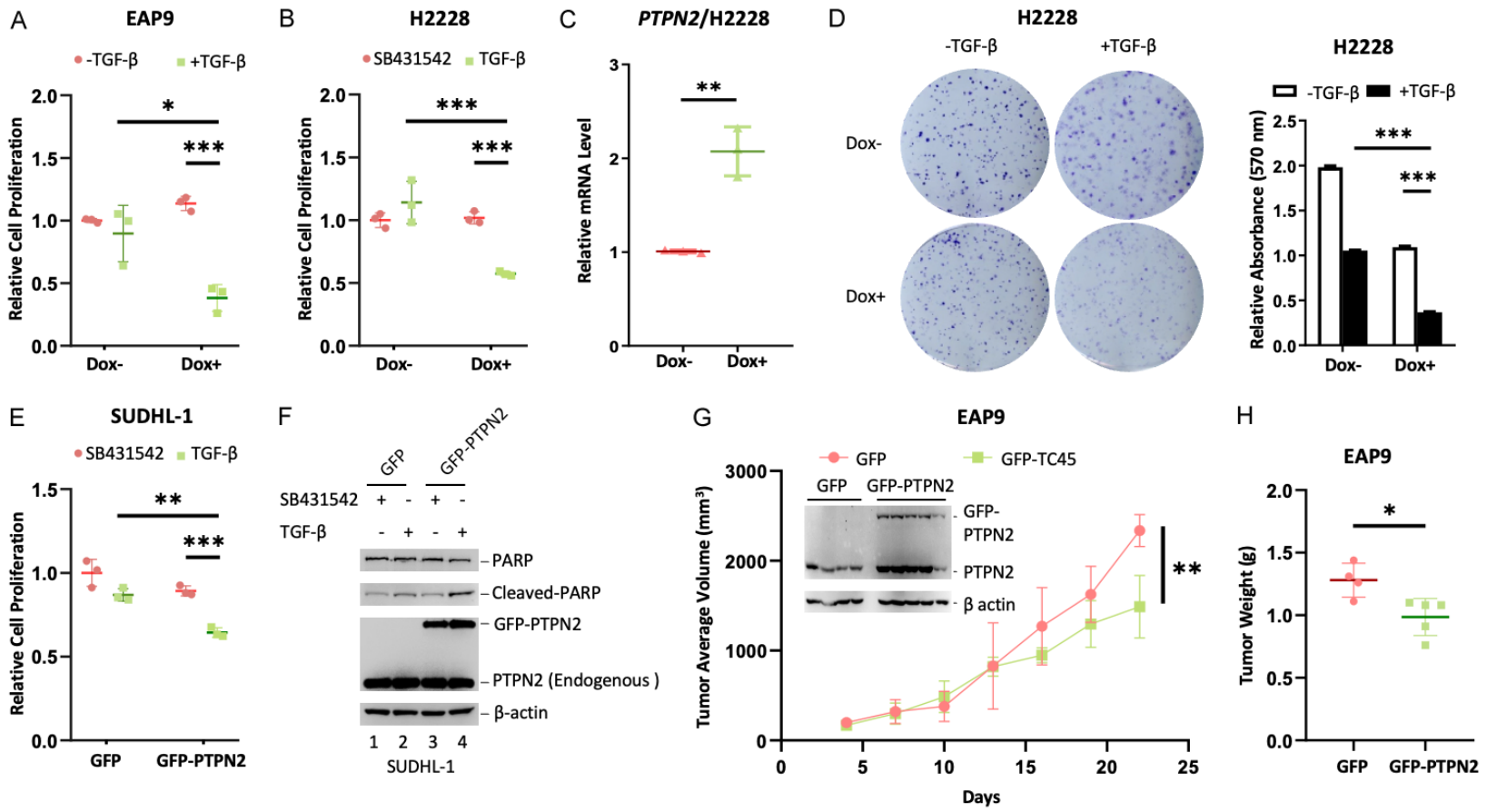

Figure 3. PTPN2 restores the anti-proliferative activity of Smad4. (A) Inducible expression of PTPN2 restores TGF-β-mediated anti-proliferation of EAP9 cells. The expression of PTPN2 was induced by 1000 ng/ml Dox in EAP9 tet-on stable cells, and the stable cell lines were treated with or without 2 ng/ml TGF-β. Cell proliferation was examined by Cell Counting Kit-8 at 96 h after treatment. (B) Inducible expression of PTPN2 restores TGF-β-mediated anti-proliferation of H2228 cells. The expression of PTPN2 was induced with or without 1000 ng/ml Dox in H2228. Cells were treated by 5 μM SB431542 or 5 ng/ml TGF-β, and cell proliferation was examined by Cell Counting Kit-8 at 48 h. (C) The overexpression of PTPN2 was induced by Dox in H2228 tet-on stable cells. H2228 stable cells were treated with or without 1000 ng/ml Dox for 24 h and subjected to qRT-PCR. (D) The combination of inducible PTPN2 expression and TGF-β treatment suppresses the proliferation of H2228 cells. H2228 stable cells were plated in the 6-well plates. Cells were treated by 2 ng/ml TGF-β with or without Dox for 5 days. Crystal violet was used to stain the colonies, and the absorbance at 570 nm was measured. (E) PTPN2 enhances TGF-β-mediated anti-proliferation of SUDHL-1 cells. SUDHL-1 cells stably expressing GFP or GFP-PTPN2 were treated by 5 μM SB431542 or 2 ng/ml TGF-β, and cell proliferation was examined by Cell Counting Kit-8 at 96 h after treatment. (F) Overexpression of PTPN2 promotes TGF-β-induced apoptosis in SUDHL-1 cells. PTPN2 stable expression cells were generated by lentivirus. SUDHL-1 stable cells were treated with 5 μM SB431542 or 2 ng/ml TGF-β for 24 h and subjected to western blot analysis. (G, H) Overexpression of PTPN2 suppressed tumorigenesis by decreasing tumor sizes (G) and weight (H). GFP or GFP-PTPN2 was stably overexpressed in EAP9 cells. EAP9 cells (1×10^7 cells) were subcutaneously injected into 6-week-old nude mice. Tumor volumes were measured at indicated days. At day 23 after cell implant, tumors were dissected, and tumor weights were measured. Tumor samples were analyzed by western blot to confirm GFP or GFP-PTPN2 overexpression.

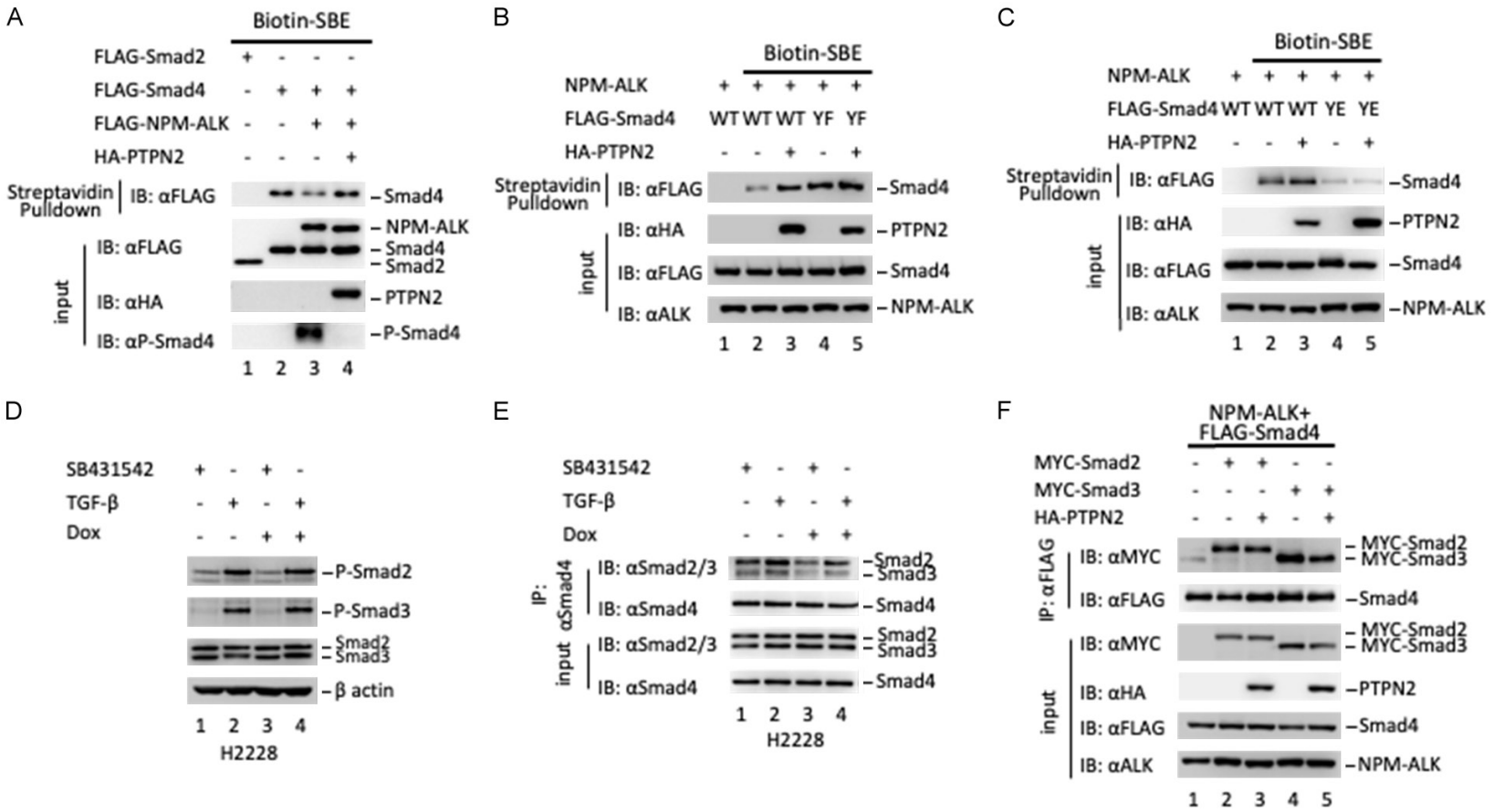

Figure 4. PTPN2 reverses the effect of tyrosine phosphorylation on the DNA-binding activity of Smad4. (A) PTPN2 restores the DNA-binding ability of Smad4 in HEK293T cells. Expression plasmids encoding HA-PTPN2 or FLAG-Smad4, as well as NPM-ALK, were transfected to HEK293T cells as indicated. Cell lysates were collected and incubated with biotin-labeled SBE oligo and streptavidin beads. The pulldown protein and whole cell lysate input were examined by western blot. Smad2 was used as a negative control. (B) PTPN2 is unable to facilitate the binding between biotin-SBE and Smad4-Y94F, a phosphorylation-defective mutant of Smad4. Expression plasmids encoding HA-PTPN2 or FLAG-Smad4, as well as NPM-ALK, were transfected in HEK293T cells as indicated. Co-IP and western blot were carried out as previously described. (C) PTPN2 does not affect the binding between biotin-SBE and Smad4-Y94E, a phospho-mimic mutant of Smad4. Expression plasmids encoding HA-PTPN2 or FLAG-Smad4, as well as NPM-ALK, were transfected in HEK293T cells as indicated. Co-IP and western blot were carried out as previously described. (D) Inducible expression of PTPN2 doesn't affect the TGF-β-induced phosphorylation of Smad2 or Smad3. H2228 cells expressing PTPN2 by Dox induction were treated with 5 μM SB431542 or 2 ng/ml TGF-β for 2 h. (E) Ectopic expression of PTPN2 does not affect Smad2/3/4 complex formation. Expression of PTPN2 was induced by 1000 ng/ml Dox. Endogenous Smad4 was immunoprecipitated, and the precipitated complex was analyzed by western blot. (F) The expression of PTPN2 does not affect the assembly of Smad2/4 complex nor Smad3/4 complex. HEK293T was transfected as indicated. Co-IP was performed, and the immunoprecipitated products were analyzed by western blot.

Spermidine activates PTPN2 and enhances TGF-β responses

As a member of the classical PTP family, PTPN2 is a non-transmembrane protein consisting of a conserved catalytic domain and a noncatalytic C-terminal domain [21]. It has been reported that the non-catalytic C-terminal segment of PTPN2 regulates its phosphatase activity via an intramolecular mechanism [22]. Further study has shown that spermidine (SPD) is an agonist of PTPN2 and activates PTPN2 by disrupting the inhibitory intramolecular bond in the protein itself [23, 24]. Hence, we were wondering if spermidine could affect TGF-β response via regulating the activity of PTPN2.

We first confirmed the activation of PTPN2 by spermidine in Karpas 299 cells (**Figure 5A**), an anaplastic large cell lymphoma (ALCL) cell line harboring the fusion protein NPM-ALK [16]. Next, we examined the effect of spermidine on Smad4 Tyr95 level in SUDHL-1 cells and found that spermidine treatment reduced the Tyr95 phosphorylation of Smad4 (**Figure 5B**), suggesting the activation of PTPN2 by spermidine.

We further explored the roles of PTPN2 and spermidine in TGF-β regulated cell proliferation in Karpas 299 cells. We found that combined TGF-β and spermidine treatment led to reduced proliferation of Karpas 299 cells (**Figure 5C**). Spermidine treatment also significantly enhanced TGF-β-mediated apoptosis in Karpas 299 cells (**Figure 5D**). Furthermore, we examined the DNA replication in spermidine treated Karpas 299 cells by using EdU assay. Flow cytometry analysis showed that low concentration of spermidine treatment did not affect the DNA replication; however, while the combination of TGF-β and high concentration of spermidine treatment markedly inhibited the DNA replication (**Figure 5E**). Collectively, we found that the activation of PTPN2 restored the antiproliferative activity of Smad4 by enhancing TGF-β-induced apoptosis and inhibiting DNA replication.

Spermidine promotes TGF-β-induced transcription by rescuing Smad4 DNA-binding activity

As spermidine activated PTPN2 and enhanced TGF-β response, we wondered if spermidine could enhance TGF-β-induced transcription in ALK-positive cells. To test this, we examined the expression of TGF-β target genes by qRT-PCR and found that activation of PTPN2 by spermidine enhanced the TGF-β-induced transcription of *CDKN2B*, *CDKN1A*, *SERPINE1*, and *SMAD7* (**Figure 6A-D**) in Karpas 299 cells as well as *SMAD7* in H2228 cells (**Figure 6E**). Consequently, spermidine also enhanced the TGF-β-induced regulations of p21, PAI-1, and cMyc proteins in Karpas 299 cells (**Figure 6F**). The DNA-pulldown assay was also performed in the H2228 cells, and the results suggested that activation of PTPN2 by spermidine facilitated the binding of Smad4 and biotin-labeled SBE (**Figure 7A**).

As a transcription factor, Smad4 binds directly to the promoter of TGF-β target genes [25-27]. We then assessed if PTPN2 affected the Smad4 occupancy on the promoter of TGF-β target genes by ChIP assay. Our data showed that the activation of PTPN2 by spermidine increased the TGF-β-induced occupancy of Smad4 on the promoter of *CDKN2B*, *CDKN1C*, and *SERPINE1* (**Figure 7B-D**) in Karpas 299 cells, suggesting that PTPN2 could restore the suppressed DNA binding activity of Smad4 in ALK-positive cells.

Discussion

In this study, we discovered that Smad4 is a substrate of the protein tyrosine phosphatase PTPN2. As a multifunctional phosphatase that regulates diverse signaling pathways, PTPN2 has been reported to attenuate oncogenic protein tyrosine kinase (PTK) signaling, thereby acting as a tumor suppressor. Here we demonstrated that PTPN2 antagonized the oncogenic function of ALK on another tumor suppressor Smad4. We revealed that PTPN2 directly interacted with Smad4 and dephosphorylated Smad4 at the Tyr95 site. By dephosphorylating Smad4, PTPN2 restored the DNA-binding ability of Smad4 and thus reactivated the TGF-β-induced transcriptional response (**Figure 7E**).

As the only co-Smad for all R-Smads, Smad4 mediates canonical TGF-β-Smad signaling of the entire TGF-β family and plays a central role in TGF-β response as a tumor suppressor. Therefore, Smad4 activity regulation is critical for the adequate function of TGF-β. Our current study revealed that a post-transcriptional modification of Smad4, dephosphorylation at Tyr95, regulated its DNA-binding activity and its func-

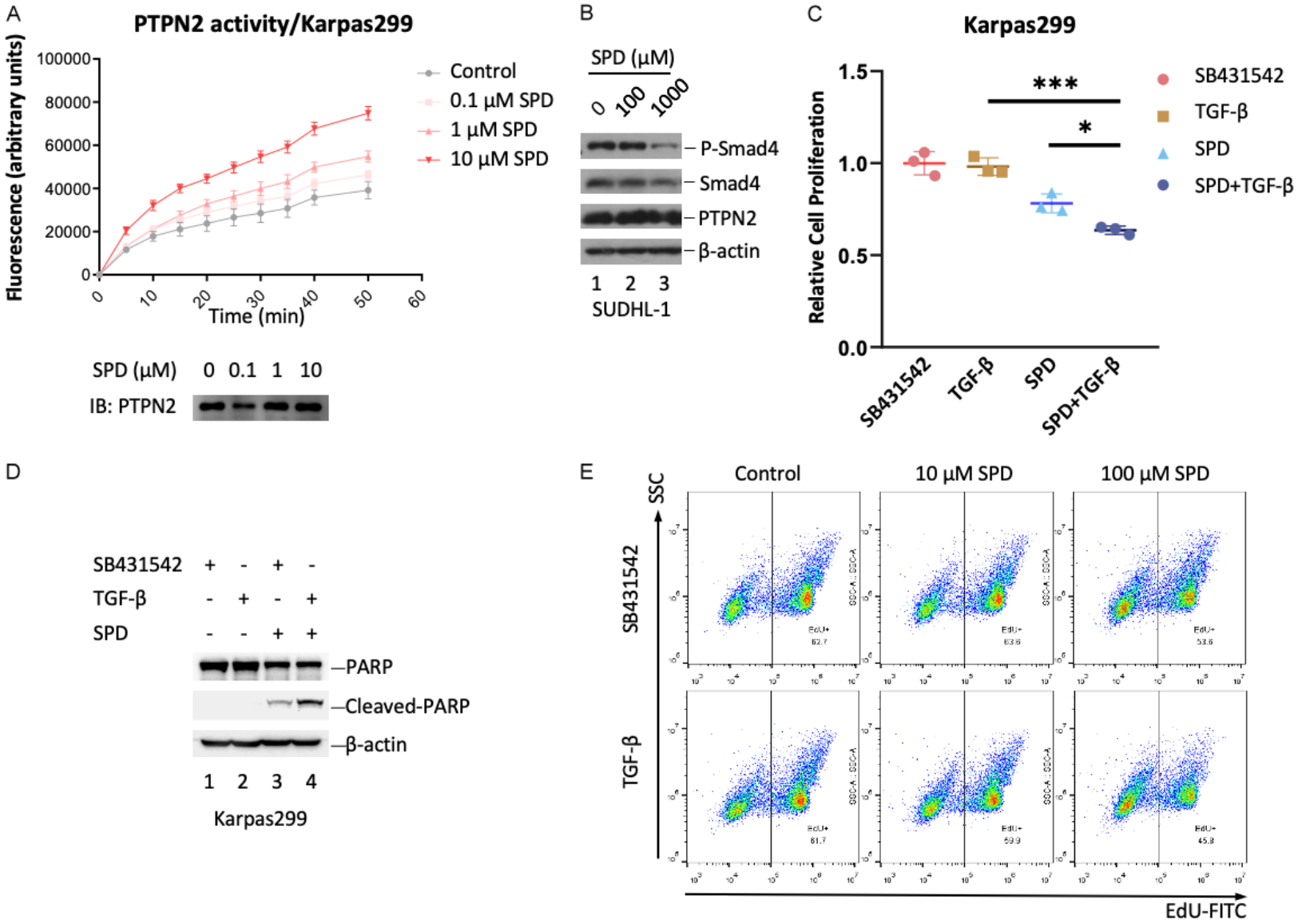

Figure 5. Spermidine activates PTPN2 and enhances TGF-β responses. (A) Spermidine (SPD), a specific agonist of PTPN2, increases the PTPN2 activity in a dose-dependent manner in Karpas 299 cells. Karpas 299 cells were treated by SPD at different concentrations for 8 h, and cell lysates were collected and immunoprecipitated with PTPN2 antibodies. The phosphatase activity of immunoprecipitated PTPN2 was detected by the EnzChek Phosphatase Assay Kit. (B) Treatment of

SPD reduces the phosphorylation of Smad4. SUDHL-1 cells were treated by SPD as indicated for 1 h, and cells were collected and subjected to western blot analysis. (C) SPD restores TGF-β-inhibited proliferation of Karpas 299 cells. Karpas 299 cells were treated with 5 μM SB431542, 2 ng/ml TGF-β or 10 μM SPD, as well as a combination of 2 ng/ml TGF-β and 10 μM SPD. The proliferation of Karpas 299 cells was examined by Cell Counting Kit-8 at 48 h after treatment. (D) Activation of PTPN2 by SPD restores TGF-β-induced apoptosis in Karpas 299 cells. Karpas 299 cells were treated by 5 μM SB431542 or 2 ng/ml TGF-β with or without the presence of 10 μM SPD for 24 h. Cell lysates were collected and subjected to western blot analysis. (E) SPD results in a reduction of active DNA synthesis in Karpas 299 cells. Karpas 299 cells were treated with 5 μM SB431542 or 2 ng/ml TGF-β with or without SPD for 24 h, and then EdU was added to culture medium for 2 h. Cells were collected and subjected to flow cytometry analysis.

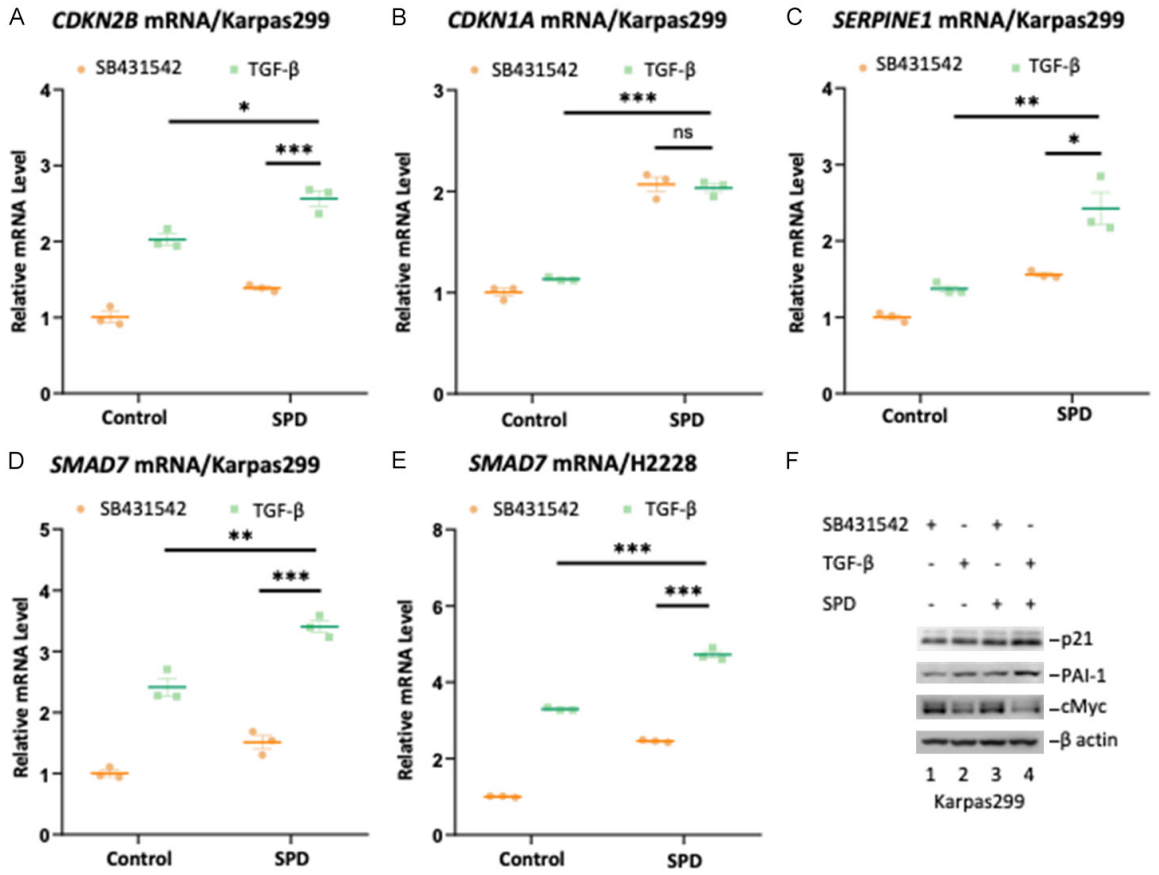

Figure 6. Spermidine promotes TGF-β-induced transcription. (A-D) SPD increases the TGF-β-induced transcription of *CDKN2B* (A), *CDKN1A* (B), *SERPINE1* (C), and *SMAD7* (D) in Karpas 299 cells. Karpas 299 cells were pre-treated by 10 μM SPD for 24 h followed by 5 μM SB431542 or 2 ng/ml TGF-β treatment with or without the presence of 10 μM SPD for 4 h. Cell lysates were collected and subjected to qRT-PCR. (E) SPD increases the TGF-β-induced transcription of *SMAD7* in H2228 cells. H2228 cells were pre-treated by 10 μM SPD for 18 h followed by 5 μM SB431542 or 2 ng/ml TGF-β treatment with or without the presence of 10 μM SPD for 8 h. Cell lysates were collected and subjected to qRT-PCR. (F) SPD increases TGF-β-induced p21, PAI1 expression and cMyc reduction in Karpas 299 cells. Karpas 299 cells were treated by 5 μM SB431542 or 2 ng/ml TGF-β with or without the presence of 10 μM SPD for 24 h. Cell lysates were collected and subjected to western blot analysis.

tion as a transcription factor. Significantly, we identified PTPN2 as the phosphatase that dephosphorylated Smad4 and modulated Smad4 DNA binding activity.

It has been well known that aberrant kinase activity of ALK attributes to the tumorigenesis. Smad4 is also found to be inactivated in various cancers [28]. Previously, we reported that the TGF-β pathway is repressed in the ALK fusion gene positive tumors, in which Smad4 Tyr95 is phosphorylated by constitutively active ALK, leading to the attenuated Smad4 DNA binding and the suppressed expression of TGF-β target genes [7]. This finding suggests that Smad4 Tyr95 phosphorylation may serve

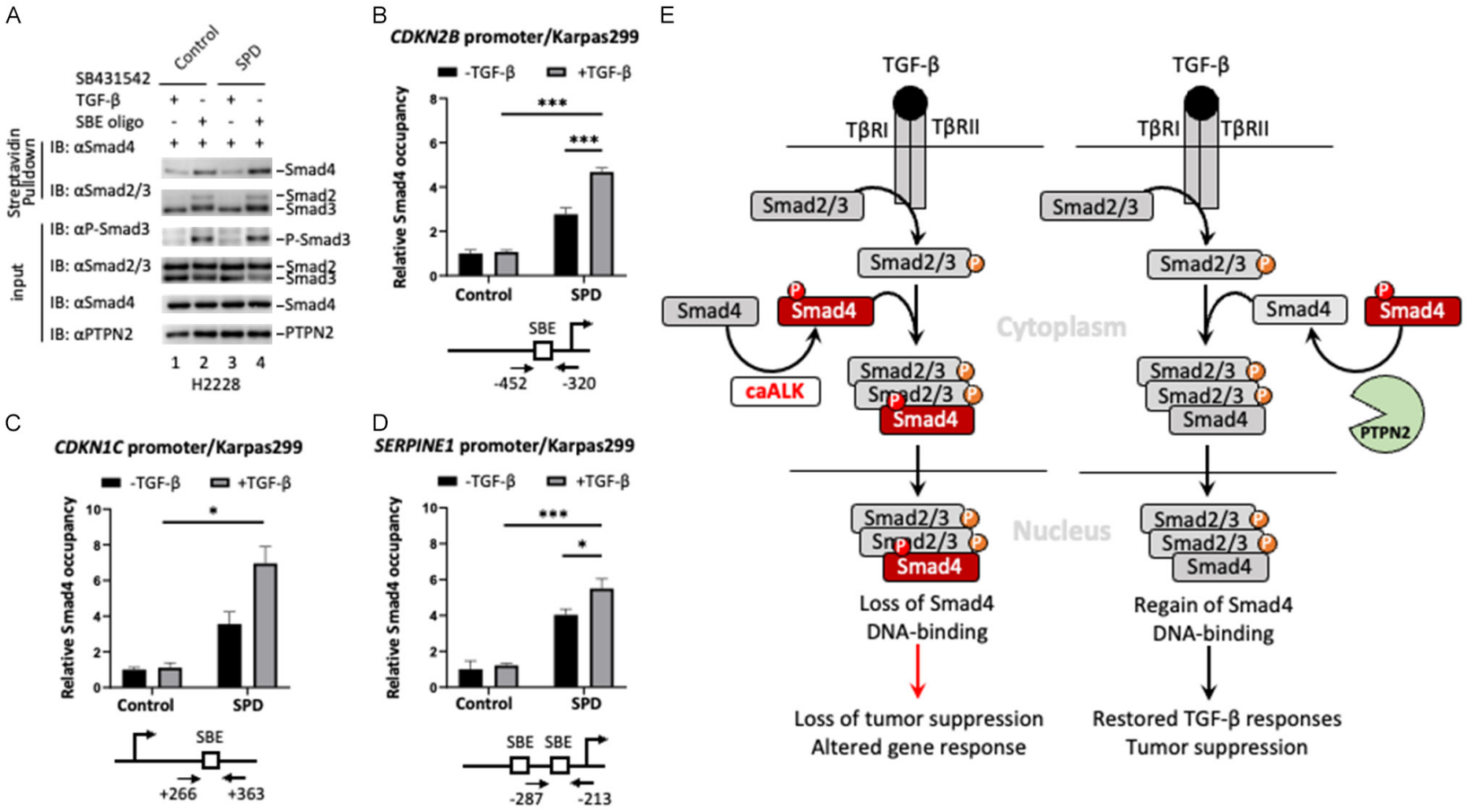

Figure 7. Spermidine rescues Smad4 DNA-binding activity. (A) Treatment of SPD enhances the DNA-binding ability of Smad3 and Smad4 in H2228 cells. H2228 cells were treated by SB431542 or TGF-β with or without 5 μM SPD for 2 h. Cell lysates were collected and incubated with biotin-labeled SBE oligo and streptavidin beads. The pulldown protein and whole cell lysate input were examined by western blot. (B-D) The activation of PTPN2 by SPD increases the TGF-β-induced occupancy of Smad4 on the promoter of *CDKN2B* (B), *CDKN1C* (C), and *SERPINE1* (D). Karpas 299 cells were pre-treated with 100 μM spermidine for 2 h followed by 2 ng/ml TGF-β treatment for 2 h. Chromatin immunoprecipitation (ChIP) was carried out with anti-Smad4 antibody, and the amount of co-precipitated DNA was determined by qPCR using primers specific to the promoters of *CDKN2B*, *CDKN1C*, and *SERPINE1*. (E) A working model of PTPN2 regulation on TGF-β signaling pathway in constitutively active ALK (caALK) positive tumor cells. In tumor cells expressing caALK, Smad4 is phosphorylated on Tyr 95 and fails to bind to DNA, leading to the loss of TGF-β signaling. Protein tyrosine phosphatase PTPN2 dephosphorylates Smad4 and rescues its DNA binding ability, thereby restoring the TGF-β-induced transcriptional response and tumor suppression.

as a therapeutic target in ALK active tumors. Our identification of PTPN2 as a Smad4 Tyr95 phosphatase provided the rationale for targeting PTPN2 for the treatment of ALK positive tumors, especially in ALK inhibitor-resistant tumors.

Furthermore, our study also characterized the function of spermidine, an agonist of PTPN2 in ALK-positive tumor cells. We found that the activation of PTPN2 by spermidine also enhanced the DNA binding of Smad4 and the TGF-β response in tumor cells. These results indicate that spermidine treatment can be a viable therapeutic approach for tumors in which Smad4 is inactivated due to Tyr95 phosphorylation.

Nevertheless, the role of PTPN2 in cancer treatment is complex. Deletion of PTPN2 was found in T-cell acute lymphoblastic leukemia (T-ALL) [29], and PTPN2 protein was absent in a large proportion of triple-negative breast cancers [30], suggesting that PTPN2 acts as a tumor suppressor. However, PTPN2 was identified as a cancer immunotherapy target, and deletion of PTPN2 in tumor cells increased the efficacy of immunotherapy [31]. In addition, Wiede et al. reported that deletion of PTPN2 in T cells promotes anti-tumor immunity and CAR T-cell efficacy in solid tumors [32]. These findings suggest that inhibition of PTPN2 in tumor cells or T cells might be favorable in specific diseases. Therefore, further investigation on the function of PTPN2 in tumorigenesis and immune regulation is required.

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Disclosure of conflict of interest

None.

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