A Short Loop on the ALK-2 and ALK-4 Activin Receptors Regulates Signaling Specificity but Cannot Account for All Their Effects on Early Xenopus Development*

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Activin, a member of the transforming growth factor β (TGF-β) superfamily, signals through a heteromeric complex of type I and type II serine-threonine kinase receptors. The two activin type I receptors previously identified, ALK-2 (ActR-I) and ALK-4 (ActR-IB), have distinct effects on gene expression, differentiation and morphogenesis in the Xenopus animal cap assay. ALK-4 reproduces the effects of activin treatment including the dose-dependent induction of progressively more dorso-anterior mesodermal and endodermal markers, whereas ALK-2 induces only ventral mesodermal markers and counteracts the effects of ALK-4. To identify regions of the receptors that determine signaling specificity we have generated chimeras of the constitutively active ALK-2 and ALK-4 receptors (termed ALK-2* and ALK-4*). The effects of these chimeric receptors on gene expression and morphogenetic movements implicate the loop between kinase subdomains IV and V in mediating the strong dorsal gene-inducing properties of ALK-4*; when the seven amino acids comprising this loop are transferred from ALK-4* to ALK-2*, the resulting chimeric receptor is capable of inducing the expression of dorsal-specific genes. In contrast, when the equivalent region of ALK-2* is transferred to the ALK-4* backbone it cannot effectively counteract the dorsalizing effects of ALK-4*, suggesting that other regions of type I receptors are also involved in determining signaling specificity.

The TGF-β family comprises a large number of secreted signaling polypeptides implicated in a diverse set of functions (1). TGF-β family members act by binding to two classes of transmembrane serine-threonine kinases termed the type I and type II receptors, thereby stabilizing their interaction (2). Type II receptors are constitutively active kinases capable of binding ligand alone, whereas type I receptors can only bind ligand co-operatively in the presence of type II receptors. Following ligand-induced association of the two receptor classes, the type II receptor phosphorylates type I receptors on serine and threonine residues in a critical regulatory region termed the GS domain (3, 4). This phosphorylation event activates the type I receptor, which then mediates downstream signaling events (4).

The TGF-β family member activin induces animal pole tissue of Xenopus embryos to form mesoderm rather than ectoderm (5), and recent evidence using a dominant-negative activin receptor suggests that activin acts as an endogenous mesoderm-inducing factor in the early amphibian embryo (6). We have shown previously that constitutively active forms of the type I activin receptors ALK-2 and ALK-4 (designated ALK-2* and ALK-4*) also induce prospective ectodermal tissue to form mesoderm, but that the two receptors elicit different responses (7). The effects of ALK-4* resemble those of activin itself, in that it can, in a dose-dependent manner, induce expression of the general mesodermal marker Xbra (8) as well as the dorso-anterior marker goosecoid (9). The response to ALK-2*, however, more resembles that of BMP-4 (10) in that it induces only ventral mesodermal markers and causes ventralization of intact embryos (7). ALK-2* is also capable of counteracting the dorsalizing effects of ALK-4*, an activity reminiscent of the ability of BMP-4 to ventralize the response to activin (10), but ALK-2* and BMP-4 differ in their ventralizing effects because the action of ALK-2* is immediate (7), whereas that of BMP-4 is delayed (11).

Three regions were identified that might determine the specificity of type I receptors, and use of chimeras-containing combinations of these regions has led us to focus on a short loop on the small lobe of the kinase, the β4-β5 loop. The 7 amino acids comprising this loop are capable, when transferred from ALK-4* to ALK-2*, of carrying with them the ability to induce dorsal markers. An analogous result has recently been obtained using the TGF-β receptor TbR1/ALK-5 and Tsk7L/ALK-2* in a tissue culture system (12). Significantly, however, the reverse transfer of the β4-β5 loop from ALK-2* to ALK-4* does not completely change the activity of the chimeric ALK-4* to that of ALK-2*. Although the chimeric receptor retains the ability to induce Xbra and loses the ability to induce dorsal genes, it is unable effectively to counteract the dorsalizing effects of ALK-4*.

These observations suggest that there are several signaling pathways downstream of type I receptors. One such pathway involves the Smad proteins, which associate with type I-type II receptor complexes, are phosphorylated on specific serine residues (13, 14), and translocate from the cytoplasm to the nucleus after stimulation of cells with exogenous TGF-β ligands (14, 15). The Smads possess sequence-specific DNA binding properties (16), and Drosophila Mad (16) and human Smad1 (17) have transcription activation domains, suggesting that they might act directly in the regulation of gene expression.

In Xenopus development, overexpression of Smad proteins in prospective ectodermal tissue causes mesoderm formation and, as with the type I receptors, different Smads elicit different

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1 The abbreviations used are: TGF, transforming growth factor; IB, interest boxes.
responses. Smad2, like ALK-4*, acts in a concentration-dependent manner to induce the expression of dorsal genes (18), whereas Smad1 and Smad5, like ALK-2*, induce ventral mesoderm (17, 19–22). These results suggest that the specificity of the signal regulated by the β4-β5 loop is realized by activation of specific Smad signaling pathways.

However, Smad1, in contrast to ALK-2*, cannot counteract the early effects of ALK-4* (or activin) at all, whereas Smad5 produces only a very weak interference. The dominant ventralizing effects of ALK-2* are therefore unlikely to be mediated by Smad competition, a conclusion consistent with the observation that these ventralizing effects are not mediated by the β4-β5 loop, which we suggest does act through Smads. We have not yet identified the region of ALK-2* which causes ventralization, but we do find that susceptibility to ventralization resides in ALK-4* sequences that are not within the β4-β5 loop, because dorsal gene induction mediated by a chimeric receptor with an ALK-2* backbone cannot be inhibited by co-expressed ALK-2*.

EXPERIMENTAL PROCEDURES

Cloning and in Vitro Transcription of Chimeric Activin Receptors—Constitutively active forms of the ALK-2 and ALK-4 receptors (ALK-2* and ALK-4*) have been described previously (23). Chimeras between the receptors were generated using a polymerase chain reaction technique in which DNA fragments were amplified using primers possessing 5' extensions with homology to the sequence to which they were to be fused. Reactions were then mixed and re-amplified. The sequences that were exchanged are shown in Fig. 1. For the region IB1, 7 amino acids were exchanged between the receptors, namely residues 269 to 275 in ALK-4* and 270 to 276 in ALK-2* (NKDNGTW versus MTSRH-SST). For IB3, 15 amino acids were exchanged between the receptors: that is, residues 359 to 373 in ALK-4* and residues 361 to 375 in ALK-2* (RDHVTDDTIDAPNQ versus MHSQSTNQLDVGNNP). The sequences exchanged in the GS domain regions were slightly more extensive than those shown in Fig. 1, including some sequences more N-terminal than those illustrated. Specifically these were residues 163 to 213 inclusive in ALK-4* (DMEDP to LQR1I) and residues 158 to 214 inclusive in ALK-2* (NPDRV to LECV). The clone containing substitutions in the IB1 region of ALK-4* (ALK-4*IB1(Mut)) was also generated by polymerase chain reaction, and the substitutions were NKNGTW to NTGNGTW, one residue (K to T) deriving from the equivalent position of ALK-3.

Clones were transcribed in vitro as described (24). RNAs were synthesized side-by-side on the same day with the same reagents and quantitated on agarose gels and by UV absorption. Xenopus Smad1 and Smad2 (Xmad1 and Xmad2) were gifts of Doug Melton (20). A mouse Smad5 cDNA (21) was a gift of Ali Hemmati-Brivanlou. Xenopus Embryos, Microinjection and Dissection—Fertilization, staging, culture, microinjection, and dissection of Xenopus embryos were as described (24, 25). A crude preparation of activin A was prepared side-by-side on the same day with the same reagents and synthesized and used for RNase protection assays. For IB3, 15 amino acids were exchanged between the receptors; that is, residues 270 to 276 in ALK-2* (NKDNGTW) and one residue (K to T) derived from the equivalent position of ALK-3.

RNA Isolation and RNase Protection Assays—RNase protection analysis was carried out as described (24). Probes include cardiac actin (27), Ef-1α (28), goosecoid (9), Xbra (8), Pintallavis (29), and Xhox3 (30).

RESULTS

Identification by Sequence Analysis of Regions That May Determine ALK-2* and ALK-4* Signaling Specificity—The specificities of the signals transduced by ALK-2* and ALK-4* are likely to be defined by peptide binding sites within their intracellular domains. The intracellular domains of all type I TGF-β receptors comprise a short poorly conserved juxtamembrane domain, a highly conserved GS domain, and finally a well-conserved serine-threonine kinase. A multiple sequence alignment was generated between the sequences of human ALK-1 to ALK-5 (31, 32) and Drosophila tkv, sax (33), and Atr-1 (34). A number of divergent regions exist between the vertebrate receptors, notably the juxtamembrane domain as well as a number of regions within the kinase domain itself, but only three divergent regions also retain homology with tkv, sax, and Atr-1 (Fig. 1). The most N-terminal region encompasses the GS domain itself; although the central core of this motif is highly conserved, the sequences directly flanking it are considerably more divergent. Other divergent regions were classified as interest boxes (IB), and two of these were also conserved between the Drosophila orthologs. IB1 is a short loop between kinase subdomains β4 and β5, and IB3 corresponds to the activation loop of the enzyme.

The IB1 Sequence of ALK-4* Can Confer Activin-like Responses to ALK-2*—Activated ALK-2* and ALK-4* receptors (ALK-2* and ALK-4*) elicit different responses in the Xenopus animal cap assay (7). ALK-4* induces a wide range of tissues including notochord, muscle, and endoderm, whereas ALK-2* induces only ventral mesodermal derivatives. Furthermore, ALK-4* induces, like activin, expression of the midline and anterior markers Pintallavis and goosecoid, and at intermediate concentrations it activates transcription of the more ventral
marker *Xbra*. ALK-2* induces expression only of *Xbra*. These markers are induced by activin in an immediate-early fashion and are easily detectable by the early gastrula stage, only 5 h after the mid-blastula transition (8, 9, 35). Expression of these genes is thus an ideal assay for primary signaling events and allows us to distinguish between signals deriving from ALK-2* and ALK-4*.

Chimeric receptors were generated in which each of the three candidate specificity regions was replaced with that from the other receptor in all possible combinations (Fig. 2). Receptors C0 to C7 correspond to constructs that contain an ALK-2* backbone, retaining either all ALK-2* sequences (C0) or containing various portions of the ALK-4* receptor in C1 to C7. Previous work has shown that ALK-2* itself cannot induce significant levels of *Pintallavis* or *goosecoid*, but it induces *Xbra* efficiently. We show here that some of the chimeric receptor constructs in the ALK-2* backbone behave very differently from ALK-2* in this respect (Fig. 3A). Most strikingly, exchange of the IB1 sequence from ALK-4* to ALK-2* results in strong expression of both *goosecoid* and *Pintallavis*. This was the case whether it was exchanged alone (C2) or in combination with the GS domain and flanks (C4), in which case the response was slightly but reproducibly stronger. By contrast, exchange of the GS domain and flanks alone or exchange of the IB3 region did not reproduce an ALK-4*-like response. These results indicate that the IB1 region of ALK-4* is a critical determinant of signaling specificity that is capable of transducing a signal that activates expression of dorsal-specific genes. This conclusion was confirmed by assaying expression of muscle-specific actin at stage 17; C4 was capable of inducing strong expression of muscle actin, and C2 had this effect in one of five experiments (not shown). The reason for this variability in C2 behavior is not clear.

Surprisingly, some chimeric receptors elicited no response. In particular C7, containing all three regions of ALK-4*, was consistently and reproducibly inactive in these assays and did not appear to interfere with the normal development of whole embryos expressing it (data not shown). In addition, the responses of animal caps expressing C3, C5, and C6 were rather variable. Sometimes C5 caused induction of *Xbra*, whereas on other occasions (such as the experiment shown in Fig. 3A) it elicited no response. Most oddly, C6 usually showed no activity but occasionally caused a full ALK-4*-like response, including induction of *Pintallavis* and *goosecoid*. The reason for this

![FIG. 2. Schematic representation of the 16 chimeric clones used in this study. Regions derived from ALK-4* are shown in green, and regions from ALK-2* are shown in red.](image)

![FIG. 3. Induction of stage 10 markers by the chimeric receptors.](image)
variability is not known, although additional experiments demonstrate that all chimeric receptors are capable, at least to some extent, of inducing Xhox3 at stage 17, suggesting that they retain some signaling activity (not shown). Despite these peculiarities, the consistency of the data obtained with chimeras C2 and C4 highlights the importance of IB1 in characterizing the receptor signal.

The b4/b5 Loop (IB1) of ALK-4* Is Required for the ALK-4*-like Response—Receptor constructs C8 to C15 contain the ALK-4* receptor backbone with various combinations of ALK-2* sequences replacing those of ALK-4*. ALK-4* induces expression of Xbra, Pintallavis, and goosecoid, as previously demonstrated (7) (Fig. 3B). Injection of RNA encoding C9 to C15 demonstrated that only chimeras retaining the IB1 region of ALK-4* mediate induction of goosecoid and/or Pintallavis (Fig. 3B) or, when assayed at stage 17, muscle-specific actin (not shown). In contrast, receptors containing the ALK-2* IB1 are capable only of an ALK-2*-like response (C10) or of no response at all at this stage (C12, C14, and C15). In contrast to the results obtained with chimeras possessing an ALK-2* backbone, the results were consistent and reproducible in all cases, and as with C0 to C7, the chimeras containing the ALK-4* backbone all induced expression of Xhox3 at stage 17 (not shown).

Interestingly, chimeras C14 (containing the IB1 and IB3 regions of ALK-2*) and C15 (containing all three regions) were similar to C6 and C7 in their inability to induce expression of early mesoderm-specific genes (Fig. 3B) and their concomitant failure to cause severe defects to embryonic development (not shown). This suggests either that progressive replacement of regions of one receptor with another results in structural perturbation and receptor inactivity or that there is a requirement for intramolecular compatibility between receptor regions. Additional evidence for a need for intramolecular compatibility comes from the observation that transfer of the ALK-2* GS domain and its flanking sequences into ALK-4* (C9) or transfer of the ALK-2* IB3 activation loop (C11), both, result in loss of goosecoid activation. Simultaneous transfer of both regions, however, (C13) has little effect on the receptor, which can both induce goosecoid strongly and cause caps to invert at the early gastrula stage.

The b4/b5 Loop (IB1) of ALK-4* Mediates Signals That Cause Cap Inversion at the Early Gastrula Stage—We have previously observed that injection of 1 ng of ALK-4* RNA induces strong morphological movements in Xenopus animal caps. A similar effect has been observed following overexpression of activin by RNA injection (23) and by overexpression of Smad2 (20). These movements, which occur at early gastrula stage 10, precede those that occur when caps extend in response to lower doses of activin when protrusions are formed at mid-gastrula stage 11. Caps that invert at stage 10 do not form protrusions and proceed to form tissue that we suspect is endodermal in nature (7). In this case the cells may be mimicking the earliest gastrulating cells on the dorsal side of the embryo, which form pharyngeal endoderm and/or head mesoderm. Migration of these cells may be temporally and mechanistically different from that of other mesodermal precursors in vivo.

We tested whether any of the chimeric receptors were capable of inducing morphological movements characteristic of ALK-4* overexpression. Fig. 4 shows that the chimeric receptors C2 and C4 both elicit cap inversion like that induced by ALK-4*. This response is also seen with the ALK-4* backbone-derived chimera C13. All the constructs that cause this effect therefore contain the IB1 region of ALK-4*, with transfer of this region alone into ALK-2* (C2) being sufficient to mediate
the response. Because the rapid cap inversion effect is also seen in response to Smad2 overexpression (20), these results suggest that the signal specified by the IB1 region of the receptor feeds directly into the Smad signaling pathway.

**Mutation of the IB1 Region of ALK-4**a Disrupts Receptor Signaling—To confirm that the β4/β5 loop (IB1) region of ALK-4a is responsible for the activin-like responses of the receptor, we generated a receptor clone containing a 2-amino acid substitution in the IB1 region of ALK-4, with one amino acid substituted from the equivalent position in the ALK-2a receptor and the other from the ALK-3 receptor, hence generating a chimeric sequence within the IB1 domain itself (NKDNGTW to NGTNGTW). We predict that this would disturb interaction of candidate downstream signaling components with the peptide sequence. Indeed this substitution resulted in complete ablation of the early gastrula responses seen normally with ALK-4a, consistent with a requirement for this region for effective signaling (Fig. 5).

**The Ability of ALK-2a to Interfere with ALK-4a-mediated Gene Induction Cannot Be Recapitulated by Transfer of the IB1 Region of ALK-2a into the ALK-4a Backbone—**ALK-2a interferes with the ability of ALK-4a to induce expression of goosecoid and Pintallavis (7). This is unlikely to result purely from competition for common signaling components at the membrane; low doses of ALK-2a will interfere very efficiently with ALK-4a-like responses, and kinase-inactive ALK-2a constructs interfere only at much higher doses, producing a qualitatively different type of interference (7). We asked whether the β4/β5 loop (IB1) from ALK-2a would confer this seemingly active inhibitory property to ALK-4a, a reasonable hypothesis as the equivalent region of ALK-4 is so potent in signal specificity. Surprisingly, this did not appear to be the case. The chimeras C3 and C5 (which contain the ALK-2a IB1 in the ALK-4a backbone) and C10, C12, C14, and C15 (which contain the ALK-2a IB1 in the ALK-4a backbone) all failed to demonstrate effective suppression of ALK-4a induction of goosecoid and Pintallavis (Fig. 6). Co-injection of these constructs lowered goosecoid and increased Xbra levels slightly, but effective titration of dorsal markers did not occur, and the observed effects were reminiscent of the partial suppression mediated by co-injection of kinase inactive ALK-2a (7). We therefore believe that the active inhibitory action of ALK-2a derives predominantly from signals elsewhere in the ALK-2a receptor.

**The Ability of ALK-2a to Interfere with Dorsal Gene Induction Requires the ALK-4a Receptor Backbone—**The ability of ALK-2a to interfere with dorsal gene induction by ALK-4a requires sequences that are not contained within the ALK-2 β4/β5 loop, because transfer of this region to ALK-4a does not confer interference activity. If other regions of ALK-2a are responsible for interference with dorsal gene induction, however, the question of how the C2 chimeric receptor activates dorsal genes is raised. With the exception of the ALK-4 β4/β5 loop (which comprises just 7 amino acids), C2 is identical to ALK-2a (Fig. 2). Why does it not inhibit its own dorsalizing activity? One possibility is that susceptibility to inhibition requires ALK-4 sequences that are not present in C2. This was tested by co-expressing C2 with ALK-2a. Fig. 7A (lanes 1 and 2) shows that ALK-2a is unable to inhibit induction of goosecoid and Pintallavis by C2, indicating that ALK-4 sequences are indeed required to respond to the inhibitory effects of ALK-2a. This result also indicates that the inhibitory effects of ALK-2a do not involve competition for molecules downstream of type I receptors; if they did, ALK-2a should inhibit dorsal gene induction by C2.

To confirm that susceptibility to inhibition requires sequences present in ALK-4, ALK-2a was co-expressed with C9, C11, and C13, all of which are capable of activating expression of Pintallavis and (to some extent) goosecoid, and all of which are based on an ALK-4a backbone (Figs. 2 and 3). Fig. 7B shows that ALK-2a is capable of inhibiting dorsal gene induction by all these constructs.

**Overexpression of Smad1 or Smad5 Does Not Interfere with Induction of Dorsal Genes by ALK-4a**—We suggest above that the IB1 region of the type I receptor feeds directly into the Smad signaling pathway. If this is so, then because the dominant ventralizing effects of ALK-2a are not specified by the IB1 region, overexpression of ventralizing Smad family members like Smad1 and Smad5 should also fail to counteract the effects of ALK-4a. Consistent with this suggestion, Fig. 8A shows that Smad1 is completely ineffective at suppressing the ability of ALK-4a to induce Pintallavis and goosecoid at stage 10.5. Smad1 is also unable to suppress the ability of activin to induce expression of goosecoid and Pintallavis (Fig. 8B), although a recent report suggests that Smad1 is capable of reducing the induction of goosecoid by activin by stage 11 (17).

Similar experiments reveal that Smad5 is also capable of eliciting little interference with dorsal gene induction, producing only a weak inhibition that resembles that obtained with the C10 chimera (Fig. 8C). We conclude that the rapid ventralizing effects of ALK-2a are not mediated solely through Smad competition.

**DISCUSSION**

**Classification of Type I TGF-β Receptors—**Our analysis allows the type I TGF-β superfamily receptors to be grouped into three classes (Fig. 1). ALK-4 and ALK-5, receptors for activin and TGF-β, respectively, fall into one group and are highly related. The IB1 domains of these receptors are identical to that of Drosophila Atr-1, although the GS and IB3 regions of

![Image](https://example.com/image.png)
Atr-1 differ from ALK-4 and ALK-5. ALK-3 (a BMP 2/4 receptor) and tkv fall into another class, defined particularly by sequences in their IB1 regions as well as in the C-terminal flanks of their GS domains. Finally ALK-1, ALK-2, and saxophone can be grouped together by similar criteria, although this class is also characterized by strong homology in the N-terminal flank of the GS domain and the C-terminal half of the IB3 activation loop. Because, with the exception of Atr-1, the receptors fall into the same classes at each of the three regions, it seems likely that tkv and sax are derived from genes duplicated before the divergence of insect and vertebrate lineages and that they may be true orthologs of ALK-3 (and ALK-6, not shown here) and ALK-1/ALK-2, respectively. We note that neither the daf-1 receptor (36) nor another putative type I activin receptor from *Caenorhabditis elegans* (SwissProt data base entry Q09488) possess homology with the Drosophila and vertebrate type I receptors in the IB1 region of the kinase. This is slightly unexpected as other components of the TGF-β signaling system appear to be intact in *C. elegans*, including the ligands, the type I and type II receptors, and the Smads.

Finally, although our sequence analysis defines three groups of type I TGF-β superfamily receptor, the L3 loop of the Smad proteins, which determines the specificity of receptor-mediated Smad activation, falls into only two classes (37). It is possible that additional Smads remain to be discovered or that other regions of the Smads also define receptor specificity.

The β4/β5 Loop Confers Activin- and Smad2-like Responses to ALK-2*—The dorsal-inducing properties of constitutively active ALK-4 can be conferred on ALK-2 by transfer of the β4/β5 loop from the kinase domain of one receptor to the other (creating C2). The dorsal-inducing effects of the chimeric ALK-2 receptor resemble those of Smad2, and we postulate that the β4/β5 loop interacts with the Smad signaling pathway.

Attempts to confirm this by immunoprecipitation of tagged Smad2 after receptor activation has not, however, revealed a simple relationship between receptor subtype and Smad2 phosphorylation. ALK-2* and ALK-4*, both, cause phosphorylation of Smad2 (data not shown). The functional significance of this phosphorylation is not yet clear, because the sites of phosphorylation are unknown.

Analysis of other chimeric receptors suggests that there is a requirement for compatibility between sequences in the IB3 activation loop and sequences in the juxtamembrane region, in or around the GS domain. In particular, we note that transfer into ALK-4* of the ALK-2* GS domain and its flanking seq-
quences (creating C9) or transfer of the ALK-2* IB3 activation loop (creating C11), both, result in loss of goosecoid activation. Simultaneous transfer of both regions, however, (creating C13) has little effect on the receptor, which can both induce goosecoid strongly and cause caps to invert at the early gastrula stage. It is possible that these regions lie close to each other in the tertiary structure of the receptor, with the activation loop being required for phosphorylation of the juxtamembrane region. Consistent with this idea, point mutation of threonines in a region N-terminal to the juxtamembrane sequences of TbR1/ALK-5 results in defective receptor signaling (38). If true the idea would explain why the two regions of the type I receptor show orthologous conservation.

The β4/β5 Loop Does Not Define All Signaling Properties of Type I TGF-β Receptors—Although transfer of the β4/β5 loop from ALK-4* to ALK-2* causes the latter to acquire dorsal gene inducing properties and to behave apparently identically to ALK-4*, the same is not true of the reverse transfer. The C10 chimera, which contains the ALK-2* β4/β5 loop in the ALK-4* backbone, is capable of inducing expression of Xbra but is unable to counteract the dorsalizing effects of ALK-4*. It differs in this respect from ALK-2*, and the results therefore demonstrate that the β4/β5 loop is not sufficient to account for all the signaling properties of the type I receptors.

Our data suggest that the β4/β5 loop regulates the activation of specific Smad family members, and consistent with this idea, we observe that the “ventralizing” Smads 1 and 5, like C10, cannot inhibit dorsal gene induction by ALK-4* (Fig. 8). This conforms with previous work demonstrating that although Smad1 can induce expression of ventral mesodermal markers, it causes little reduction in levels of Smad1, because we cannot detect stable interactions between Smad1 and ALK-2*, and tagged Smad2 (data not shown).

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