The Role of FAST-1 and Smads in Transcriptional Regulation by Activin during Early Xenopus Embryogenesis*

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Smads are signal transducers for the transforming growth factor-β superfamily of factors. In early Xenopus embryos, the transforming growth factor-β member activin induces the gene Mix.2 by stimulating the formation of a multiprotein complex, activin-responsive factor (ARF). This complex contains Smad2 or Smad3, Smad4, and a novel forkhead transcription factor, FAST-1, and binds to an enhancer (activin-responsive element; ARE) that confers activin regulation of Mix.2 transcription. Both FAST-1 and Smads can bind directly to the ARE; we have investigated 1) the role of FAST-1 and Smad DNA binding sites in ARF recognition of the ARE, 2) the contributions of FAST-1 and Smad binding to ARF binding in vitro and to ARE regulation in early Xenopus embryos, 3) the extent to which different Smads can replace Smad4 in regulation of the ARE. We find that ARF binds to ARE through both FAST-1 and Smad binding sites. FAST-1 recognition of the ARE is essential both for ARF binding in vitro and activin regulation in vivo. In contrast, Smad binding of ARE is unnecessary for ARF binding or activin regulation but does enhance the binding and regulatory activity of ARF. Also, Smad3 can partially substitute for Smad4 in the regulation of the ARE. These observations elucidate how broadly expressed signal transducers (Smads) regulate a developmentally specific transcriptional response in conjunction with a temporally restricted transcription factor, FAST-1.

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The TGF-β1 superfamily of signaling molecules regulates diverse events during development including meso-endoderm formation in Xenopus embryos (1, 2). TGF-β factors transduce signals via type I and type II serine/threonine kinase receptors, and their biological effect is achieved in part by transcriptional regulation of target genes (3–6). Ligand induces formation of a heteromeric receptor complex and phosphorylation of the type I receptor by the type II receptor. Genetic and biochemical studies have identified Smad proteins as intracellular signal transducers of TGF-β signaling (7). Smad proteins contain two conserved domains, the N-terminal MH1 domain and the C-terminal MH2 domain, separated by a divergent, proline-rich linker region. Smad2 and Smad3 are phosphorylated by cognate type I receptor in response to activin/TGF-β subfamily members at a carboxy-terminal SSXS motif in their MH2 domains. The receptor-regulated Smads bring a common signal mediator Smad4 into the nucleus, and the heteromeric complex activates target gene expression (8).

In early Xenopus embryos, activin, a TGF-β superfamily member, induces an early meso-endodermal response gene, Mix.2 (9). An activin-induced multiprotein complex, activin-responsive factor (ARF), binds to an enhancer (activin-responsive element; ARE) that confers activin regulation of Mix.2 transcription (10). ARF has been shown to be composed of Smad2, Smad4, and a novel forkhead DNA binding domain transcription factor, FAST-1 (8, 11, 12). FAST-1 is a nuclear protein containing an N-terminal forkhead DNA binding domain and a C-terminal Smad interaction domain that is responsible for activin-regulated association with the Smads (12). Smad3, like Smad2, can interact with the FAST-1 SID (13). The extent to which Smad2 or Smad3 participates in ARF complex formation in vivo has not been determined.

mRNA expression of Xenopus FAST-1, as well as of a mouse FAST-1 homologue referred to as Fast1 or FAST-2, is restricted to early embryogenesis (11, 13–15). FAST expression is essential for activin/TGF-β regulation of the ARE; cells that do not express FAST will not activate an ARE luciferase reporter in response to activin/TGF-β stimulation but will do so when transfected with cDNA encoding either FAST-1 or Fast1/FAST-2 (13–15). Furthermore, expression of a constitutively activated form of FAST-1, in which the SID has been replaced by a VP16 transcriptional activation domain, is sufficient to specifically and directly induce the full range of early mesodermal genes induced by activin. These observations indicate that the DNA binding activity of FAST-1 can specifically recognize a range of activin-responsive promoters in the early embryo. However, overexpression of full-length FAST-1 in the absence of a TGF-β signal does not activate gene expression, suggesting that FAST-1 either lacks a transcriptional activation domain or that the full-length protein is negatively regulated with respect to DNA binding.

Smad4 contains an intrinsic transcriptional activation domain in its C-terminal half. It may, therefore, be recruited by Smad4 into the ARF complex that makes the complex transcriptionally active (8, 16). In addition, the N-terminal region of Smad4 is necessary to enhance stability of DNA binding by ARF, suggesting a dual role for Smad4 in transcriptional regulation by ARF (8). In contrast, the primary role of Smad2 may be interaction with the upstream regulatory apparatus. Smad2 associates with and is phosphorylated by the type I receptor ActRIB (17); this phosphorylation stimulates a direct association of Smad2, through its MH2 domain, with both FAST-1 and Smad4 (8, 12, 18). There is no evidence that Smad4 can asso-
associate directly with FAST-1. Smad2 may therefore serve as an adapter, providing a receptor-regulated linkage between Smad4 and FAST-1. Smad3, which has an MH2 domain very similar to that of Smad2, can also participate in the ARF complex (12), but its functional role in the complex has not been characterized.

Recently, intrinsic DNA binding activity has been reported for the MH1 domains of Mad, the Drosophila homologue of Smad1, Smad3, and Smad4 (19–23). Smad binding sequences have been identified in the promoter regions of a variety of TGF-β superfamily-responsive genes, including the Dpp-responsive gene vestigial, the TGF-β-responsive PAI-1 and collagenase genes, and the mouse goosecoid gene (14, 21–25). In general, these sites appear to be necessary for TGF-β responsiveness, although in the case of an element from the collagenase promoter a Smad3/4 binding site is not necessary for TGF-β regulation (21). While concatenated Smad3/4 binding sites have been shown to be sufficient to confer TGF-β responsiveness on a reporter (20, 26), it is not clear that Smads act on endogenous promoters in the absence of interaction with additional transcription factors.

FAST-1 alone can bind tightly to the ARE; however, a Smad3/Smad4 binding site has also been identified in the ARE (25, 27), raising the question of the relative contribution of FAST-1 and Smads to recognition and activation of the ARE. In Xenopus embryos, FAST-1 and the Smads bind to the ARE as a preformed complex, ARF; we have now investigated the relative importance of FAST-1 and Smad binding in the recognition of ARE by ARF formed in vivo as well as for transcriptional regulation of the ARE by activin in vivo. FAST-1 and Smad4 or Smad3, but not Smad2, bind to distinct sites within the ARE, and ARE binding by the full ARF complex formed in vivo in response to an activin signal accurately reflects the binding activities of its DNA binding components, FAST-1 and Smad4. The FAST-1 binding site is essential for regulation of this element by TGF-β signaling both in cultured cells and in the Xenopus embryo; the Smad binding site enhances responsiveness but is not essential for regulation. The DNA binding activity of Smad4 is localized to the MH1 domain, which appears to have additional functions in ARF stabilization. We also find that high level expression of Smad3 can significantly substitute for Smad4 in the FAST-1-dependent activation of the ARE. Our observations provide a basis for understanding how broadly expressed signal transducers (Smads) participate in the regulation of a developmentally specific transcriptional response in conjunction with a temporally restricted transcription factor, FAST-1.

**EXPERIMENTAL PROCEDURES**

ARE Oligonucleotides—Sequences of upper strands are as follows. ARE is 5’-ACAGACACT TATCTGCTGC CCTAAAATGT GTATTCCATG GAAATGTCTG CCCTTCT-3’. Smad binding sites are added as a nucleotide, and this site is methylated with DMS. After electrophoresis of the probe at 5°C in 5% polyacrylamide gels containing 0.5× TBE.

Electrophoretic Mobility Shift Assay—0.1–0.25 pm 32P-labeled probe was used for each DNA binding assay. DNA binding assays using Xenopus extracts were performed as described previously (10). DNA binding assays using GST fusion proteins were performed in binding buffer containing 25 mM Tris (pH 7.5), 10% glycerol, 80 mM NaCl, 35 mM KCl, 1 mM dithiothreitol, 100 μg/ml poly(dA-dT)8, and complexes were separated in 5% polyacrylamide gels containing 0.5× TBE.

Metathylation Interference Assay—Metathylation interference assays were performed as described (28). Briefly, ARE oligonucleotide was single end-labeled with 32P and methylated with DMS. After electrophoretic mobility shift assay (EMSA), free probes and protein-bound probes were recovered and cleaved with piperidine. Equal cpm of free probes and protein-bound probes were separated in 15% sequencing gels.

Luciferase Reporter Assay—A luciferase reporter vector was generated by subcloning the luciferase coding region (from HindIII to Sall) of pGL3 promoter (Promega) between EcoRI and HpaI sites of pBCAT (9). ARE luciferase reporter constructs were generated by subcloning a single copy of wild type or mutant ARE oligonucleotides into HindIII site of the reporter vector, and the constructs were confirmed by sequencing.

All mammalian expression constructs are in pCS2+ or pCS2+MT vectors. Flag-FAST-1 H208 contains codons 61–518 of Xenopus FAST-1, 6MyC-Smad2 and 6MyC-Smad3 contain codons 2–467 and 12–424 of human Smad2 and Smad3, respectively. 6MyC-Smad4 and 6MyC-Smad4 MH2 contain codons 4–552 and codons 233–552 of mouse Smad4, respectively.

**RESULTS**

The MH1 domains of Smad3 and Smad4 Bind to the ARE Directly—To test the ARE binding by Smad proteins proposed to function in activin/TGF-β signal transduction, bacterial GST fusion proteins containing regions of Smad2, Smad3, or Smad4 were generated, and binding of the fusion proteins to double-stranded ARE oligonucleotide probe was examined by EMSA. A Smad4 MH1 domain fusion protein bound to ARE (Fig. 1, lanes 3–5), while a mutant Smad4 MH1 fusion protein with an
amino acid substitution of Arg100 to Thr (Smad4 MH1 R100T) or a mutant Smad4 fusion protein missing the MH1 domain (Smad4 D MH1) failed to bind to ARE (Fig. 1, lanes 6–8 and lanes 12–14). A full-length Smad4 fusion protein (Smad4) caused retarded migration of ARE; however, the shifted probe did not resolve as a discrete band (Fig. 1, lanes 9–11). Poor resolution of full-length Smad4 fusion protein shifted probe has also been reported for an oligonucleotide containing Smad binding sequence from the promoter region of the PAI-1 gene (23). A Smad3 MH1 domain fusion protein (Smad3 MH1) also bound to ARE (Fig. 1, lanes 18–20). A Smad2 MH1 domain fusion protein (Smad2 MH1) and fusion proteins containing different regions of Smad2 (MH1 plus linker, full-length, or linker plus MH2) all failed to bind to ARE (Fig. 1, lanes 15–17, data not shown). The results indicate, consistent with other recent reports (20, 23, 25), that the MH1 domains of Smad3 and Smad4, but not Smad2, directly bind to the ARE.

Methylation Interference on ARE Associated with Binding of ARF from Frog Embryos Is a Composite of Smad3/Smad4 and FAST-1 Binding—DMS methylation interference assays were performed to permit a comparison of the sites on the ARE necessary for binding of purified Smads and FAST-1 with those necessary for binding of ARF formed in response to an activin signal in early embryos. FAST-1 overexpressed in Xenopus embryos bound to ARE probe, and methylation interference identified the sequence ATGTGTATTC as being critical for binding (Fig. 2, A, lanes 1–4, and B). An identical interference pattern was obtained with GST-FAST-1 fusion protein purified from Escherichia coli (data not shown). We refer to this sequence as the FAST-1 binding site. A single base substitution (G to C) in this sequence has previously been shown to eliminate FAST-1 and ARF binding to the ARE (11), and this site is consistent with a consensus binding site reported for a human FAST-1 homologue (TGT(G/T)(T/G)ATT) (27).

Methylation interference confirmed the sequence GTCTGCCC as being critical for binding of the Smad4 MH1 fusion protein to ARE probe (Fig. 2, A, lanes 5–8, and B). The Smad3 MH1 fusion protein bound to the same sequence (data not shown, summarized in Fig. 2B). We refer to this sequence as...
the Smad binding site of the ARE. The binding region for Smads in ARE is generally consistent with the Smad binding sites observed in other vertebrate promoters (GTCT) (21, 23).

To investigate whether ARE recognition by the ARF complex that is formed in embryos in response to activin stimulation reflects the binding specificities of its components, methylation interference analysis of ARF binding to ARE probe was performed. The methylation interference pattern for ARF binding to ARE probe was roughly a composite of the patterns observed for the FAST-1 and the Smad3/4 MH1 domains alone (Fig. 2, A, lanes 9–12, and B), indicating that the complex formed in vivo uses the DNA binding activities of both FAST-1 and Smad3/4 in target site recognition.

Relative Contributions of FAST-1 and Smad DNA Binding to ARF Binding of the ARE—To assess the relative contributions of DNA binding of Smad and FAST-1 to ARF binding of the ARE, ARE probes mutated in the FAST-1 or Smad binding sites were generated and tested in EMSA (Fig. 3A), mSB-ARE (○), ARE-L (■), and ARE-R (□). The ARF binding in the absence of competitor is considered as relative ARF binding of 100%. The average and S.D. of three experiments are shown.

The results indicate that the FAST-1 binding site is absolutely required for ARF recognition of the ARE and that the Smad binding site is functional but not completely abolished compared with ARF binding to wild type ARE probe (Fig. 3C, lanes 6 and 8). ARF binding to ARE-R probe was 3- and 5-fold less efficient, respectively, than ARF binding to wild type ARE probe. These results indicate that the FAST-1-binding site is absolutely required for ARF recognition of the ARE and that the Smad binding site enhances this recognition but is not essential.

The relative contributions of FAST-1 binding and Smad4/Smad3 binding to the total affinity of ARF for ARE were examined by comparing wild type or mutant AREs as competitors of ARF binding to ARE. ARF binding to wild type ARE probe was competed by ARE, mSB-ARE, and ARE-L competitors (Fig. 3D, lanes 3–14), while ARE-R oligonucleotide failed to reduce the ARF binding to ARE probe at 50-fold molar excess (Fig. 3D, lanes 15–18). Binding of the Smad4 MH1 fusion protein to ARE was reduced by ARE-R competitor in a concentration-dependent manner (data not shown). Competition by wild type or mutant AREs was quantitated and is presented in Fig. 3E. Mutant AREs that lack the Smad binding site or region but contain the FAST-1 binding site (mSB-ARE, ARE-L) compete roughly 5-fold less well than does wild type ARE for ARF binding. This observation indicates that in the context of the...
ARF complex, Smad binding enhances the affinity of the complex for its target on the order of 5-fold. In the absence of the FAST-1 binding site (ARE-R), competition for ARF binding is not observed, confirming the observations with mutant probes (Fig. 3C) that FAST-1 binding is essential for ARF binding of ARE.

**Full Transcriptional Response of ARE Requires both the FAST-1 Binding Site and the Smad Binding Site**—We next examined the relative importance of the FAST-1 and Smad binding sites in trans-activation of the ARE in embryos and cultured cells using luciferase reporter constructs. In *Xenopus* embryos, the response of an ARE-luciferase reporter (ARE-Lux) to activin RNA injection was significantly greater than that of mSB-ARE-Lux (Fig. 4A), indicating that the Smad binding site contributes to the endogenous response to mesoderm-inducing signals, but is not required for this responsiveness. mFB-ARE-Lux did not respond to activin stimulation in embryos, indicating that FAST-1 binding is essential for activin responsiveness in vivo. Similar results were obtained when wild type or mutant ARE-Lux reporter constructs were expressed in isolated animal caps in the presence or absence of activin stimulation (Fig. 4B). We then examined trans-activation of wild type and mutant ARE reporters in the presence and in the absence of FAST-1 and Smad4. Smad4-deficient cell lines SW480.7 and MDA-MB-468 also lack detectable endogenous FAST-like activity. In SW480.7 cells, ARE-Lux did not respond to transfection of ActRIB*, a constitutively active form of activin type IB receptor, or to transfection of Smad4/ActRIB* (data not shown), confirming previous reports that FAST-1 is required for the trans-activation of ARE (8, 13). Transfection of FAST-1/Smad4 caused a basal activation of ARE-Lux, and the activation of ARE-Lux was further increased by co-transfection of ActRIB* (Fig. 4C). Although the level of response was significantly reduced compared with that of ARE-Lux, transfection of FAST-1/Smad4/ActRIB* induced activation of mSB-ARE-Lux. In contrast, mFB-ARE-Lux did not respond to transfection of FAST-1/Smad4/ActRIB*. The increase in response by transfection of FAST-1/Smad4/ActRIB* over the response observed by transfection of FAST-1/ActRIB* was significantly greater with ARE-Lux (80-fold) than with mSB-ARE-Lux (16-fold). The results both from embryos and SW480.7 cells are consistent with the data obtained from *in vitro* ARE binding assays, indicating that the FAST-1 binding site is absolutely required for trans-activation of the ARE by activin signaling, and Smad DNA binding can enhance this trans-activation.

Mutation of the Smad binding site of ARE provides one approach to assessing the role of Smad4 DNA binding in ARF recognition of the ARE. A complementary approach is to eliminate the DNA binding domain of Smad4 and assess how this affects ARF activation of the ARE. Transfection of Smad4 lacking the MH1 domain and much of the linker domain (Smad4 MH2) resulted in activation of ARE-Lux over the control lacking Smad4, but the response of ARE-Lux to expression of Smad4 MH2 was significantly smaller than the response to full-length Smad4 (Fig. 4D). Smad4 MH2 caused a similar -fold activation of ARE-Lux and mSB-ARE-Lux, consistent with the expectation that elimination of the DNA binding activity of Smad4 should reduce the importance of the Smad4 binding site in the ARE. Smad4 MH2 was less efficient, however, at supporting activation of mSB-ARE-Lux than was full-length Smad4. This suggests that there may be a requirement for the MH1 domain of Smad4 in complex formation/stabilization or transcriptional activation in addition to its recognition of the Smad binding site. It is also possible that while the mutation introduced into the Smad binding site eliminates detectable recognition by the free Smad4 MH1 domain in solution, the MH1 domain retains some significant affinity for the mutant Smad4 binding site in the context of the assembled complex. The reduced effectiveness of Smad4 lacking the MH1 domain would in this case reflect the loss of the contribution of a relatively non-site-specific DNA binding affinity of this domain in the context of the intact ARF complex.

**Smad3 Can Partly Substitute the Function of Smad4 as the Transcriptional Activator of ARE**—The MH1 domains of Smad3 and Smad4 bind to the ARE, whereas the MH1 domain of Smad2 cannot (14, 20, 23). The MH2 domains of Smad3 and Smad2 interact with FAST-1, whereas the MH2 domain of Smad4 cannot (8, 12), and the MH2 domain of Smad3, when fused to the LexA DNA binding domain, can trans-activate a reporter construct in yeast (29). Smad3 therefore fulfills the known functions of both Smad2 and Smad4 in ARF, raising the question of whether Smad3 might substitute for Smad4 in the absence of endogenous Smad4. To examine this, we tested the ability of ectopically expressed Smad2 and Smad3 to substitute for Smad4 in the FAST-1-dependent regulation of ARE-

![Fig. 4](image-url)

**Fig. 4. DNA binding by Smad4 enhances the transcriptional response of ARE to activin in embryos and cultured cells.** A and B, ARE luciferase reporters (100 pg/embryo) were injected with (■) or without (□) activin RNA (200 pg/embryo) to the animal hemisphere of *Xenopus* embryos at stage 3 and stage 2, respectively. Embryos were harvested at stage 10 (A), or animal cap explants were dissected at stage 8 and harvested at stage 10 (B). C, the indicated DNAs were transfected into SW480.7 cells with wild type or mutant ARE reporter constructs. D, SW480.7 cells were transfected with cDNA encoding full-length Smad4 or the Smad4 MH2 domain alone in the presence of FAST-1, ActRIB*, and the indicated ARE luciferase reporters.
We next tested if Smad3 can cooperate with Smad2 and Smad4 for trans-activation of ARE. In SW480.7 cells, the activation of ARE-Lux by Smad2 or Smad4 was further increased by expression of increasing amounts of Smad3 (Fig. 5B). This is in contrast to a previous report that co-transfection of Smad3 can inhibit the FAST-2/TGF-β-dependent activation of the Mix.2 ARE or mouse goosecoid TARE (14). Positive cooperation between Smad2 and Smad2/4 was also observed in MDA-MB-468 cells using ActRIB* or TGF-β1 and in HepG2 cells using ActRIB* (data not shown). In SW480.7 cells and MDA-MB-468 cells (data not shown). Kretzschmar et al. (30) and Yagi et al. (25) have also observed Smad3-mediated/enhanced activation, rather than inhibition, of an ARE reporter construct. The basis for these differing observations on the effect of Smad3 co-expression on regulation of the ARE is not clear; clarification of the conditions under which Smad3 may support or antagonize FAST-dependent activation of the ARE will require further investigation.

**DISCUSSION**

We have used DNA binding and reporter activation studies to examine the relative contribution of FAST-1 binding and Smad4 or Smad3 binding to the activin/TGF-β-regulated trans-activation of the ARE by ARF. FAST-1 recognition of its binding site in ARE is essential both for ARF binding and trans-activation by TGF-β signals. Binding of the ARE by the MH1 domain of Smad4 enhances ARF binding and trans-activation but is not essential for ARF formation or trans-activation. These observations differ somewhat from the report of Zhou et al. (27), using a human homologue of FAST-1, that both FAST-1 and Smad binding sites are necessary for transcriptional regulation. The Zhou et al. study differed from ours in that it used an artificial rather than a naturally occurring target site for human FAST-1 and Smad3/4 and used an artificial expression system in which human FAST-1 is expressed at a level that may differ significantly from that which occurs in vivo. Here we are testing an enhancer element (ARE) derived from an activin-regulated *Xenopus* gene, Mix.2, using the signal transduction apparatus endogenous to early embryos.

A single set of type II receptors and signaling Smads can mediate quite different transcriptional and phenotypic responses in different cell types, highlighting the question of what determines the specificity of these responses. Genes induced by activin during the induction of mesoderm in the pregastrula embryo are not inducible later in development, although embryos continue to express both activin receptors, Smad2, and Smad4. Levels of FAST-1 mRNA, on the other hand, drop rapidly during the same period that activin responsiveness of early mesodermal genes is lost (11). These observations suggest that FAST-1 might be a determinant of the specificity of early embryonic responses to activin, and the present work provides a molecular basis for this specificity. The Mix.2 ARE is bound by a complex containing the general activin signal transducers, Smad2 and Smad4, and the early embryo-specific transcription factor FAST-1. FAST-1 is an essential determinant of recognition of the ARE; in the absence of either the protein or its target site, no regulation of the ARE is observed (8, 13). While the general transducer Smad4 can also bind to the ARE, this does not seem to be sufficient for activation, since co-overexpression of Smad2, Smad4, and activated type I activin receptor cannot activate the ARE in cells that lack FAST. Smad4 binding does enhance both ARF formation and trans-activation, however, indicating that Smad DNA binding is a significant component of ARF recognition of the ARE.

Smad4 lacking the MH1 domain supports FAST-1-dependent activation of a mutant ARE lacking the Smad binding site less effectively than does full-length Smad4, suggesting that the MH1 domain may have a role in complex formation/trans-activation in addition to DNA binding. The MH1 domain of Smad4 previously has been shown to have a role in enhancing homomeric Smad4 complexes (31); it is possible that such homomeric interactions are important for full stabilization or activation of ARF. Alternatively, it is possible that the MH1 domain may contribute significantly to the overall DNA binding of the ARF complex even in the absence of a specific Smad target site; i.e. this domain may have sufficient non-site-spe-
cific DNA binding activity to contribute to the DNA binding affinity of the ARF complex.

Demonstration of a requirement for introduction of Smad4 in cell lines that lack endogenous Smad4 for TGF-β signaling has established the current model for obligate Smad4 heteromerization with receptor-regulated Smads (32, 33). While numerous examples of such heteromerization have now accumulated, several experiments at least raise the possibility that Smad4 may not always be required for responses to TGF-β superfamily stimuli. In Drosophila wing, imaginal discs containing clones that have lost the function of Medea, the Drosophila Smad4 homologue, reporters responsive to Dpp can still be activated by high doses of Dpp signal (34). Also, chimeric mouse embryos in which Smad4 has been eliminated from the embryonic, but not the extraembryonic, tissues develop normally through gastrulation (35). Because there is independent evidence for a requirement for BMP signaling for normal pregastrula development (36), there is compelling evidence that different promoter enhancer elements may be differentially regulated depending on the Smad composition of the ARF complex. That Smad3 can form an active signaling complex with FAST-1 in the absence of Smad4 suggests that the combinatorial possibilities for the formation of active signaling complexes in response to TGF-β superfamily factors are more complex than previously recognized.

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