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Correspondence and requests for materials should be addressed to D.B.N. (e-mail: Dimitar@ximpact3.ski.mskcc.org). Coordinates have been deposited with the Brookhaven Protein Data Bank under accession number 1nuk.

## erratum

# Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TFG-β-induced transcription

**Ying Zhang, Xin-Hua Feng & Rik Derynck**

*Nature* **394,** 909–913 (1998)

In Fig. 1 of this Letter, the key in all panels was incorrect: black bars should be +TGF-β, and white bars should be −TGF-β; in addition, the c-Fos Smad3/4 bar colours were transposed in panel **b**. The correct figure is shown here.

In Fig. 3f, right panel, the symbols should all have been plus signs, as shown here. There were also two typographical errors in Fig. 3a (1NL, not 31NL; 4NL, not 34NL). □

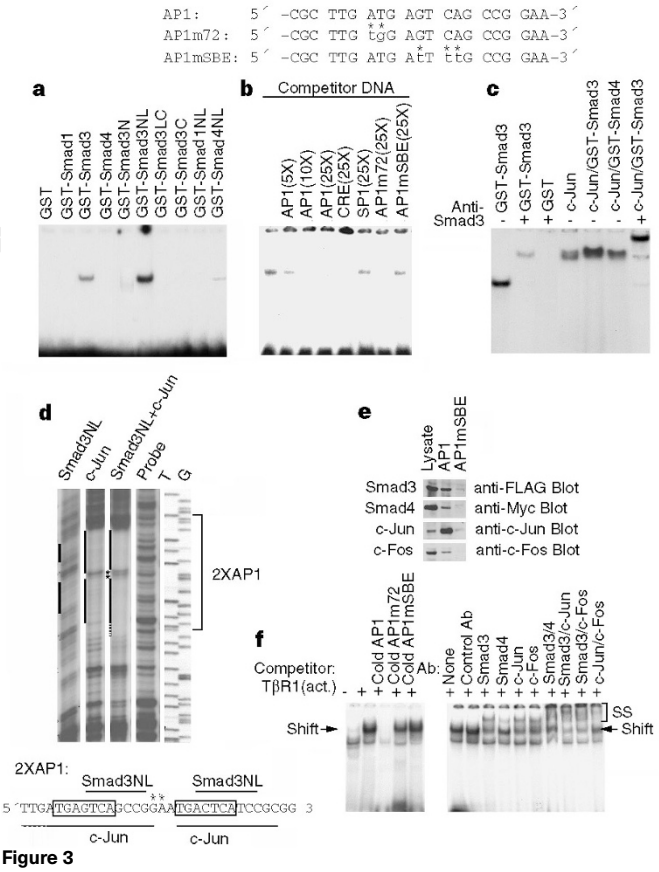


**Figure 3**

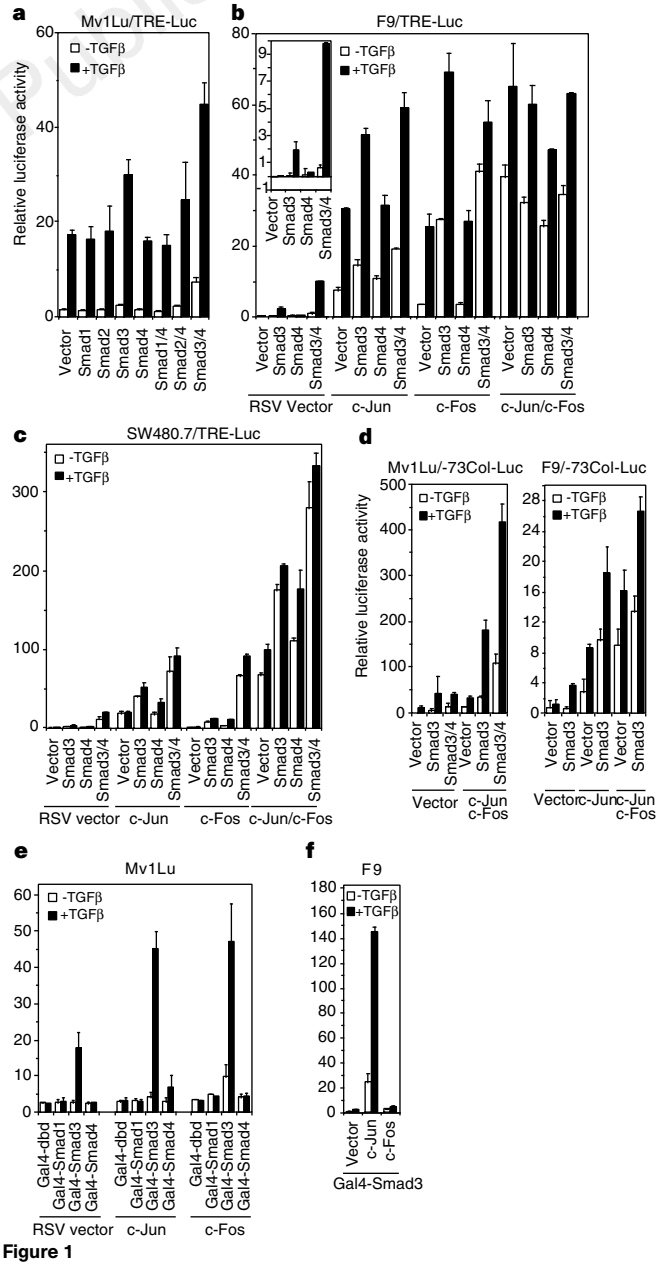


**Figure 1**

# Smad3 and Smad4 cooperate with c-Jun/c-Fos to mediate TGF-β-induced transcription

**Ying Zhang, Xin-Hua Feng & Rik Derynck**

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**Smad proteins transduce signals for transforming growth factor-β (TGF-β)-related factors[1]. Smad proteins activated by receptors for TGF-β form complexes with Smad4. These complexes are translocated into the nucleus and regulate ligand-induced gene transcription[2–4]. 12-*O*-tetradecanoyl-13-acetate (TPA)-responsive gene promoter elements (TREs) are involved in the transcriptional responses of several genes to TGF-β (refs 5–8). AP-1 transcription factors, composed of c-Jun and c-Fos, bind to and direct transcription from TREs, which are therefore known as AP1-binding sites[9]. Here we show that Smad3 interacts directly with the TRE and that Smad3 and Smad4 can activate TGF-β-inducible transcription from the TRE in the absence of c-Jun and c-Fos. Smad3 and Smad4 also act together with c-Jun and c-Fos to activate transcription in response to TGF-β, through a TGF-β-inducible association of c-Jun with Smad3 and an interaction of Smad3 and c-Fos. These interactions complement interactions between c-Jun and c-Fos, and between Smad3 and Smad4. This mechanism of transcriptional activation by TGF-β, through functional and physical interactions between Smad3–Smad4 and c-Jun–c-Fos, shows that Smad signalling and MAPK/JNK signalling converge at AP1-binding promoter sites.**

Several AP1-regulated promoters are transcriptionally induced by TGF-β (refs 5–8, 10) and Smad2 or Smad3 with Smad4 (refs 2, 3, 11). We therefore tested the effect of TGF-β and Smad proteins on transcription from a synthetic reporter, TRE-Luc[12], which contains four tandem AP1-binding sites from the collagenase I promoter[13]. Transcription from this reporter in TGF-β-responsive Mv1Lu cells was induced by TGF-β, occurred as early as 2 h after TGF-β treatment (data not shown), and reached 15–20-fold induction after 12 h (Fig. 1a). Among the Smad proteins tested, only Smad3 induced moderate ligand-independent transcription and enhanced the response to TGF-β (Fig. 1a). Smad3 and Smad4 acted together to induce marked ligand-independent and ligand-dependent transcriptional activation, consistent with the required cooperation of both Smad proteins in a heteromeric complex[2,3,14]. Co-expression of Smad2 and Smad4 also conferred transcriptional activation, albeit to a lesser extent than Smad3 and Smad4, whereas the promoter was not responsive to Smad1 and Smad4.

We next studied the roles of c-Jun or c-Fos in the transcriptional activity of Smad3 and Smad4. In F9 cells, which lack c-Jun and c-Fos[15,16] yet have endogenous Smad3 and Smad4 (data not shown),

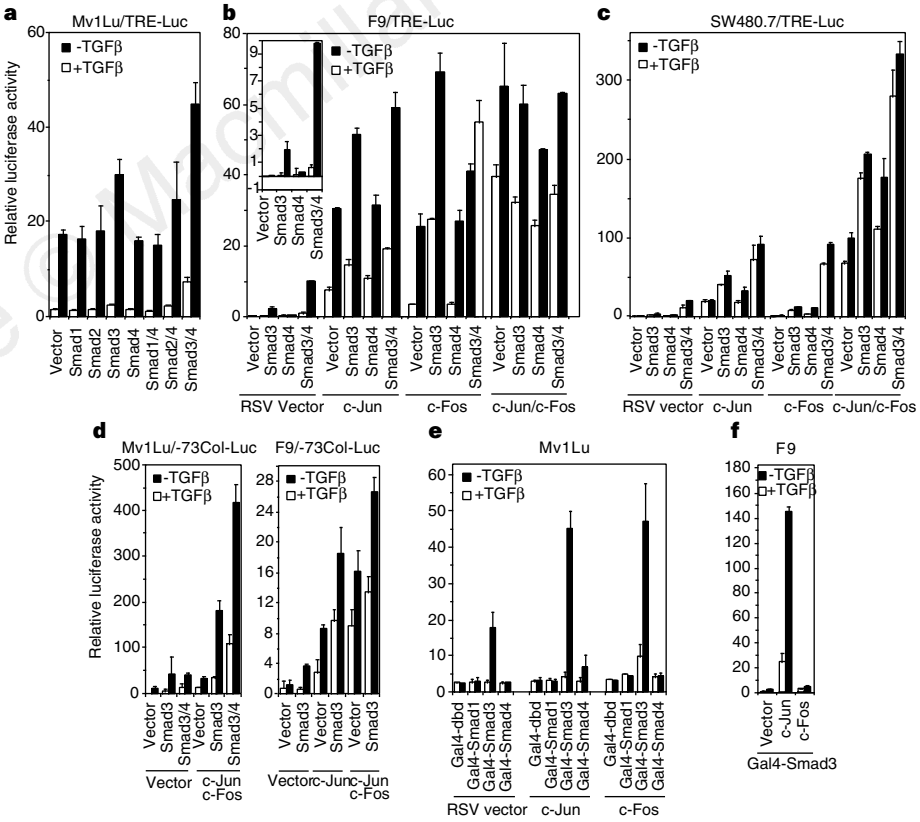


**Figure 1** Smad3/Smad4 and c-Jun–c-Fos cooperate to induce transcription from the AP-1 promoter. We measured the effects of Smads, c-Jun, and c-Fos on luciferase expression from AP-1 promoters in the presence or absence of TGF-β. Values are relative to control cells in the absence of TGF-β. The reporter plasmid TRE-Luc was used in **a**–**c**, whereas the -73Col-luc reporter was used in **d**. **a**, TGF-β-responsive Mv1Lu cells. **b**, F9 cells. The inset shows on an extended scale the effects of Smad3 and/or Smad4 on transcription. **c**, TGF-β-unresponsive, Smad4-defective SW480.7 cells. Note the difference in scale compared with **b**. **d**, Smad3 and Smad3–Smad4 synergized with c-Jun and c-Fos to induce transcription from the -73Col-luc reporter, which contains a single AP1-binding site, in Mv1Lu and F9 cells. **e**, **f**, we used pFR-Luc, with its five Gal4 DNA-binding sequences, as a reporter for transcription mediated by Gal4-fusion proteins. Values are relative to control cells transfected with Gal4–dbd (DNA-binding domain) in the absence of TGF-β. **e**, In Mv1Lu cells, the transcription by Gal4–Smad3, but not Gal4–Smad4, was induced by TGF-β and further enhanced in a TGF-β-dependent way by c-Jun or c-Fos. **f**, In F9 cells, c-Jun increased transcription by Gal4–Smad3, which was strongly enhanced by TGF-β. In contrast with Mv1Lu cells, co-expression of c-Fos alone had little effect on transcription by Gal4–Smad3 in F9 cells.

Smad3 overexpression activated transcription in response to TGF-β, and Smad4 synergistically enhanced this activity in the absence of c-Jun and c-Fos (Fig. 1b, inset). c-Jun and c-Fos conferred a high level of TGF-β-inducible transcription from this promoter (Fig. 1b). Smad3, but not Smad4, acted with either c-Jun or c-Fos to induce strong ligand-independent transcription, which was further enhanced by TGF-β. Finally, the high level of TGF-β-independent transcription mediated by c-Jun–c-Fos was further enhanced by TGF-β. The strong transcriptional activation by co-expressed c-Jun and c-Fos was not further increased by Smad3 and/or Smad4, possibly because a saturating level of stimulation was already reached (Fig. 1b). We also observed a synergy of Smad3/4 with c-Jun or c-Fos in TGF-β-responsive Mv1Lu cells (data not shown).

In SW480.7 cells, which lack Smad4 and, consequently, responsiveness to TGF-β (ref. 3), and in contrast with F9 and Mv1Lu cells, c-Fos alone did not allow transcription from the TRE (Fig. 1c), which is consistent with the inability of c-Fos to bind DNA directly[9] and indicates that the effect of c-Fos in F9 and Mv1Lu cells depended on endogenous Smad3 and Smad4. The synergy of Smad3 and Smad4 with c-Jun and c-Fos was more apparent in SW480.7 cells than in F9 and Mv1Lu cells, and co-expression of Smad4 enhanced the already high level of synergy of Smad3 with c-Jun or c-Fos or both. Finally, Smad3–Smad4 acted together with c-Jun–c-Fos at the 73-base-pair (bp) human collagenase I promoter with its single consensus TRE[13] (Fig. 1d), consistent with the results using TRE-luc (Fig. 1a–c). These results indicate that the transcriptional cooperativity between Smad3–Smad4 and c-Jun–c-Fos also occurs at a single AP1-binding site.

We also characterized the synergy between Smad3–Smad4 and c-Jun–c-Fos in Gal4 transactivation assays, in which we assessed the effects of c-Jun or c-Fos on the ability of Smad proteins, fused to a Gal4 DNA-binding domain, to drive transcription from a promoter with five tandem Gal4-binding sites (Fig. 1e, f). In Mv1Lu cells, TGF-β activated transcription by Gal4–Smad3, but not by Gal4–Smad1, and c-Jun and c-Fos enhanced the TGF-β-dependent transcription by Smad3 (Fig. 1e). In F9 cells, c-Jun, but not c-Fos, enhanced transcription by Gal4–Smad3 (Fig. 1f). This indicates that the effect of c-Fos on Gal4–Smad3 activity in Mv1Lu cells (Fig. 1e) is due to its synergy with endogenous c-Jun, whereas c-Jun does not require c-Fos to transactivate Smad3. In contrast to Smad3, the activity of Gal4–Smad4 was not induced by TGF-β and was increased only minimally by c-Jun or c-Fos (Fig. 1e). As Smad4 alone does not activate transcription[14,17], this minimal increase is probably due to synergy with endogenous Smad3.

The transcriptional cooperation between Smad3 and c-Jun correlates with a physical interaction between these proteins. In Mv1Lu cells, mammalian two-hybrid assays showed only low-level interactions of Smad3 with c-Jun or c-Fos in the absence of TGF-β. Addition of TGF-β induced a strong interaction of Smad3 with c-Jun and a weaker interaction of Smad3 with c-Fos (Fig. 2a). In F9 cells, the low-level interaction of Smad3 with c-Fos was not increased by TGF-β, whereas, as in Mv1Lu cells, the interaction of Smad3 and c-Jun was strongly enhanced by TGF-β (Fig. 2b). These results indicate that the TGF-β-induced interaction of Smad3 with c-Fos in Mv1Lu cells may be due to stabilization by endogenous c-Jun. In transfected cells expressing Smad3, c-Jun co-immunoprecipitated with Smad3 following TGF-β-receptor activation (Fig. 2c, d). Only a small amount of c-Fos co-precipitated with Smad3, and

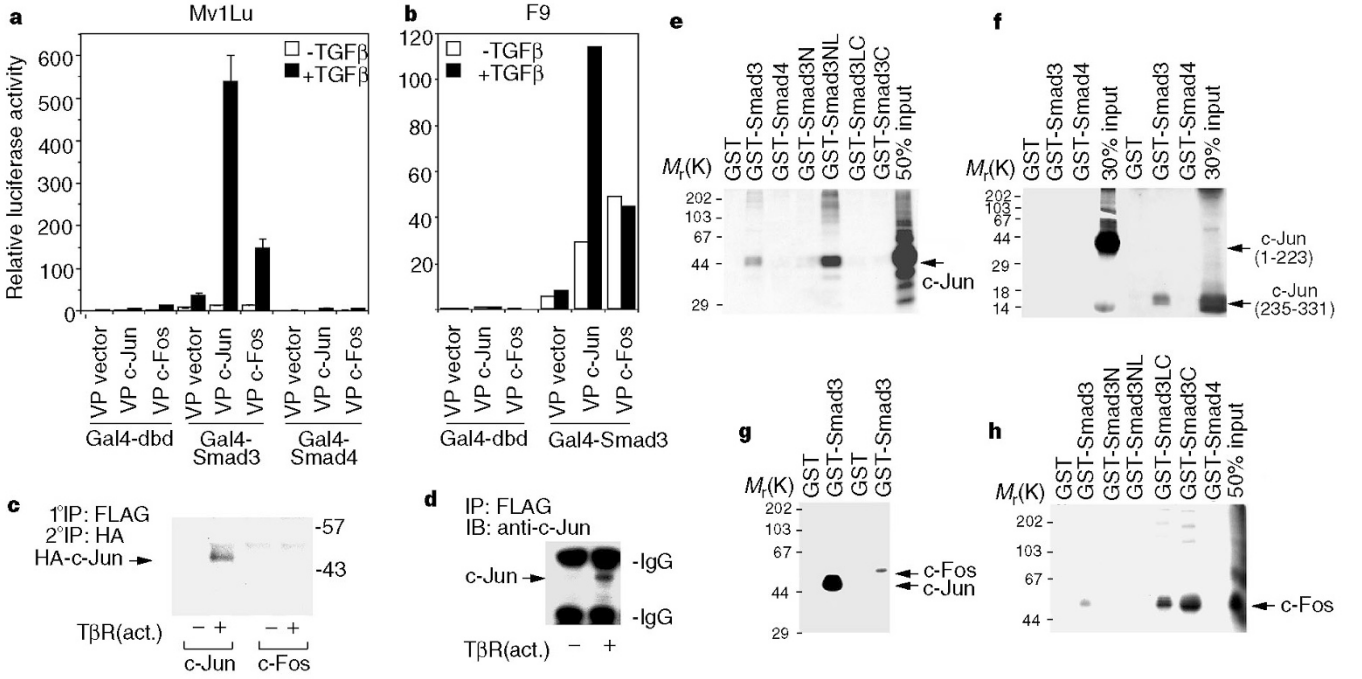


**Figure 2** Interaction between Smad3 and c-Jun or c-Fos. **a**, Mammalian two-hybrid assays in Mv1Lu cells reveal TGF-β-inducible interactions between c-Jun or c-Fos and Smad3, but not Smad4. Smad3 and Smad4 were expressed as fusions with the GAL4 DNA-binding domain and c-Jun and c-Fos were expressed as fusions with the VP16 transactivation domain. Interactions were scored as transcription from pFR–luc; dbd represents the DNA-binding domain as control. **b**, Mammalian two-hybrid assays were performed as in **a**, except that F9 cells were used. Note the difference in scale compared with **a**. **c**, Ligand-dependent association of transfected c-Jun and Smad3. Flag-tagged Smad3 and HA-tagged c-Jun or c-Fos were co-expressed in $^{35}$S-labelled Cos-1 cells and sequential immunoprecipitations (IP), first with anti-Flag and, following dissociation, with anti-HA antiserum, were performed. c-Jun precipitated with Smad3 only following receptor (TβR) activation. The upper band shows co-precipitated Smad3, which, in the case of the c-Fos immunoprecipitaitons, may comigrate wit c-Fos. **d**, Endogenous c-Jun interacts with Smad3 following receptor (TβR) activation. Flag-tagged Smad3, expressed in Cos-1 cells, was immunoprecipitated and the association of c-Jun with Smad3 was visualized by western blotting using anti-c-Jun antibodies. **e**, Smad3 interacts directly with c-Jun. The c-Jun-interaction domain of Smad3 is localized at its N-L segment. **f**, c-Jun interacts with Smad3 through its C-terminal bZIP domain. **g**, The interaction of c-Fos with Smad3 is much weaker than the interaction of c-Jun with Smad3. Equal mounts of *in vitro*-translated $^{35}$S-labelled c-Jun or c-Fos, with similar specific radioactivity, were incubated with GST or GST–Smad3. **h**, Direct association of c-Fos with the conserved C-domain of Smad3.

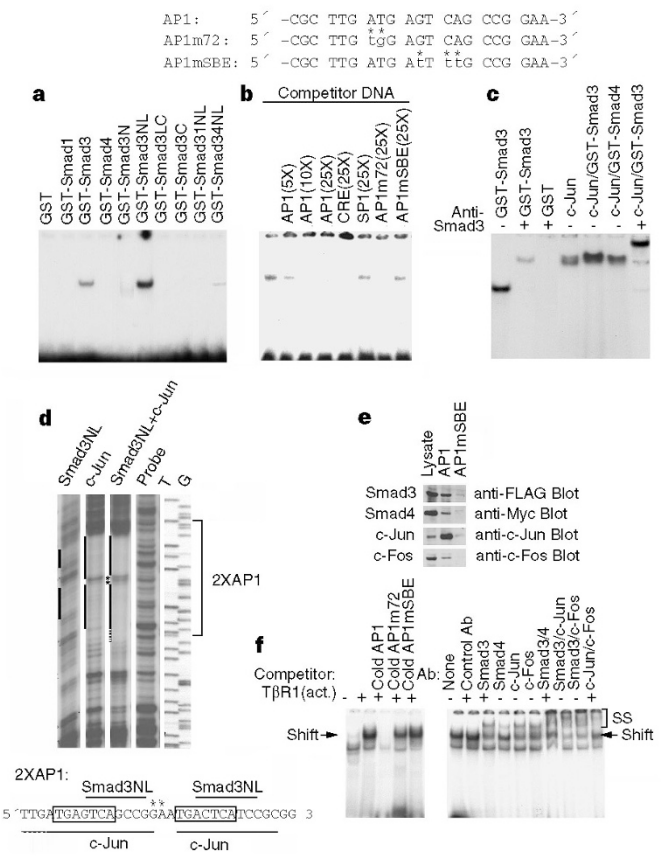


**Figure 3** Smad3–Smad4 and c-Jun–c-Fos participate in a nucleoprotein complex with the consensus AP1-binding sequence. **a**, Direct binding of Smad3, but not Smad1 or 4, to the AP-1 consensus sequence shown at the top. We used semipurified GST or GST-fused Smad proteins in gel shift assays. The DNA-binding sequence of Smad3 is found in its N-L segment. The N-domain showed minimal DNA binding. The C-domain with or without the L segment did not bind DNA. **b**, Excess unlabelled AP-1 or CRE (agagattgccTGACGTCAgagagctag) probes, but not Sp1 (attcgatCGGGGCGGGCcgagc) probe, competes with GST–Smad3 for binding to the AP-1 sequence. A mutated AP-1 probe, AP1m72, which has mutations in the AP-1 consensus sequence and does not bind c-Jun, but not another mutant AP-1 probe, AP1mSBE, which has mutations in both AP1- and Smad3-binding sequences, competes with Smad3 for binding to the AP-1 sequence. **c**, GST–Smad3 without or with purified c-Jun was incubated with the [$^{32}$P]-AP-1 probe, and gel shift analyses were performed. Anti-Smad3 antibodies supershifted GST–Smad3–DNA and c-Jun–GST–Smad3–DNA complexes, indicating that c-Jun and Smad3 bind to the AP-1 probe together. **d**, Footprinting analysis of the interaction of Smad3 and/or c-Jun with the AP1-binding site. The protected sequences (determined from parallel sequencing reactions using ddT and ddG) are marked next to the lanes and on the sequence shown below. **e**, Smad3, Smad4, c-Jun and c-Fos interact with the AP1-binding consensus oligonucleotide. Immobilized AP-1 or mutated AP1mSBE oligonucleotides were incubated with cell lysates from Cos-1 cells transfected with expression plasmids for Flag–Smad3, Smad4–Myc, c-Jun, c-Fos and activated TGFβ receptors (TβRI (act.)). The affinity-purified complex was analysed by immunoblotting using the indicated antibodies. The lysate control lanes showed that all four proteins were expressed. **f**, Gel shift and supershift analyses using the [$^{32}$P]-AP-1 probe in nuclear extracts from 293 cells transfected with expression plasmids for Flag–Smad3, Smad4–Myc, HA–c-Jun or c-Fos, with or without activated TGF-β receptors. In the left panel, excess unlabelled AP-1 or mutant AP-1 probe was included in the reaction as indicated. In the right panel, antibodies were added as indicated. The TGF-β-dependent protein–DNA (shift) and supershifted (SS) complexes are indicated.

this was not always observed. These results show a ligand-dependent association of Smad3 with c-Jun, and indicate that receptor-induced phosphorylation and nuclear translocation of Smad3 may be essential for association of Smad3 with c-Jun.

To characterize the interaction of Smad3 with c-Jun and c-Fos, we used glutathione *S*-transferase (GST) binding assays. c-Jun associated directly with Smad3, but not with Smad4; c-Jun associated more efficiently with the N-L segment of Smad3 and not with the C-domain (Fig. 2e). Smad3 interacted with the carboxy-terminal segment of c-Jun, which contains the bZIP domain that mediates DNA binding and dimerization[9], and not with its amino-terminal segment (Fig. 2f). Consistent with the weak interactions between c-Fos and Smad3 in mammalian cells (Fig. 2a, b), c-Fos interacted much less with Smad3 than did c-Jun; the c-Fos–Smad3 interaction was mediated by the C-domain of Smad3, and the L or N-L segment decreased this association (Fig. 2g, h). Together, our results indicate that decreased affinity of the C-domain for the N-domain[18] following receptor-induced C-terminal phosphorylation of Smad3 exposes the N-L segment, allowing binding to c-Jun, and the C-domain, allowing binding to c-Fos. This complex can then activate transcription more efficiently.

As shown in gel mobility-shift assays, Smad3 associated directly with the AP-1 sequence from the collagenase I promoter, and this interaction was mediated by the N-L segment of Smad3 (Fig. 3a). The C- and L-C domains, which can activate transcription when fused to a DNA-binding domain[14,19], did not bind the AP-1 sequence. The N-L domain of Smad4 showed very weak binding to the AP-1 sequence and full-length Smad4 did not bind. Full-length Smad1 and its N-L domain did not bind the AP-1 sequence. The Smad3–DNA interaction competed with unlabelled AP-1 probe, but not with the unrelated Sp1-binding sequence GGCGGGG. The cyclic-AMP-response element (CRE) TGACGTCA, which differs from the TPA-responsive AP-1 site only in having one extra cytosine, also competed efficiently for Smad3 binding (Fig. 3b). TGF-β and Smad3 and Smad4 repress the promoter activity of the cyclin A promoter[3,20]: this downregulation by TGF-β requires the ATF-1/CREB(CRE-binding protein)-binding CRE sequence[21]. Thus, direct binding of Smad3 to the CRE site may mediate this downregulation by TGF-β.

c-Jun homodimers and c-Jun–c-Fos dimers activate transcription through their ability to interact directly with the AP1-binding site[9]. Smad3 and c-Jun interacted individually with the AP-1 oligonucleotide, as expected. Incubation of both Smad3 and c-Jun with this oligonucleotide resulted in a more slowly migrating, more intense complex than the complex formed with c-Jun alone, and this complex was supershifted using an anti-Smad3 antibody. These results indicate that Smad3 and c-Jun can bind simultaneously to the TRE and cooperate to form a more stable complex than would be formed with either protein alone. Footprinting analysis showed that the N-L domain of Smad3 protected the sequence GTCAGCC, which overlaps with the AP1-binding sequence TGAGTCA[13] (Fig. 3d) and resembles the Smad3/4-binding sequence GTCTAGNC[22,23]. Incubation of Smad3 and c-Jun with the AP-1 oligonucleotide conferred a protection pattern that reproducibly differed to some extent from the c-Jun pattern and was distinct from the Smad3 pattern. Consistent with the different footprinting patterns, Smad3 and c-Jun showed differential sequence requirements for binding at the TRE. Thus, a mutant sequence, AP1m72, which fails to bind c-Jun[13], still competed with the $^{32}$P-labelled AP-1 probe for Smad3 binding, whereas another oligonucleotide, AP1mSBE, which also does not bind c-Jun[13] because of mutations in both AP1- and Smad3-binding elements, did not compete with the AP-1 probe for Smad3 binding (Fig. 3b). Overlapping AP1 and Smad3-binding sequences, as found in several TGF-β-regulated promoters, may be important for TGF-β-induced transcription. Thus, not all AP1-binding sites may have equal affinities for Smad3, and this affinity

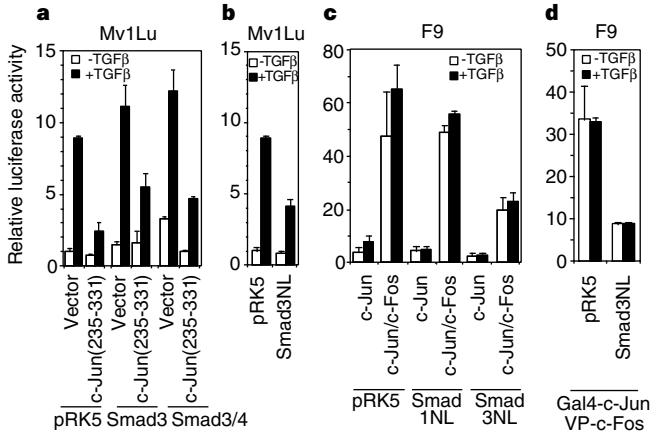


**Figure 4** Dominant-negative inhibition of TGF-β- and Smad3/4-induced transcription from the AP1-binding site by truncated c-Jun or Smad3. **a**, Overexpression of the dominant-negative c-Jun mutant, c-Jun(235–331), inhibits transcriptional activation from the TRE promoter by TGF-β, Smad3 or Smad3–Smad4 in Mv1Lu cells. c-Jun(235–331) contains only the C-terminal bZIP domain of c-Jun. **b**, Overexpression of Smad3NL, that is, the N-L segment of Smad3, inhibits TGF-β-induced transcription from the TRE promoter in Mv1Lu cells. **c**, Overexpression of Smad3NL but not Smad1NL inhibits c-Jun- or c-Jun–c-Fos-induced transcription from the TRE promoter in F9 cells. **d**, Smad3NL inhibits the physical interaction between Gal4–c-Jun and VP–c-Fos as scored in mammalian two-hybrid assays.

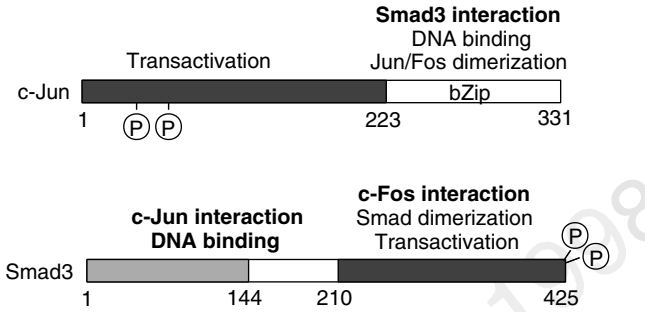


**Figure 5** Domains of c-Jun and Smad3. The functions shown in bold have been assigned on the basis of our present results.

may be determined by a few nucleotides that flank the AP1-binding heptanucleotide.

Smad3 and Smad4 also participated with c-Jun and c-Fos in a multimeric complex at the AP-1/Smad3-binding site *in vivo*. Oligonucleotides containing the AP-1/Smad3-binding sequence interacted with c-Jun, c-Fos, Smad3 and Smad4 in transfected cell lysates (Fig. 3e), although only Smad3 and c-Jun can bind this sequence directly. The interaction of c-Jun–c-Fos and Smad3–Smad4 at the AP-1/Smad-binding sequence was further supported by supershift analyses using different antibody combinations (Fig. 3f). TGF-β-receptor activation induced the formation of a DNA–protein complex, which was absent in unstimulated cells. This TGF-β-dependent complex competed with excess unlabelled AP-1/Smad3-binding oligonucleotide, but competed inefficiently with two different mutated oligonucleotides that do not bind c-Jun, suggesting a key role of c-Jun in the formation of this complex (Fig. 3f). The TGF-β-inducible complex was supershifted using antibodies against Smad3, Smad4, c-Jun or c-Fos (Fig. 3f). The incomplete supershift of the DNA–protein complex by the antibodies is not surprising, as several other bZIP transcription factors, including TGF-β-inducible JunB, can form complexes with different transcriptional activities at the AP1-binding site[24]. A combination of two different antibodies resulted in a supershift band of even slower electrophoretic mobility (Fig. 3f). The interaction data and supershift analyses shown in Figs 2 and 3, combined with the functional interactions of c-Jun–c-Fos and Smad3–Smad4 (Fig. 1), indicate that all four components, that is, Smad3, Smad4, c-Jun and c-Fos, form a multimeric protein complex at the AP-1/Smad-binding sequence.

We studied the role of c-Jun in TGF-β signalling further by using a truncated version of c-Jun. c-Jun(235–331) contains the bZIP domain of c-Jun and acts as a dominant–negative inhibitor of AP-1 activity[25], presumably by sequestering c-Jun and c-Fos or by occupying the AP-1 DNA-binding site. c-Jun(235–331) inhibited TGF-β- and Smad3/4-induced transcription from TRE-luc (Fig. 4a). As this segment of c-Jun interacts with Smad3, c-Jun(235–331) may inhibit Smad3/4 and TGF-β signalling by directly binding and sequestering Smad3, or by interfering with the binding of Smad3 to the DNA.

Overexpression of the N-L segment of Smad3 also inhibited TGF-β-induced transcription from the TRE promoter in Mv1Lu cells (Fig. 4b). Although this inhibition may be due to ability of the N-L domain to interact with the C-domain of Smad3 and thus prevent Smad3 association with Smad4 (ref. 18), it may also be due to its interaction with c-Jun and/or the TRE, thus creating an inactive complex. Accordingly, Smad3N-L, but not Smad1N-L, inhibited the transcriptional activity of c-Jun and c-Jun–c-Fos (Fig. 4c) and interfered with the interaction between c-Jun and c-Fos in mammalian two-hybrid assays (Fig. 4d).

Our results suggest a model for how Smad3 and Smad4 mediate TGF-β-induced transcription at AP1-binding sites through physical and functional interactions with the TRE and with c-Jun/c-Fos. Thus, Smad3 and Smad4 associate with each other and interact directly with the Smad-binding sequence at the AP1-binding site in a conceptually similar manner to c-Jun-c-Fos heteromers[9,24]. The resulting Smad3/4-mediated transcription is highly TGF-β-inducible and does not require c-Jun or c-Fos, as shown in F9 cells (Fig. 1b). In the presence of c-Jun and c-Fos, the heteromeric Smad complex interacts in a ligand-dependent fashion with the AP-1 complex, primarily through interaction of Smad3 with c-Jun, but probably also stabilized by the affinity of Smad3 for the DNA and for c-Fos and Smad4. The resulting synergy of Smad3–Smad4 and AP-1 then confers strong, highly TGF-β-inducible transcription from the AP1-binding site. Our results also indicate that the functional organization of Smad3 resembles that of the bZIP transcription factors (Fig. 5), which have a basic region that mediates DNA binding closely preceding the leucine-zipper dimerization domain, and a transactivation domain located at the other end of the protein[9,24]. Whereas bZIP transcription factors function by dimerization, further functional diversity can be achieved from combinatorial pairwise interactions between Smad proteins and AP-1 transcription factors.

Finally, mitogenic activation of tyrosine kinase receptors, stress and ultraviolet irradiation all activate MAP kinase cascades and JNK kinase, leading to transcriptional activation of the AP-1 complex[24]. Our results show that these signals converge with TGF-β-induced Smad signalling at the AP-1 promoter site. The phosphorylation of Smad1 and Smad2 (refs 26, 27) in response to mitogenic stimulation of MAP kinase raises the possibility that such phosphorylationof Smad3 may regulate its interaction with the AP1-binding site and the AP-1 complex. □

#### Methods

**Expression plasmids and reporter constructs.** N-terminal Flag-tagged Smads1–3, haemagglutinin (HA)-tagged or VP16-fused c-Jun and c-Fos, C-terminal Flag-tagged Smad1NL (amino acids 1–245), and truncated c-Jun (amino acids 1–223) and c-Jun (amino acids 235–331) were generated by polymerase chain reaction (PCR)-based subcloning into plasmid pRK5 (ref. 28). Gal4(1–147)-fused Smad3 was made by inserting the Smad3 complemen-

tary DNA into pSG424 (ref. 29). Details can be provided on request. pGal4–Smad1 and pGal4–Smad4 were provided by J. Massagué, and pRSV–c-Jun, pRSV–c-Fos and the pRSV vector plasmids were provided by R. Tjian and K. Yamamoto. The C-tagged pRK5–Smad4F (ref. 3), pRK5-Smad3NL (ref. 4) and constitutively active cytoplasmic TGF-β-receptor (TβR)II–TβRI have been described[30]. The -73Col-Luc reporter plasmid contains the luciferase gene under control of a truncated collagenase I promoter with a single AP-1 consensus sequence[13], whereas TRE-Luc contains four tandem copies of the AP-1 consensus sequence in front of the luciferase gene[12]. pFR-Luc has five copies of a Gal4-binding element, followed by the luciferase gene (Stratagene).

**Transient transfections and functional assays.** Transient transfections, TGF-β treatment, Gal4 transactivation, mammalian two-hybrid assays and luciferase assays were done as described[17]. For each transfection, 0.5 μg of each expression plasmid, 0.5 μg luciferase reporter plasmid, and 0.25 μg β-galactosidase plasmid were used. For the Gal4 transactivation assay and mammalian two-hybrid assays, 50 ng Gal4(1–147) fusion plasmids were used. The total plasmid concentration was kept constant, and, when needed, vector DNA was added.

**GST-fusion proteins and *in vitro* protein-binding assays.** Plasmids pGEX–Smad3 and pGEX–Smad4 have been described[3] and all other GST–Smad fusion were made by subcloning the Smad cDNAs from corresponding pRK5–Smad plasmids[4] into pGEX (Pharmacia). Equal amounts of GST or GST–Smad fusion protein bound to glutathione–Sepharose beads were incubated with $^{35}$S-labelled, *in vitro*-translated proteins (TNT translation kit, Promega) with similar specific radioactivity. associated $^{35}$S-labelled proteins were detected by SDS–PAGE and autoradiography.

**DNA affinity purification of associated proteins, immunoprecipitation, western blotting.** For affinity purification of proteins bound to the AP1-binding oligonucleotide, 200 ng of a biotinylated AP-1 probe were immobilized to streptavidin-conjugated magnetic beads (Promega). The beads were then incubated with lysates of transiently transfected COS-1 cells. After extensive washing, the AP-1 probe and associated proteins, immobilized on magnetic beads, were removed using a magnet and resuspended in SDS sample buffer. Flag-tagged Smad3 and Myc-tagged Smad4 were detected by immunoblottng using anti-Flag M2 antibody (Kodak, IBI) or anti-Myc 9E10 antibody. c-Jun was detected using a mixture of two anti-c-Jun antibodies, c-Jun(D) and c -Jun(N), and c-Fos was detected by using c-Fos(4)–horseradish peroxidase (HRP) (Santa Cruz Biotech). Immunoprecipitations were performed as described[30]. Sequentially immunoprecipitated complexes were visualized by autoradiography and Smad3-associated endogenous c-Jun was visualized by immunoblotting as described above.

**Electrophoretic mobility-shift assays.** Mobility-shift assays were done using a Promega gel-shift assay kit. The reactions contained 0.4–0.5 μg glutathione–Sepharose-purified GST fusion protein or purified c-Jun (Promega). Excess unlabelled competitor oligonucleotide (Promega) was added to the reaction mixture before preincubation, when required. For gel mobility shift and supershift assays using cell nuclear extracts, 1 μl extract containing about 1 μg $\mu l^{-1}$ protein, prepared as described[22], was used. For supershift analyses, 1 μl antibody, HA(12A5) against HA–c-Jun (Babco) and antibodies against c-Fos(K-25) (Santa Cruz Biotech), affinity-purified Smad3 (ref. 11) and Smad4 (ref. 22), were incubated at 4 °C for 90 min after adding $^{32}$P-labelled probes.

**DNA footprinting.** The DNA probe for DNaseI footprinting containing two tandem AP1-binding sequences (sequence shown in Fig. 3d) were excised from pBSK2XAP-1, a derivative of pBS2SK$^+$ (Stratagene), and 5′-$^{32}$P-labelled at only one end. Footprinting was carried out using the core footprinting system (Promega).

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