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Yes-Associated Protein (YAP65) Interacts with Smad7 and Potentiates its Inhibitory Activity Against TGF-β/Smad Signaling

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Abbreviations: TGF-β, transforming growth factor-β; YAP65, Yes-Associated Protein

Running title: YAP65 and Smad7 co-repress TGF-β/Smad signaling

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SUMMARY

Members of the TGF-β family of growth factors signal from the cell surface through serine/threonine kinase receptors. Intracellular propagation of the signal occurs by phosphorylation of intracellular proteins of the Smad family. Smad7 belongs to the subclass of inhibitory Smads that function as antagonists of TGF-β signaling. A yeast two-hybrid screen of a human placental cDNA expression library using full-length mouse Smad7 as bait identified Yes-Associated Protein (YAP65) as a novel Smad7-interacting protein. The association of Smad7 with YAP65 was confirmed using co-expressed tagged proteins in COS-7 cells. Deletion of the PY motif of Smad7 reduced but did not abolish YAP65-Smad7 association, suggesting the existence of several interacting domains. We demonstrate that YAP65 potentiates the inhibitory activity of Smad7 against TGF-β-induced, Smad3/4-dependent, gene transactivation. Furthermore, YAP65 augments the association of Smad7 to activated TGF-β receptor type I (TβRI), whereas YAP65(1-301), which exerts a dominant-negative effect against Smad7-driven inhibition of TGF-β signaling, reduces these interactions. Together, these data provide the first evidence that YAP65 is a Smad7 partner that facilitates the recruitement of the latter to activated TβRI, and enhances the inhibitory activity of Smad7 against TGF-β signaling.

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INTRODUCTION

The Transforming Growth Factor-β (TGF-β) family of polypeptidic growth factors plays important roles during embryonic development, maintenance of adult tissue homeostasis, and various pathological conditions, ranging from abnormal tissue repair states to cancer (Branton & Kopp, 1999; de Caestecker et al., 2000). TGF-βs signal via serine/threonine kinase transmembrane receptors that phosphorylate cytoplasmic mediators of the Smad family (Attisano & Wrana, 2000). The receptor-associated Smads (R-Smads), such as Smad1, Smad2, Smad3 and Smad5, interact directly with, and are phosphorylated by, activated TGF-β receptors type I (TβRI), and are ligand specific. Upon phosphorylation, they form heteromeric complexes with Smad4, a common mediator for all Smad pathways. These heterocomplexes are then translocated into the nucleus where they function as transcription factors, binding DNA either directly or in association with other proteins (Massagué & Wotton, 2000). Inhibitory Smads, such as Smad6 and Smad7, bind activated TβRI, thereby preventing phosphorylation of R-Smads (Massagué & Wotton, 2000; Wrana, 2000).

Aside from its inhibitory activities against TGF-β signaling (Nakao et al., 1997), Smad7 has also been shown to exert broader functions in the cell, such as inducing apoptotic cell death (Lallemand et al., 2001; Landstrom et al., 2000), and activating Jun N-terminal kinase (Mazars et al., 2001), the latter contributing to the former effect.

Among proteins known to bind Smad7 are Smurf1 and Smurf2, two ubiquitin-ligases that are recruited to the activated TβRI by Smad7, leading to proteasomal degradation of the receptor (Ebisawa et al., 2001; Kavsak et al., 2000). Another protein called STRAP,

originally identified as a TβRI-interacting protein (Datta et al., 1998), also associates with Smad7 (Datta & Moses, 2000). STRAP recruits Smad7 to activated TβRI, and stabilizes the Smad7-TβRI association. This phenomenon prevents R-Smad phosphorylation by TβRI and subsequent intracellular signaling. STRAP is thus thought to assist Smad7 in inhibiting TGF-β-dependent transcription.

In an attempt to identify novel Smad7 partners, we screened a human cDNA expression library with the yeast two-hybrid system, using a human full-length Smad7 cDNA as bait. We report the identification of the Yes-Associated Protein (YAP65) as a Smad7-interacting protein, capable of recruiting Smad7 to activated TβRI, and potentiating the capacity of the former to block Smad signaling.

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RESULTS AND DISCUSSION

Identification of YAP65 as a Smad7-Interacting Protein-To identify proteins that could be involved in the negative regulation of TGF-β signaling, we screened a human placental cDNA expression library with a LexA fusion encoding the full-length mouse Smad7. Several positive clones encoding putative WW domains were identified and sequenced. WW domains are present in several unrelated proteins, including Pin1, dystrophin, or Nedd4, and consist of ~40 amino acids, of which two, tryptophan and proline, are highly conserved (Rotin, 1998). These WW domains can recognize certain proline-rich modules known as PY motifs, found in numerous proteins, including Smad7 or interleukin receptors, as well as in the transactivation domain of several transcription factors including c-Jun, AP-2, KROX-20 and -24, and C/EBP-α, for example (reviewed in (Sudol et al., 2001)).

One of the clones identifed in the two-hybrid screen encoded the WW domain of YAP65, a proline-rich phosphoprotein originally identified as a protein binding to the SH3 domain of the Yes proto-oncogene product (Sudol, 1994). To investigate the association of YAP65 with inhibitory Smads *in vivo*, COS-7 cells were transfected with either Smad6-Myc or Smad7-Myc, alone or together with His-YAP65. As shown in Fig. 1A, comparable amounts of Smad6 and Smad7 were expressed in each cell lysate (no IP, blot Myc, central panel). Similarly, YAP65 was equally expressed in samples containing either Smad6 or Smad7 (no IP, Blot His, lower panel). His-YAP65 from cell lysates was pulled down with Ni-NTA (nickel-nitrilotriacetic acid) magnetic agarose beads. Western blot analysis of associated proteins with an anti-Myc antibody revealed that YAP65 interacts only with Smad7, not with Smad6 (upper panel).

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Smad7 contains a PY motif, a conserved domain recognized by WW domains. To determine whether the Smad7 PY domain was involved in the interaction with YAP65, COS-7 cells were transfected with either Flag-Smad7 or Flag-Smad7ΔPY, a truncated form of Smad7 lacking the PY motif (Ebisawa et al., 2001), in absence or presence of His-YAP65. Following Ni-NTA magnetic agarose beads pull down, Smad7 was revealed with an antibody to Flag. As shown in Fig. 1B, removal of the PY domain of Smad7 reduced, but did not eliminate, the association with YAP65, suggesting that the PY motif is not the only domain of Smad7 involved in the interaction with YAP65.

YAP65 and Smad7 Co-Repress TGF-β Signaling-The functional relevance of the Smad7/YAP65 interaction was examined in the context of TGF-β-induced Smad-dependent gene transcription in HaCaT keratinocytes, using the artificial $(CAGA)_9$-lux promoter/reporter gene construct (Dennler et al., 1998). As shown in Fig. 2A, and as expected from the literature, TGF-β-induced transactivation was inhibited by Smad7. Under the same experimental conditions, YAP65 slightly reduced TGF-β action, and exerted an additive activity with Smad7 to further reduce TGF-β effect. On the other hand, the mutated construct $(CAGA)_9$MUT-lux, in which the critical CAGA motifs have been mutated into TACA and do not bind Smad3 and Smad4 (Dennler et al., 1998), showed no modulation by either TGF-β, Smad7, or YAP65 (Fig. 2B).

Functional Analysis of YAP65 Domains in the Context of TGF-β Signaling-In addition to WW domains, YAP65 possesses several other modules potentially involved in protein-protein interactions, such as SH3- and PDZ-binding motifs. A schematic representation of YAP65 structure is shown in Fig. 3A. The SH3 domain recognizes Src homology domains

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within other proteins (Musacchio et al., 1994), and the PDZ domain is responsible for cell membrane association (Songyang et al., 1997). YAP65 also exhibits an activation domain that allows it to function as transcriptional co-activator of PEBP2α (Yagi et al., 1999).

To examine the functionality of the domains of the YAP65 protein in the context of Smad7-driven inhibition of TGF-β signaling, truncated forms of YAP65 were tested for their ability to modulate the inhibitory function of Smad7. Overexpression of YAP65(1-301) and YAP65(156-275) (left and central panels, respectively), both of them containing the WW domain of YAP65, resulted in a slight enhancement of TGF-β effect on $(CAGA)_9$-lux in HaCaT keratinocytes (Fig. 3B), a phenomenon that may be the consequence of an interaction of the truncated YAP65 proteins with endogenous Smad7. Most importantly, expression of both these mutants prevented the inhibition of TGF-β signaling by Smad7. In contrast, co-expression of full-length YAP65 together with Smad7 (right panel) significantly potentiated Smad7 inhibitory effect.

To understand the mechanisms by which these truncated YAP65 proteins interfere with Smad7 activity, their ability to bind Smad7 was examined in immunoprecipitation experiments. Several Gal4 binding domain (Gal4BD)-fused YAP65 constructs were transfected into COS-7 cells in the presence of Flag-Smad7, cell lysates were subjected to immunoprecipitation with an anti-Flag antibody, and immunoprecipitates probed for the presence of YAP mutants with an anti-Gal4BD antibody. As shown in Fig. 3C, both YAP65(1-301) and YAP65(156-275) interacted with Smad7, consistent with the notion that the WW domains of YAP65 are involved in the association with Smad7, as suggested from

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our yeast two-hybrid screen and from our data presented in Fig. 1B, where removal of the PY motif of Smad7 reduced Smad7-YAP65 association.

We next compared the ability of full-length YAP65 vs. that of YAP65(1-301) to modulate Smad7 association with activated TβRI. The latter interaction is critical for Smad7-driven inhibition of Smad signaling, as it prevents R-Smad-TβRI association and subsequent R-Smad phosphorylation (Nakao et al., 1997). COS-7 cells were transfected with Flag-Smad7 and constitutively active TβRI(TD)-HA, in the absence or presence of either full-length YAP65 or YAP65(1-301). Following immunoprecipitation of the cell lysates with the anti-Flag antibody, TβRI(TD) was revealed with anti-HA antibody. As shown in Fig. 4, full-length YAP65 dose-dependently increased the amount of TβRI(TD) bound to Smad7. Inversely, YAP65(1-301) reduced the amount of TβRI(TD) bound to Smad7. These data are consistent with the additive effect of YAP65 with Smad7 to block TGF-β signaling, likely by stabilizing the association of Smad7 with activated TβRI, whereas YAP65(1-301) may exert a dominant-negative effect against Smad7 by preventing the association of the latter with activated TβRI.

Conclusions-We have identified YAP65 as a novel Smad7-interacting protein involved in the association of Smad7 to activated TβRI. This phenomenon is accompanied by the potentiation of the inhibitory activity of Smad7 against TGF-β signaling. YAP65 appears therefore to function as an inhibitor of TGF-β/Smad signaling, in a manner similar to that of the previously described STRAP protein (Datta et al., 1998; 2000). Whether STRAP and YAP65 may cooperate for this activity remains to be elucidated. YAP65, originally described as a c-Yes-associated protein, may transmit signals from Yes/Src/Crk to the

nucleus and function as a transcriptional co-activator. Further investigations are required to understand the mechanisms responsible for the recruitment by YAP65 of Smad7 to TβRI, and to establish a possible link between Src/Yes-related YAP65 activities and the interference with the TGF-β/Smad pathway.

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MATERIALS AND METHODS

Yeast Two-Hybrid Screening-A human placental cDNA expression library in yeast L40ΔGal4 was used for two-hybrid screening, as described (Vojtek et al., 1993). Full-length mouse Smad7 cDNA fused to LexA was used as bait. Briefly, library-containing yeast cells were tranformed with LexA-Smad7 and plated in galactose-containing medium without histidine. Positive clones were picked after 3-5 days and tested again on medium containing LacZ to identify real positive clones.

*Cell Cultures***-**HaCaT keratinocytes and COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 2 mM glutamine, and antibiotics (100 units/ml penicillin, 50 µg/ml streptomycin-G and 0.25 µg/ml Fungizone™). Human recombinant TGF-β1 was purchased from R&D Systems Inc. (Minneapolis, MN).

Plasmid Constructs-$(CAGA)_9$-lux and $(CAGA)_9$MUT-lux (Dennler et al., 1998) were gifts from S. Dennler and J.-M. Gauthier (Glaxo Wellcome, Les Ulis, France). pCMX-Gal4(1-93) containing murine YAP65(1-301), YAP65(156-275) and YAP65(276-472), pcDNA3.1/HisC(YAP65) and pcDNA3.1/HisCYAP65(1-301), described in (Yagi et al., 1999), were provided by Y. Ito (Kyoto University, Kyoto, Japan). Human pcDNA3-Myc-Smad6 (Topper et al., 1997) was a gift from J.N. Topper (Stanford University, Stanford, CA). Human pcDNA3-Myc-Smad7 has been described previously (Mazars et al., 2001). Smad7ΔPY (Ebisawa et al., 2001) and TβRI(TD)-HA (Wieser et al., 1995) expression vectors were gifts from K. Miyazono (Cancer Institute of the Japanese Foundation for

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Cancer Research, Tokyo, Japan) and J.L. Wrana (Mount Sinai Hospital, Toronto, Canada), respectively.

Transient Cell Transfections and Reporter Assays-Transient cell transfections were performed with a liposome-based protocol (Fugene™, Roche Diagnostics, Indianapolis, IN) in fresh medium containing 1% FCS. Following appropriate incubation periods (see Figure legends), the cells were rinsed twice with phosphate buffered saline (PBS), harvested by scraping, and lysed in 200 µl of Reporter Lysis Buffer (Promega Corp., Madison, WI). pRSV-β-galactosidase was co-transfected in every experiment to monitor transfection efficiency: aliquots corresponding to identical β-galactosidase activities were used for each reporter assay and co-precipitation experiments. Luciferase activity was determined with a commercial assay kit (Promega Corp).

Co-precipitations and Western blotting-COS-7 cells were transfected with expression vectors encoding the tagged proteins of interest. Fourty hours later, cells were washed twice with cold PBS, scraped, and solubilized in a buffer containing 20 mM Tris -HCl, pH8, 150 mM NaCl, 5 mM $MgCl_2$, 0, 5% NP-40, 10% glycerol, 1 mM orthovanadate, 1 mM PMSF, 20 µg/ml aprotinine and 20 µg/ml leupeptine. For Flag-tagged protein immunoprecipitations, cell lysates were cleared of debris by centrifugation and incubated overnight at 4°C with anti-Flag M2 antibody (Sigma Chemical Co., St-Louis, MO), followed by incubation with protein G-Sepharose beads at 4°C for 1 h (Amersham Pharmacia Biotech, Uppsala, Sweden). After 5 washes with solubilization buffer, immunoprecipitates were eluted by boiling for 3 min in SDS sample buffer (100 mM Tris-HCl, pH8, 0.01%

bromophenol blue, 36% glycerol, 4% SDS) and subjected to SDS-polyacrylamide gel electrophoresis. In the case of 6xHis-tagged proteins, purifications were performed using Ni-NTA (nickel-nitrilotriacetic acid) magnetic agarose beads (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol for protein-protein interactions. After electrophoresis, proteins were electrotransferred to nitrocellulose filters, immunoblotted with HRP-coupled anti-HA (Roche Diagnostics), anti-Myc (Roche Diagnostics), anti-Flag (Sigma), or anti-His (Sigma) antibodies, or anti-Gal4 (Santa-Cruz Biotechnologies, Santa Cruz, CA) antibody, the latter coupled with a secondary, HRP-coupled, anti-rabbit antibody (Santa Cruz Biotechnologies), and revealed using a chemiluminescence detection system (ECL^+, Amersham Pharmacia Biotech).

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FIGURE LEGENDS

FIG. 1: ***In vivo* association of YAP65 with Smad7**. *A*, COS-7 cells were transfected with Myc-tagged Smad6 or Smad7, in the absence or presence of 6xHis-YAP65, as indicated. His-tagged proteins were pulled down from lysates using Ni-NTA magnetic agarose beads (Ni-NTA pull-down). Smads were detected by immunoblotting (Blot) with anti-Myc antibodies (top panel). Expression of Smads and YAP65 in cell lysates was verified by immunoblotting with anti-Myc (middle panel) and anti-6xHis antibodies (bottom panel). *B*, COS-7 cells were transfected with Flag-tagged full-length Smad7 or ΔPY-Smad7, in the absence or presence of His-YAP65, as indicated. Cell lysates were subjected to 6xHis purification with Ni-NTA beads, and Smad7 forms detected by immunoblotting (Blot) with anti-Flag antibodies (top panel). The expression of Smad7 and YAP65 in total cell lysates was confirmed with anti-Flag (middle panel) and anti-6xHis antibodies (bottom panel). NS: non-specific.

FIG. 2: **YAP65 and Smad7 co-represses Smad signaling**. *A*, Sub-confluent HaCaT keratinocytes were co-transfected with $(CAGA)_9$-Lux, without or with YAP65 and/or Smad7 expression vectors. Six hours after transfections, TGF-β (10 ng/ml) was added where indicated (+) and incubations continued for 24 h before reporter gene activity was determined. *B*, HaCaT keratinocytes were transfected as described in panel *A*, except that $CAGA)_9$MUT-lux was used instead of $(CAGA)_9$-lux. Results represent the mean $\pm$ S.D. of at least three independent experiments performed on duplicate samples.

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FIG. 3: **Association of Smad7 with various fragments of YAP65**. *A*, schematic representation of the YAP65 protein. Putative domains and critical amino acid numberings are indicated. AD, activation domain; bm, binding motif. *B*, HaCaT keratinocytes were co-transfected with $(CAGA)_9$-Lux, without or with YAP65(1-301), YAP65(156-276) or full-length YAP65 expression vector, as indicated, in the absence or presence of Smad7 expression vector. Six hours after transfections, TGF-β was added and incubations continued for 24 h before reporter gene activity was determined. The mean relative fold activation by TGF-β ± S.D. is plotted. Experiments were performed four times with similar results. *C*, COS-7 cells were transfected with Flag-tagged Smad7, in the absence or presence of Gal4-tagged YAP65(156-276) or YAP65(1-301), as indicated. Empty Gal4 expression vector (Gal4e) was used as a control. Cell lysates were subjected to immunoprecipitation (IP) with anti-Flag antibodies, and co-precipitating YAP65 fragments detected by immunoblotting (Blot) with anti-Gal4 antibodies (top panel). Expression of Smad7 and YAP65 fragments was confirmed using anti-Flag (middle panel) and anti-Gal4 antibodies, respectively (bottom panel).

FIG. 4: **YAP65 stabilizes the interaction of Smad7 with activated TβRI**. COS-7 cells were transfected with HA-tagged activated TβRI (TβRI(TD)-HA), in the absence or presence of Flag-Smad7, without or with increasing amounts of either 6xHis-tagged full-length YAP65 or YAP65(1-301), as indicated. Cell lysates were subjected to anti-Flag immunoprecipitation (IP), and co-precipitating TβRI was detected by immunoblotting (Blot) with anti-HA antibodies (top panel). To confirm expression of TβRI, Smad7 and YAP65,

aliquotes of total cell lysates were immunoblotted with anti-HA, anti-Flag and anti-6xHis antibodies, respectively (bottom panel).

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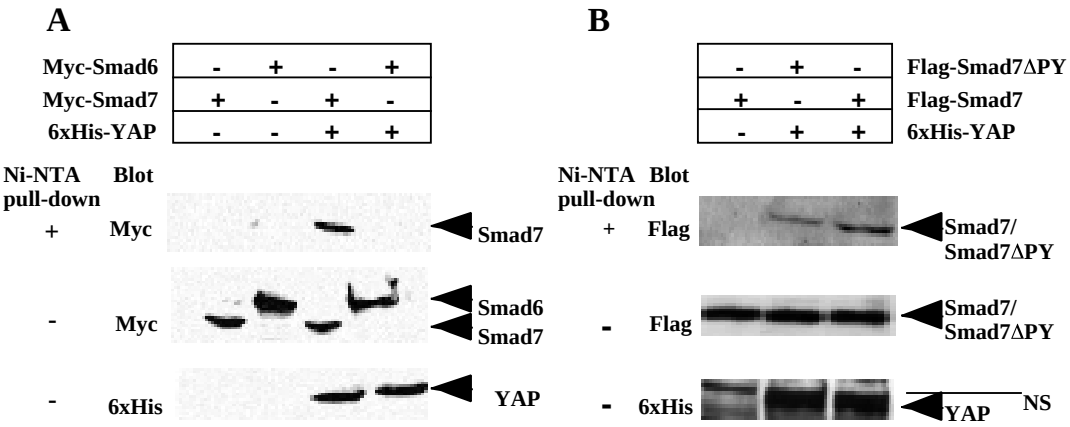

FIG. 1

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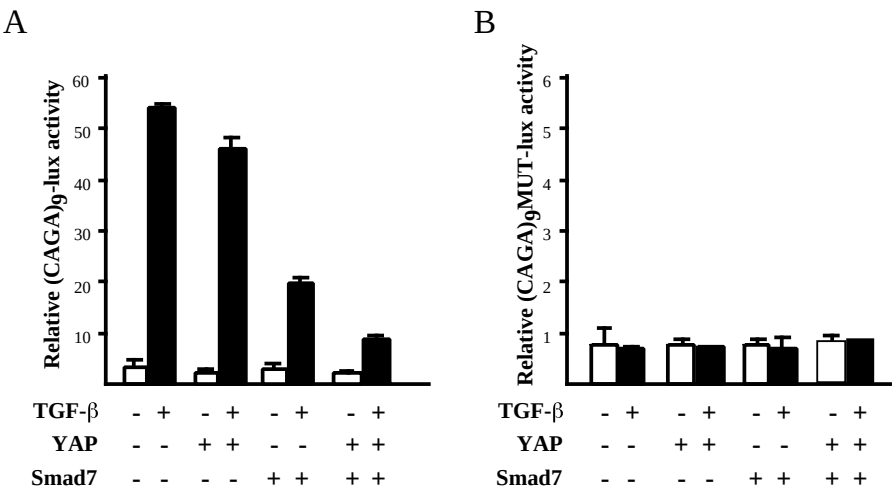

FIG. 2

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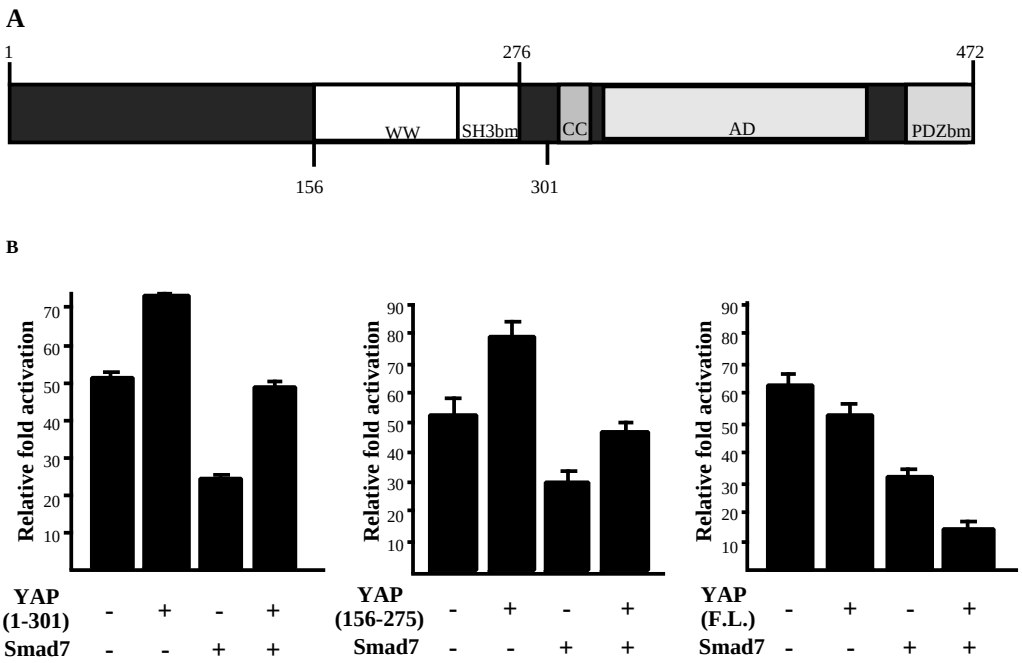

FIG. 3

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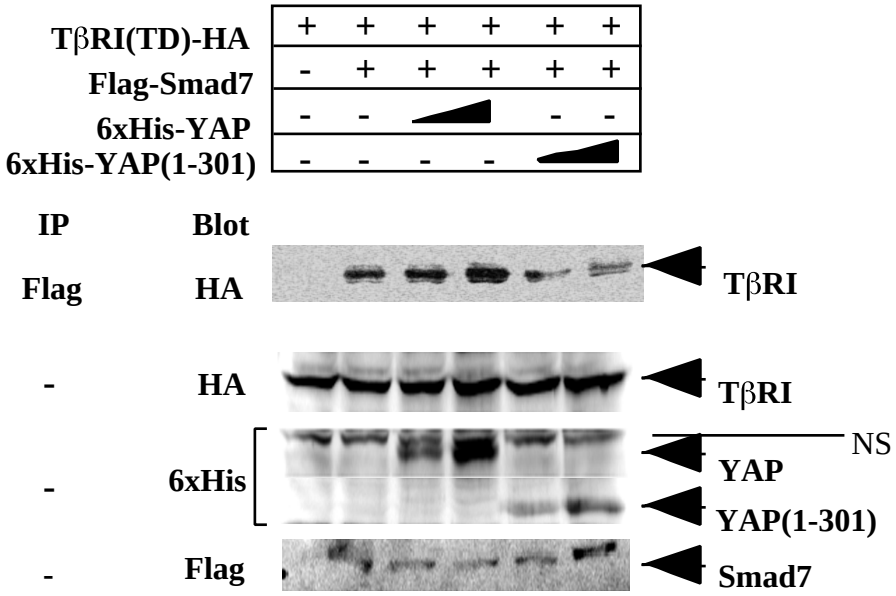

FIG. 4