

Negative regulation of Activin/Nodal signaling by SRF during *Xenopus* gastrulation

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Activin/Nodal signaling is essential for germ-layer formation and axial patterning during embryogenesis. Recent evidence has demonstrated that the intra- or extracellular inhibition of this signaling is crucial for ectoderm specification and correct positioning of mesoderm and endoderm. Here, we analyzed the function of *Xenopus* serum response factor (XSRF) in establishing germ layers during early development. XSRF transcripts are restricted to the animal pole ectoderm in *Xenopus* early embryos. Ectopic expression of XSRF RNA suppresses mesoderm induction, both in the marginal zone in vivo and caused by Activin/Nodal signals in animal caps. Dominant-negative mutant or antisense morpholino oligonucleotide-mediated inhibition of XSRF function expands the expression of mesendodermal genes toward the ectodermal territory and enhances the inducing activity of the Activin signal. SRF interacts with Smad2 and FAST-1, and inhibits the formation of the Smad2-FAST-1 complex induced by Activin. These results suggest that XSRF might act to ensure proper mesoderm induction in the appropriate region by inhibiting the mesoderm-inducing signals during early embryogenesis.

KEY WORDS: SRF, Germ-layer formation, Activin and Nodal signaling, *Xenopus*

INTRODUCTION

In the *Xenopus* early embryo, the mesodermal cell fate is established in the marginal zone between the animal and vegetal poles by inductive signals from the underlying endoderm. This induction cooperates with the independent patterning signals from the organizer to define the dorsoventral and anteroposterior pattern of the body axis (Harland and Gerhart, 1997; Heasman, 2006). Several members of the TGF- β growth factor superfamily, including Activin, Vg1 and Nodal-related proteins are responsible for the induction of mesoderm and endoderm germ layers as well as for the subsequent patterning of the embryo (Schier, 2003). These ligands bind to a serine-threonine kinase receptor complex leading, intracellularly, to the phosphorylation and activation of the receptor-Smad family of signal transducers (R-Smads) that includes Smad2 and Smad3. Upon activation, these R-Smads translocate into the nucleus where they control gene expression in association with Smad4 and certain transcription regulators (Schier, 2003; Shi and Massague, 2003; Whitman, 2001). However, a detailed understanding of how the cellular responses to TGF- β ligands are modified by the differential combination of distinct Smad partners remains elusive.

Recent studies in the mouse and frog suggest that the intra- or extracellular inhibition of mesoderm-inducing signals is crucial for appropriate germ-layer specification. Inactivation of mouse *Lefty2*, an extracellular feedback inhibitor of Nodal signaling, results in

expansion of the primitive streak and mesoderm migration defects (Meno et al., 1999). Knockdown of *Xenopus Lefty* also causes the fate domains of the organizer and dorsal mesoderm to expand, leading to exo-gastrulation (Branford and Yost, 2002; Cha et al., 2006). In the frog, the loss of function of intracellular factors such as *Ectodermin* and *Xema* expands mesoderm at the expense of ectoderm specification (Dupont et al., 2005; Suri et al., 2005). The phenotype of mice mutant for DRAP1, a transcriptional co-repressor, resembles that of *Lefty2* mutants (Iratni et al., 2002). These proteins have been shown to limit the spatial or temporal extent of the response to Activin/Nodal signaling in vertebrate embryos.

Serum response factor (SRF) is a MADS box-containing transcription factor that binds to a serum response element (SRE) found in the promoters of a variety of genes, including immediate early genes, neuronal genes and muscle genes (Shore and Sharrocks, 1995). SRF contains a highly conserved N-terminal DNA-binding and dimerization domain termed the MADS box – owing to its homology among yeast (MCM1, Agamous), plant (Deficiens) and vertebrate (SRF) proteins – and a C-terminal transactivation domain (Johansen and Prywes, 1993; Norman et al., 1988; Shore and Sharrocks, 1995). SRF controls cell growth and differentiation, neuronal transmission and muscle development, and functions by regulating the expression of its target genes (Carson et al., 2000; Castillo et al., 1997; Treisman, 1986). SRF-deficient mouse embryos display early embryonic lethality owing to the absence of mesodermal cells, and this has led to the proposal that SRF is required for mesoderm formation during mouse gastrulation (Arsenian et al., 1998). Interestingly, experiments using SRF^{-/-} embryonic stem cells suggest that the phenotype of SRF mutants may be due to a non-cell-autonomous defect in differentiation toward mesoderm, rather than any impairment in the cell-autonomous induction of the mesoderm program (Weinhold et al., 2000).

To better understand the molecular mechanisms by which SRF affects germ-layer specification during vertebrate embryogenesis, we have investigated SRF function in *Xenopus* early embryos, where

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mesoderm induction and patterning are much better characterized than, for example, in mice. SRF expression is restricted to the prospective ectoderm in *Xenopus* early embryos. Ectopic expression of SRF inhibits mesoderm formation. Conversely, loss-of-function of SRF stimulates mesendoderm induction, thereby expanding the expression of mesendodermal genes toward the ectodermal territory. In addition, SRF, a binding partner of Smad2 and FAST-1, impedes their association in Activin/Nodal signaling. These results suggest that SRF may function to restrict inappropriate germ-layer specification throughout the vertebrate embryo.

MATERIALS AND METHODS

Embryos and microinjection

Xenopus eggs were in vitro fertilized as described previously (Newport and Kirschner, 1982), and developmental stages of the embryos were determined according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994). Microinjection was carried out in 0.33×Modified Ringer (MR) containing 4% Ficoll-400 using a Nanoliter Injector (WPI). Injected embryos were cultured in 0.33×MR until stage 8 and then transferred to 0.1×MR for later culture to the appropriate stages.

Whole-mount in situ hybridization and RT-PCR

Whole-mount in situ hybridization was performed with digoxigenin (DIG)-labeled probes as described (Harland, 1991). For RT-PCR analysis, total RNA was prepared from embryos or animal cap explants with TRI reagent (Sigma) and treated with RNase-free DNaseI (Roche) to remove genomic DNA. RNA was transcribed using M-MLV reverse transcriptase (Promega). PCR amplification was performed using Taq polymerase (TaKaRa). Primers used for RT-PCR analysis are described at the homepage of the De Robertis group <http://www.hhmi.ucla.edu/derobertis/index.html>. The number of PCR cycles for each primer pair was determined empirically to maintain amplification in the linear range.

Plasmids, RNA synthesis, morpholinos and cell lines

The mammalian expression plasmid for Flag-tagged Smad2 was described previously (Lee et al., 2004). The pCGN-HA-SRF construct was kindly provided by Dr Jae-Hong Kim (Korea University, Seoul, Republic of Korea) (Johansen and Prywes, 1993). GST-tagged SRF and deletion mutant constructs were obtained by PCR and cloning into the *Bam*HI and *Spe*I sites of eukaryotic expression vector pEBG. Flag-tagged SRF was generated in the pEF-Flag vector. Myc-tagged FAST-1 deletion mutant constructs were obtained by PCR using the Myc-FAST-1 construct as template and cloning into the *Eco*RI and *Xho*I sites of pCS2-MT.

For expression in *Xenopus* embryos, XSFR constructs including pSP64T-wt XSFR and pSP64T-dn XSFR (kind gifts from Dr Harumasa Okamoto, Neuroscience Research Institute, AIST, Tsukuba, Japan) were linearized with *Xba*I and their capped mRNAs were synthesized using the SP6 mMessage mMachin kit (Ambion). The XSFR-6Myc construct was generated by subcloning the sequence containing its coding region and 5' untranslated region with MO-binding sites into the *Bam*HI and *Cla*I sites of pCS2-MT. FAST-VP16^A and FAST-En^R constructs were described previously (Watanabe and Whitman, 1999). The morpholino antisense oligonucleotides (GeneTools) directed against *Xenopus* SRF were as follows: XSFR MO1, CTGGTTACTGGGCAGCATCCCTTG; XSFR MO2, AAATTTACTAATCTGCCCTTCCTTG. The standard control MO (CO MO) was CCTCTTACCTCAGTTACAATTATA.

Mv1Lu, HepG2 and HeLa cells were all maintained in Dulbecco's Modified Eagle Medium (high glucose) supplemented with 10% fetal bovine serum and 100 µg/ml gentamicin. Mv1Lu-SRF cells that stably express SRF were generated by transfection with pCGN-HA-SRF expression plasmid. A day after transfection, cells were split and selected for neomycin resistance. Neomycin-resistant colonies were pooled after 2 weeks of selection, expanded and analyzed.

Luciferase reporter assays

HepG2 or Mv1Lu cells were transiently transfected with different combinations of plasmid DNA in 12-well plates using Lipofectamin and Plus Reagent (Invitrogen, Rockville, MD) according to the manufacturer's

instructions. The cells were transfected with SRF, reporter plasmid, ARE-Luc/FAST-1, constitutively active HA-tagged ALK4*, pCMV-Gal to normalize transfection efficiency and pcDNA3 to normalize the amount of transfected DNA. All transfections were normalized to a total of 1 µg of DNA in each well. Cells were harvested 36 hours after transfection and the luciferase activity was measured using Enhanced Luciferase Assay Kit (BD Biosciences). Values were normalized to the β-galactosidase activity and represent the mean of three independent transfections with error bars indicating the standard deviation. Similar results were obtained in three separate experiments.

Immunoblotting and immunoprecipitation

293T, Mv1Lu and HeLa cells were used for the detection of protein-protein interaction in vivo. For the treatment with Activin A, HeLa or Mv1Lu cells were starved overnight in 0.2% serum-containing medium 24 hours after transfection, and treated with 25 ng/ml Activin A for 2 hours. Cells were lysed in ice-cold RIPA buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5 mM Na₃VO₄, 50 mM NaF and Protease Inhibitor Cocktail (Complete, Roche). Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were immunoblotted with various antibodies, followed by incubation with HRP-conjugated antibodies to rabbit or mouse IgG and detected by chemiluminescence according to the manufacturer's instructions (Pierce).

For immunoprecipitation, cell lysates were incubated with the appropriate antibody for 2 hours, followed by incubation with Protein G Plus-agarose beads (Santa Cruz Biotechnology) for 1 hour at 4°C. The beads were washed four times with RIPA buffer and then boiled for 5 minutes in 2×SDS sample buffer. The eluted immunoprecipitates were analyzed by immunoblotting as described above. For GST pull-down assay, cell lysates were incubated with glutathione-agarose beads (Santa Cruz Biotechnology) for 2 hours. After washing the beads four times with RIPA buffer, immunoblotting was performed.

Antibodies used for immunoprecipitation: anti-SRF rabbit polyclonal antibody (G-20, Santa Cruz Biotechnology); anti-Flag (M2, Sigma); anti-Smad2 (Zymed). Antibodies used for immunoblotting: anti-Smad2 mouse monoclonal antibody (BD Transduction Laboratories); anti-HA (Y-11, Santa Cruz Biotechnology); anti-Smad4 (Santa Cruz Biotechnology).

RESULTS

Ectopic expression of SRF causes axial defects in *Xenopus* embryos

In order to investigate the biological function of SRF in *Xenopus* early embryos, we first examined the spatial expression pattern of *Xenopus* SRF (XSFR) during early development. From the early cleavage to gastrula stages, XSFR transcripts were observed predominantly in the animal hemisphere and at relatively low levels in the marginal zone as assayed by in situ hybridization (Fig. 1A-C). At later stages, XSFR transcripts were found in the anterior region and somites (data not shown). Consistent with this, RT-PCR analysis revealed the prominent expression of XSFR in the animal half and its low expression in dorsal and ventral marginal tissues (Fig. 1D). No expression, however, could be detected in the vegetal half (Fig. 1D).

To examine the effects of ectopic expression of XSFR on axis formation, we injected XSFR RNA into the marginal zone of two dorsal or ventral blastomeres of four-cell stage embryos. Compared with the phenotype of uninjected control embryos, dorsally XSFR-injected embryos exhibited a severely shortened body axis and the truncation of anterior structures (72%, *n*=123) (Fig. 1E,F). These phenotypes are similar to those caused by experimental conditions in which Activin or Nodal signaling is inhibited (Chang et al., 1997; Osada and Wright, 1999; Piepenburg et al., 2004). Ventral injection of XSFR, however, resulted in more modest defects in trunk and tail development (Fig. 1G). To characterize at the molecular level the events leading to these disrupted phenotypes, we next examined the

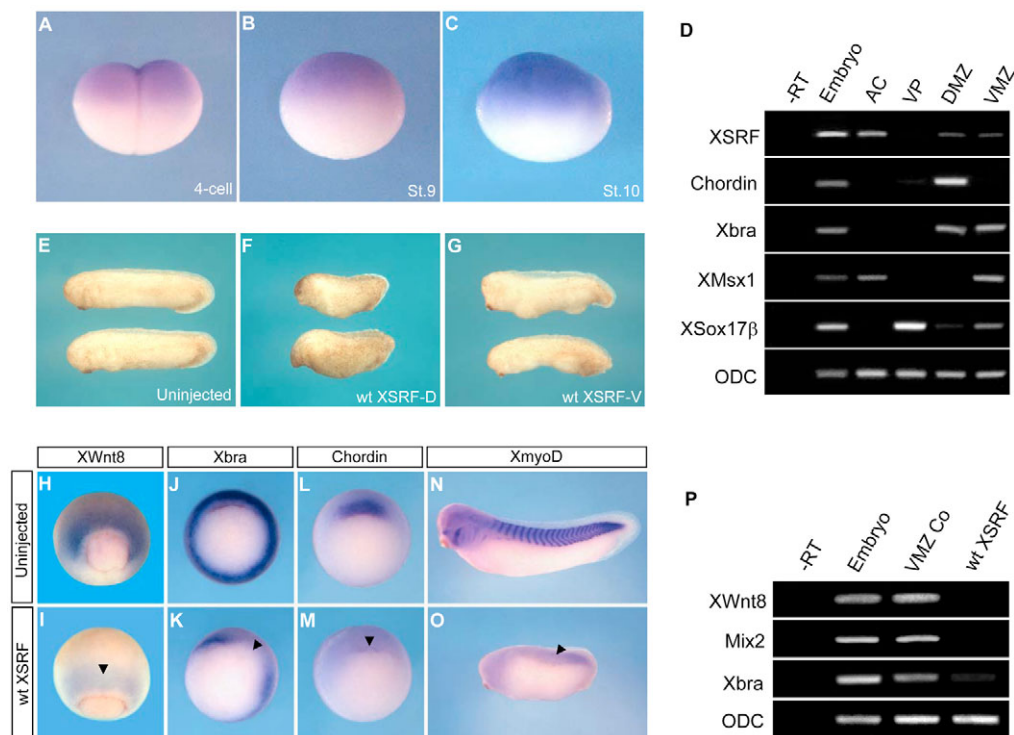


Fig. 1. Misexpression of XSRF suppresses mesodermal cell fates. (A–C) Spatial expression of XSRF at the four-cell, blastula and early gastrula stages. Lateral views are shown. (D) XSRF expression in dissected gastrula embryos. Animal cap (AC), vegetal pole (VP), dorsal marginal zone (DMZ) and ventral marginal zone (VMZ) explants were dissected from stage 10.5 gastrula embryos and subjected to RT-PCR analysis. –RT, control RT-PCR in the absence of reverse transcriptase. The following controls were included as markers for the dissected tissues: Chordin for dorsal mesoderm, Xbra for pan-mesoderm, XMsx1 for animal cap and ventral mesoderm and XSox17β for endoderm. ODC, ornithine decarboxylase loading control. (E–G) Phenotypic effects of overexpression of wild-type (wt) XSRF RNA. Four-cell stage embryos were injected in the dorsal (F) or ventral (G) marginal regions with wt XSRF mRNA (2 ng), or not injected as a control (E), and cultured until sibling embryos reached stage 30. Lateral views are shown and anterior is to the left. (H–P) Ectopic wt XSRF interferes with the expression of mesodermal genes. Four-cell stage embryos were injected in the dorsal or ventral marginal regions with wt XSRF mRNA (2 ng) and, along with uninjected controls, cultured to stage 10.25–10.5 (H–M, P) and 28 (N, O) and then analyzed by whole-mount in situ hybridization using antisense *Xenopus* Wnt8 (H, I), brachyury (J, K), chordin (L, M) and myoD (N, O) probes or RT-PCR (P). Arrowheads in I, K, M, O indicate the absence or reduction of expression of genes. (H, I) Ventro-vegetal views are shown. (J–M) Vegetal views are shown with dorsal side at top. (N, O) Lateral views are shown with anterior side to the left. (P) Ventral marginal zone explants were dissected at stage 10.25 and subjected to RT-PCR. VMZ Co, uninjected ventral marginal zone tissue.

expression of mesodermal markers in XSRF-injected embryos. Ventral or dorsal injection of XSRF suppressed the expression of ventral mesodermal marker Wnt8, pan-mesodermal marker Xbra, and dorsal mesodermal marker Chordin, in the early gastrula embryos (Fig. 1H–M). Moreover, XSRF-injected embryos showed dramatically inhibited formation of the somites, a paraxial mesoderm derivative, which was evident by the absence of MyoD expression at the tadpole stages (Fig. 1N, O). Consistently, RT-PCR analysis showed that ectopic XSRF could reduce the expression of mesodermal markers, such as Wnt8, Mix2 and Xbra, in the ventral region (Fig. 1P). Taken together, these results indicate that ectopically injected XSRF could interfere with mesoderm formation, thereby leading to the defects in axis specification.

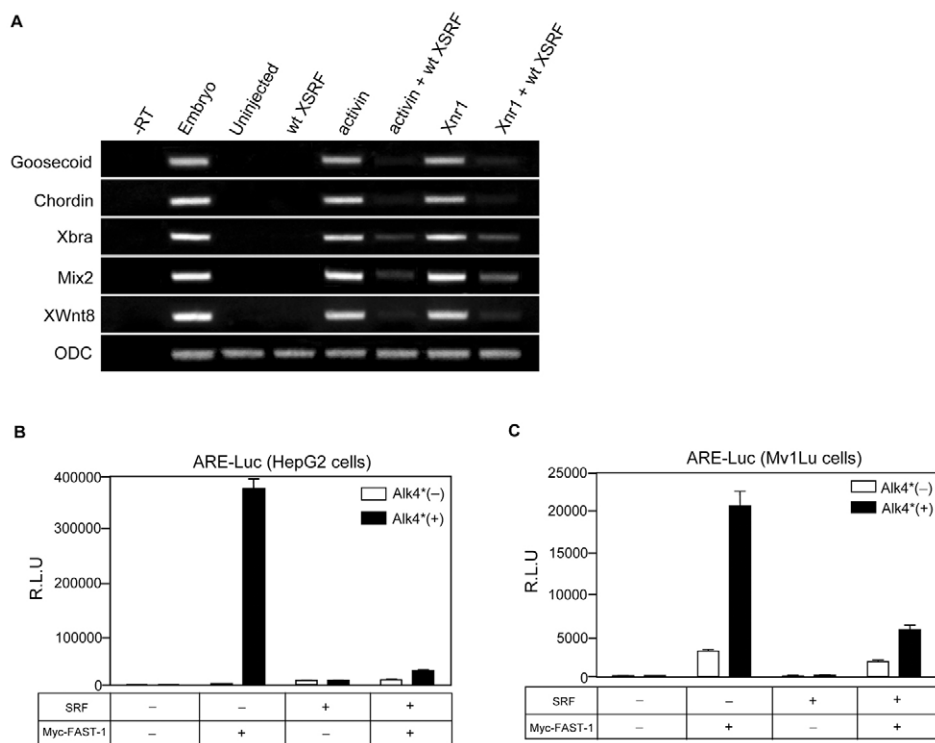
SRF inhibits Activin- and FAST-1-dependent transcription

On the basis of the inhibitory effects of XSRF on mesoderm formation shown above, we next tested whether SRF could inhibit the mesoderm-inducing activity of Activin/Nodal signaling. Functional assays in animal cap cells showed that XSRF could inhibit the induction of mesodermal markers including Goosecoid,

Chordin, Xbra, Mix2 and Wnt8 by Activin or Xnr1 ligands (Fig. 2A). To examine the effects of SRF on Activin- and FAST-1-dependent transcription in mammalian cells, we further performed the luciferase assay using the ARE-Luc reporter containing three copies of the Activin response element (ARE) from the *Xenopus* Mix2 promoter. As shown in Fig. 2B, C, ARE-Luc was activated by constitutively active Activin type-I receptor (ALK4*) in the presence of FAST-1, and this activation was suppressed by SRF in HepG2 and Mv1Lu cells. Similar results were obtained using Activin A (data not shown). Together, these results suggest that SRF may function to antagonize Activin/Nodal signaling in *Xenopus* embryos and mammalian cells.

Inhibition of XSRF function expands mesendoderm

To examine the effects of loss of XSRF function on germ-layer formation, we first employed a dominant-negative (DN) XSRF construct that mainly comprises the DNA-binding domain, lacking the C-terminal half of its wild-type form (Belaguli et al., 1997; Watanabe et al., 2005). Dorsal injection of DN XSRF at the four-cell stage resulted in embryos with the stout, shortened body axis and

**Fig. 2. SRF inhibits Activin/Nodal signaling.**

(A) Wild-type XSRF inhibited the induction of mesendoderm markers by Activin or Xnr1 in animal caps.

Embryos were injected at the four-cell stage in the animal pole region with wt XSRF mRNA (2 ng) with or without Xnr1 mRNA (50 pg), and then the animal caps isolated at stage 8.5 were cultured in the presence of Activin protein (10 ng/ml, for animal caps without Xnr1 injection) until stage 10.25 and then subjected to RT-PCR analysis. ODC, ornithine decarboxylase as a loading control; -RT, a control of RT-PCR on stage 10.25 whole embryo in the absence of reverse transcriptase; Uninjected, uninjected control. (B) HepG2 and (C) Mv1Lu cells were transiently transfected with a control vector or SRF along with ARE-Luc and FAST-1. Cells were harvested 36 hours after transfection for luciferase assays. β -Galactosidase activities were used to normalize for transfection efficiency. All luciferase assays were performed in triplicate.

microcephaly (Fig. 3A,B), which is similar to the phenotypes caused by the increase in Activin/Nodal signaling. By contrast, ventrally DN XSRF-injected embryos showed no dramatic changes in phenotype (data not shown). Interestingly, overexpression of DN XSRF in the animal region of four-cell stage embryos ectopically induced mesodermal (Chordin and VegT), endodermal (Sox17 β) and neural (Zic3) markers in ectoderm, with the reverse effect on the expression of epidermal keratin, an epidermal marker (Fig. 3C). In addition, DN XSRF enhanced the inducing activity of Activin protein in animal cap cells, increasing the expression of two dorsal markers, Goosecoid and Chordin, and decreasing that of Xbra which is dependent upon the relatively low levels of Activin signals (Fig. 3D). Together, these data suggest that inhibition of XSRF function may augment the mesendoderm-inducing signals in early embryos.

To substantiate the above effects of loss of XSRF function in *Xenopus* embryos, we next designed two kinds of antisense morpholino oligonucleotides (MO1 and MO2) capable of depleting XSRF protein. Both of the MOs specifically inhibited the production of C-terminally Myc-tagged XSRF protein as analyzed by western blotting, without affecting the levels of control β -catenin protein (Fig. 4A). Control MO, however, had no effect on the translation of this XSRF RNA. We next observed the phenotypes of XSRF-depleted embryos upon injecting the MOs into the animal or dorsal regions at the four-cell stage. Animal injection of XSRF MO1 or MO2 produced embryos with the microcephaly and shortened body axis (MO1: 96%, $n=32$; MO2: 73%, $n=48$) (Fig. 4C,F), phenotypes identical to those caused by DN XSRF. Dorsal injection of either MO resulted in not only these defects, but also gastrulation-inhibited phenotypes such as spina bifida and exo-gastrulation (MO1: 94%, $n=97$; MO2: 70%, $n=60$) (Fig. 4D,G). These defective embryos could be rescued by coexpression of wild-type XSRF RNA that lacks MO-binding sites and is resistant to its translational inhibition (MO1: 36% defective, $n=30$; MO2: 30% defective, $n=50$) (Fig. 4E,H), supporting the specific effects of XSRF MOs on the early development of *Xenopus* embryos. Next, we performed molecular

characterization of XSRF knockdown by in situ hybridization on whole embryos. MO1- or MO2-mediated depletion of XSRF caused a dorsal marker, Goosecoid, to be expressed in a wider region as compared with that in uninjected control embryos, and caused the mesoderm-specific markers Xbra and Wnt8 to spread toward the animal pole (Fig. 4I-T). DN XSRF had the same effects, expanding the expression of these mesodermal genes in whole embryos (Fig. 4U-W). Taken together, we suggest that XSRF might function to maintain ectodermal cell fate in the animal region of early embryos by preventing mesendoderm-inducing signals, such as Activin and Nodal, from expanding into this area.

SRF associates with Smad2 and FAST-1

To elucidate the molecular mechanism by which SRF suppresses Activin/Nodal signaling, we first tested whether SRF could interact with Smad2, a crucial mediator of these signaling pathways (Shi and Massague, 2003). Indeed, coimmunoprecipitation experiments using epitope-tagged proteins from 293T cells showed that SRF binds Smad2 (Fig. 5A). We also found that endogenous Smad2 coimmunoprecipitates with endogenous SRF in Mv1Lu and HeLa cells (Fig. 5B,C), showing that their interaction occurs at physiological protein levels. The interaction between Smad2 and SRF was enhanced by constitutively active Activin type-I receptor (ALK4*) or Activin A. To locate the domain within Smad2 responsible for interaction with SRF, we evaluated the abilities of a series of Smad2 deletion mutants to bind SRF using GST pull-down assay. These indicated that it is the MH2 domain of Smad2 that retains the ability to bind SRF (Fig. 5D,E).

Furthermore, we investigated whether SRF could also bind FAST-1, a DNA-binding partner of Smad2 in Activin/Nodal signaling (Whitman, 2001). GST pull-down assays demonstrated that SRF interacts with FAST-1 (Fig. 6A,B). This association was not affected by constitutively active Activin type-I receptor (data not shown). To identify the region within FAST-1 required for this interaction, we examined whether SRF could coimmunoprecipitate with a series of

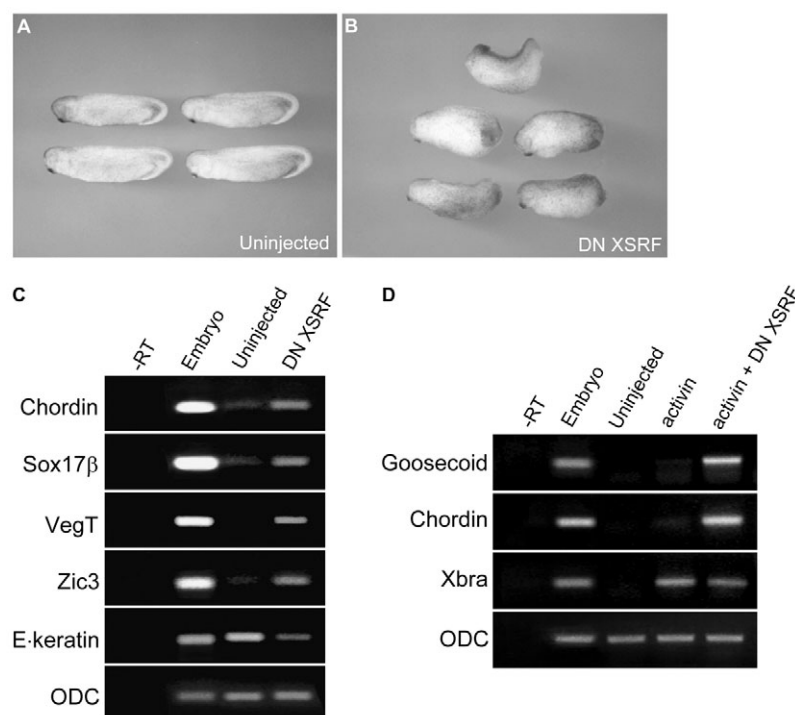


Fig. 3. Interfering with XSRF function changes cell fates. (A,B) Phenotypes of embryos injected with DN XSRF RNA dorsally as compared with uninjected embryos. (C,D) Embryos were injected at the four-cell stage in the animal pole region with DN XSRF mRNA (1–2 ng) and the animal cap explants cut at stage 8.5 were cultured to stage 10.25 in the presence or absence of Activin protein (5 ng/ml) and then analyzed by RT-PCR. (C) DN XSRF alone induced mesoderm (Chordin and VegT), endoderm (Sox17β) and neural (Zic3) markers in animal caps. Epidermal keratin (E-keratin), an epidermal marker, was reduced by injection of DN XSRF. (D) DN XSRF enhanced the ability of Activin to induce target genes in animal caps. ODC was used as a loading control; Uninjected, uninjected control animal caps.

deletion fragments of FAST-1 in GST pull-down experiments. Fig. 6B shows that the Forkhead DNA-binding domain is required for FAST-1 to associate with SRF.

SRF inhibits Activin-induced formation of FAST-1-Smad2 complex

Smad2 is phosphorylated by the TGF-β or Activin type-I receptor, associates with Smad4, and then enters into the nucleus (Schier, 2003; Shi and Massague, 2003). Thus, we checked whether SRF could suppress the phosphorylation of Smad2 by Activin. Smad2, however, was phosphorylated by Activin in stable Mv1Lu cells overexpressing SRF as strongly as in control Mv1Lu cells (Fig. 7A), indicating that SRF does not affect Activin-dependent phosphorylation of Smad2.

Since SRF associates with the MH2 domain of Smad2 that mediates its interaction with Smad4, we also examined the possible inhibitory effects of SRF on the ligand-induced formation of the Smad2-Smad4 complex that is essential for TGF-β or Activin signaling. However, formation of the Smad2-Smad4 complex induced by Activin was not reduced in Mv1Lu cells stably overexpressing SRF as compared with that in control Mv1Lu cells (Fig. 7B).

We also tested whether SRF could interfere with the ability of FAST-1 to bind Smad2, as both of them associate with SRF. As shown in Fig. 7C, Myc-tagged FAST-1 coimmunoprecipitated with Flag-tagged Smad2 and this interaction was enhanced by constitutively active Activin type-I receptor (ALK4*). However, the level of Myc-tagged FAST-1 that coimmunoprecipitated with Flag-tagged Smad2 was markedly reduced by the co-transfection of SRF (Fig. 7C), suggesting a negative role of SRF in the association of FAST-1 and Smad2. Since this indicates the possible inhibitory effects of SRF on FAST-mediated transcription, we speculated that an activated form of FAST-1 (FAST-VP16^A) would recover the defective phenotypes caused by overexpression of XSRF in *Xenopus* early embryos. Accordingly, we found that disruption of axial

structure by XSRF could be rescued by coexpression of FAST-VP16^A (29% defective, $n=52$) (Fig. 7D,E). Conversely, co-injection of an inhibitory form of FAST-1 (FAST-En^R) could rescue the gastrulation-defective phenotypes caused by XSRF MO (45% defective, $n=38$) (Fig. 7F,G). Overall, these results suggest that SRF may negatively regulate Activin/Nodal signaling by inhibiting the formation of a functional complex between FAST-1 and Smad2.

DISCUSSION

Several lines of evidence presented here show that SRF downregulates Activin/Nodal signaling in *Xenopus* embryos and mammalian cells. Ectopic overexpression of XSRF causes the embryonic malformations shown at later stages including anterior truncation, shortened body axis and defective gastrulation movements (Fig. 1). These defects are recapitulated by inhibition of components of Activin/Nodal signaling (Kofron et al., 2004; Onuma et al., 2002; Osada and Wright, 1999). Consistently, these gain-of-function phenotypes of XSRF can be rescued by coexpression of an activated mutant of the FAST-1 co-factor (Fig. 7). Furthermore, SRF inhibits Activin/Nodal signal-dependent transcription as analyzed by our reporter assays and RT-PCR (Fig. 2). These demonstrate that SRF antagonizes Activin/Nodal signaling upstream of, or in parallel to, the FAST-1 factor.

Conversely, XSRF loss-of-function results in a shift in the cellular fates along the animal-vegetal axis, causing the mesoderm that normally exists at the equator of the embryo to spread toward the animal pole at the expense of ectoderm (Figs 3, 4). This seems to be responsible for the defective embryos that show microcephaly and alterations in gastrulation movement. Recent evidence has shown that loss of inhibitors of mesoderm-inducing signals can lead to inappropriate germ-layer development. Depletion of *Xenopus* Lefty, an extracellular inhibitor of Nodal signaling, expands the organizer and mesendodermal tissues, with consequent exo-gastrulation (Branford and Yost, 2002; Cha et al., 2006). The maternal protein Ectodermin acts as a ubiquitin ligase for Smad4 to antagonize,

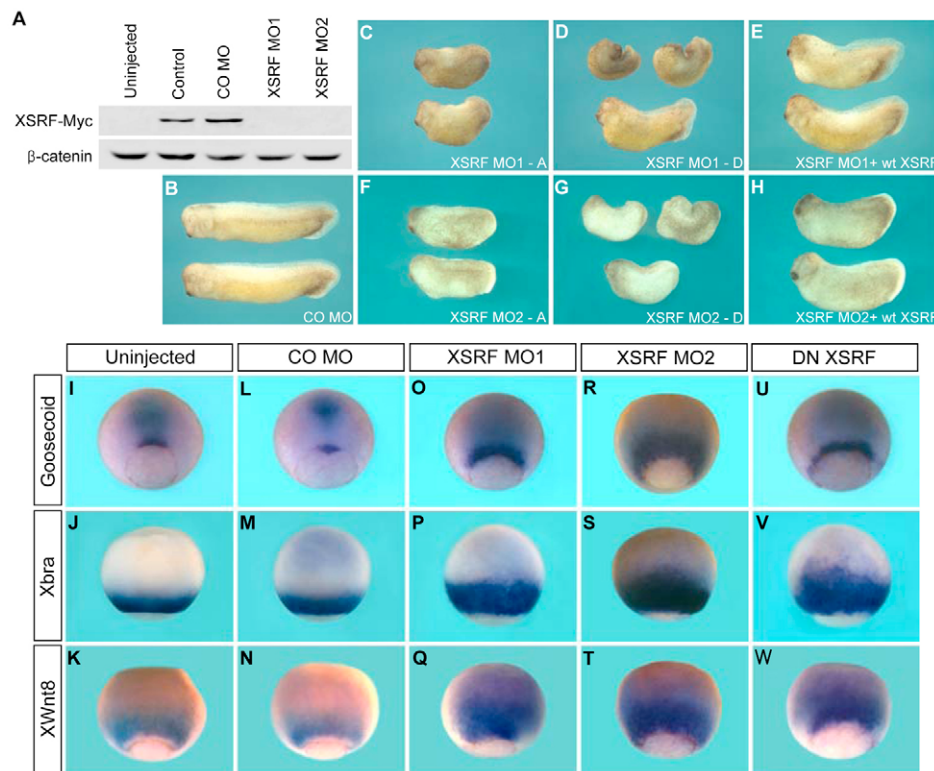


Fig. 4. Depletion of XSRF leads to expansion of mesoderm. (A) XSRF morpholino oligonucleotides (MOs) specifically knockdown the translation of C-terminally Myc-tagged XSRF protein in animal cap cells. Four-cell stage embryos were injected in the animal pole region with C-terminally 6Myc-tagged XSRF RNA (2 ng) with or without CO MO (60 ng), XSRF MO1 (60 ng) or XSRF MO2 (40 ng), and then animal cap explants dissected at late blastula stages were subjected to western blotting. Uninjected, animal caps without injection; Control, animal caps injected with XSRF-6Myc only. (B-H) Phenotypes of XSRF-depleted embryos. Embryos were injected at the four-cell stage with the indicated reagents (60 ng CO MO; 60 ng XSRF MO1; 40 ng XSRF MO2; 100 pg wt XSRF) dorsally (B,D,E,G,H) or anally (C,F) and cultured to stage 31. (I-W) Knockdown of XSRF expands the expression of mesodermal markers. Four-cell stage embryos were injected in the dorsal or ventral marginal region with CO MO (60 ng), XSRF MO1 (60 ng), XSRF MO2 (40 ng) or DN XSRF (2 ng) and then analyzed at the mid-gastrula stages by in situ hybridization against Goosecoid (I,L,O,R,U), Xbra (J,M,P,S,V) or Wnt8 (K,N,Q,T,W).

intracellularly, TGF- β signaling for ectoderm specification (Dupont et al., 2005). In addition, knockdown of maternal *Zic2* transcription factor causes the same phenotypes as excess Nodal signaling, as this protein functions to negatively regulate Nodal-related gene expression during anteroposterior patterning (Houston and Wylie, 2005). This antagonism of TGF- β /Nodal signaling for proper germ-layer formation is conserved in mice, and mutation of the transcriptional co-repressor DRAP1 or mouse Lefty2 leads to expansion of the primitive streak and severe gastrulation defects (Iratni et al., 2002; Meno et al., 1999). Given that ectopic XSRF precludes mesoderm formation, both in vivo and caused by Activin/Nodal signaling in animal caps, and that its knockdown leads to expansion of mesoderm into the prospective ectoderm, it is reasonable to suggest that XSRF has the same function of limiting the response to the mesoderm-inducing signals during germ-layer specification.

Although the predominant expression of XSRF in the animal pole region of the embryo indicates its role as an ectodermal determinant (Fig. 1), low levels of XSRF expression in the marginal zone might also suggest the requirement of XSRF for mesoderm formation. This possibility is corroborated by the observation that *Srf*-knockout mice failed to develop mesoderm (Arsenian et al., 1998). However, it could be challenged by the fact that ectopic overexpression of XSRF

has an inhibitory effect on mesoderm formation as shown in our results. A likely explanation for this paradox is that XSRF might function to establish the vegetal-to-animal gradient of the mesoderm- and endoderm-inducing signaling. In this regard, note that Activin and Nodal ligands induce target genes in a concentration-dependent manner, with dorsal mesoderm and endoderm at high concentrations and ventral mesoderm at low concentrations (Agius et al., 2000; Gurdon and Bourillot, 2001). Although these mesoderm- and endoderm-inducing molecules are induced by VegT, which is a transcription factor inherited by all vegetal cells and uniformly distributed in the vegetal region, the cell fates, mesodermal or endodermal, might be determined by either distinct transcription regulators or a gradient of inhibitors of these signals along the animal-vegetal axis (Heasman, 2006; Zhang et al., 1998). The existence of these inhibitors that counteract the activity of mesoderm- and endoderm-inducers is suggested by the phenotype of VegT-depleted embryos, in which the marginal zone cells differentiate as ectoderm at the expense of mesoderm and the vegetal cells differentiate as mesoderm and ectoderm instead of endoderm (Zhang et al., 1998). Thus, it is possible that XSRF might function to set the activity threshold of mesoderm-inducing signaling through the antagonizing mechanism to enable the marginal zone to adopt a mesodermal cell fate but not an endodermal one. Therefore, high

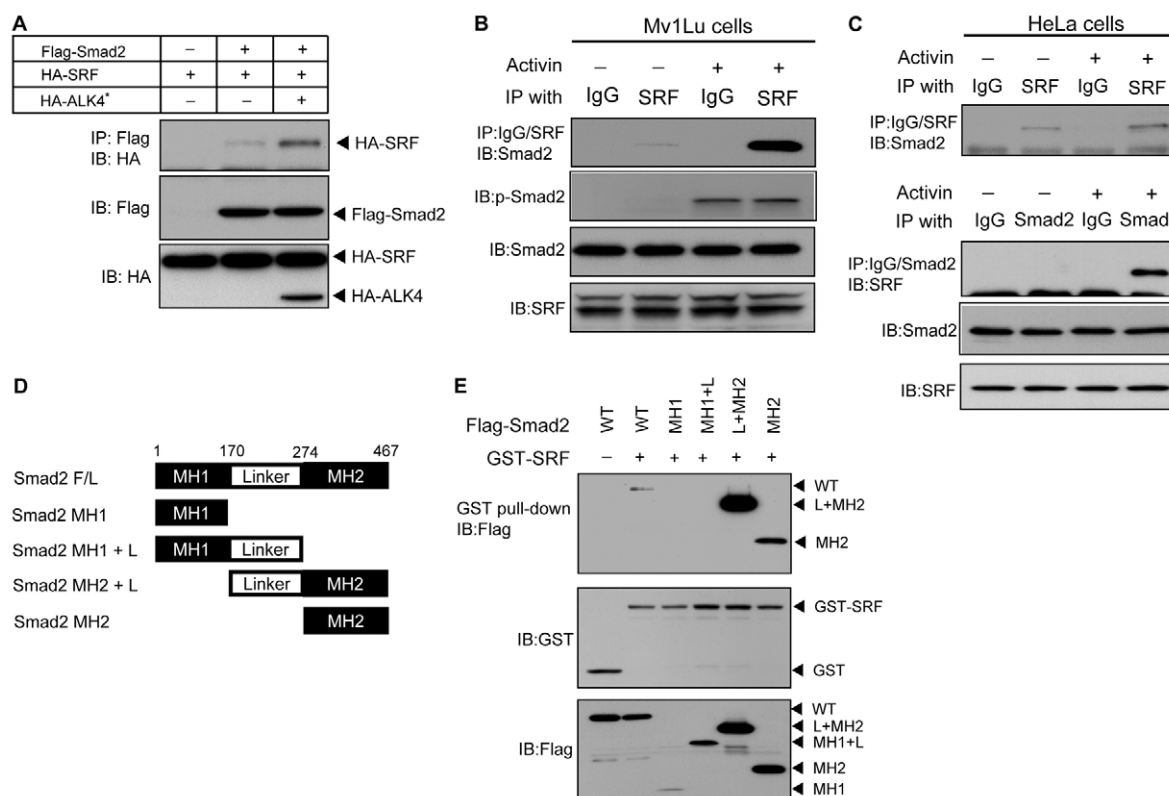


Fig. 5. SRF interacts with Smad2. (A) 293T cells were transfected with HA-tagged SRF and Flag-tagged Smad2 with or without constitutively active Activin type-I receptor (ALK4*). Cell lysates were immunoprecipitated (IP) with anti-Flag antibody, followed by immunoblotting (IB) with anti-HA antibody to detect Smad-bound SRF. (B,C) Interaction of endogenous SRF and Smad2 was examined in Mv1Lu and HeLa cells. (B) Mv1Lu cells were left untreated or treated with Activin A for 1 hour. Cell lysates were subjected to IP with anti-SRF rabbit polyclonal antibody, followed by IB with an anti-Smad2 mouse monoclonal antibody. (C) HeLa cell lysates were subjected to IP with anti-SRF antibody, followed by IB with anti-Smad2 antibody. This was repeated in reverse order. (D) Schematic of Smad2 truncation mutants. (E) Flag-tagged Smad2 deletion mutants were transfected into 293T cells together with GST-SRF. Cell lysates were pulled down by glutathione-agarose beads and then immunoblotted with anti-Flag antibody.

expression of XSRF could suppress mesodermal cell fates in the marginal zone instead, whereas its knockdown could stimulate them in the animal pole region as demonstrated in our results. Given this activity of XSRF in germ-layer specification, the absence of mesoderm in the homozygous *SRF*^{-/-} mice (Arsenian et al., 1998), which is in contrast to the expansion of mesoderm in SRF-depleted *Xenopus* embryos, may be in part due to the over-induction of endoderm at the expense of mesoderm. In support of this, strong staining for the ectodermal marker Oct-6 and for the endodermal marker HNF-3 β was observed in SRF-mutant mice (Arsenian et al., 1998). Furthermore, the phenotypic difference between frog and mice mutants might stem from the variable completeness of depletion in knockout and MO injection methods. In addition, it has been shown that loss of the ability of *SRF*^{-/-} embryonic stem (ES) cells to differentiate into mesodermal cell fates can be recovered by treatment with external factors or their subcutaneous injection into nude mice (Weinhold et al., 2000). This suggests non-cell-autonomous impairment of *SRF*^{-/-} embryonic cells in mesoderm formation, the nature of which might be partly understood through our proposal that SRF might function as a modulator of the levels of input signals to determine cell fate.

We also found that SRF acts as a Smad2-binding partner to inhibit Activin/Nodal-dependent transcription. Recent evidence points to several mechanisms by which interference with Smad

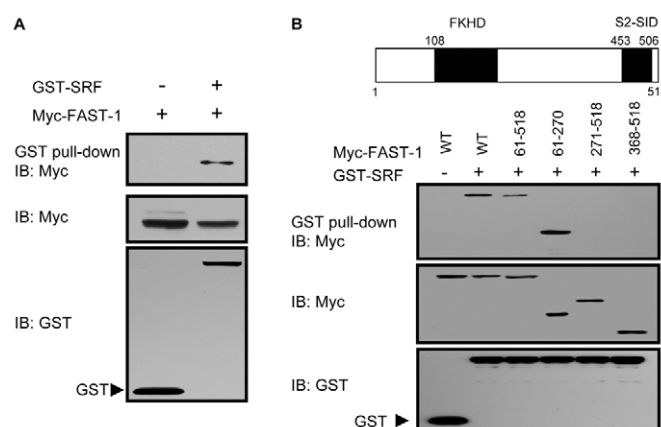


Fig. 6. SRF binds FAST-1. (A) GST-tagged SRF was transfected into 293T cells together with Myc-FAST-1. Cell lysates were pulled down by glutathione-agarose beads and then immunoblotted with anti-Myc antibody. (B) Schematic representation of the structure of FAST-1. FKHD, Forkhead DNA-binding domain. Myc-tagged FAST-1 deletion mutants were transfected into 293T cells together with GST-SRF. Cell lysates were pulled down by glutathione-agarose beads and then immunoblotted with anti-Myc antibody.

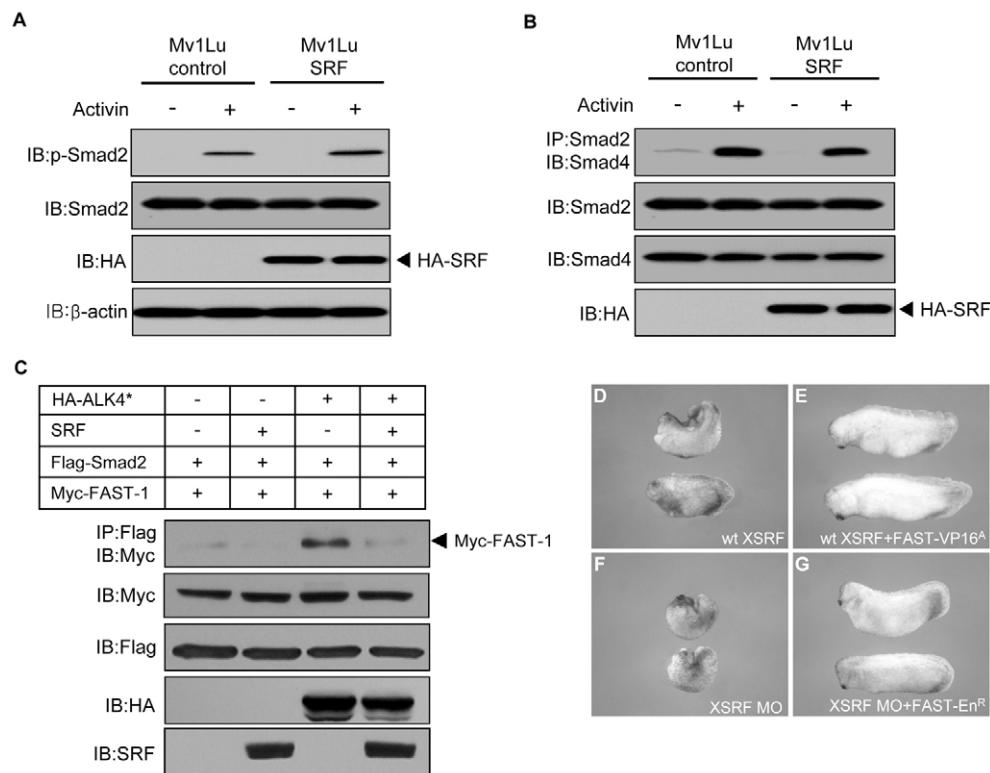


Fig. 7. SRF inhibits Activin-induced formation of FAST-1-Smad2 complex. (A) Stable Mv1Lu cells expressing exogenous SRF and control cells were incubated in the presence or absence of 25 ng/ml Activin A for 1 hour. Smad2 phosphorylation was analyzed by immunoblotting with anti-phospho-Smad2 antibody. Expression of Smad2 and SRF was monitored by immunoblotting with anti-Smad2 and anti-HA antibodies. (B) Stable Mv1Lu cells expressing exogenous SRF and control cells were treated as in A. Cell lysates were immunoprecipitated with anti-Smad2 antibody and then immunoblotted with anti-Smad4 antibody. Expression of Smads and SRF was monitored by immunoblotting with anti-Smad2, anti-Smad4 and anti-HA antibodies. (C) 293T cells were co-transfected with the indicated constructs and harvested 36 hours after transfection. Cell lysates were immunoprecipitated with anti-Flag antibody and then immunoblotted with anti-Myc antibody. Expression of Flag-Smad2, Myc-FAST-1, HA-ALK4* and SRF was monitored as indicated. (D-G) Rescue by FAST-1 mutants of the axial defects caused by gain- or loss-of-function of XSRF. Four-cell stage embryos were injected dorsally with a combination of the indicated reagents (2 ng wt XSRF; 20 pg FAST-VP16^A; 60 ng XSRF MO; 2 pg FAST-En^R) and cultured to stage 31.

transcriptional complexes negatively regulates TGF- β signaling. For instance, the oncoprotein Ski, a transcriptional co-repressor, competes with R-Smads for association with Smad4, disrupting the formation of a functional complex between Smad4 and R-Smads (Wu et al., 2002). Moreover, Ski could also repress Smads directly by recruiting the transcriptional repressor N-CoR as well as the histone deacetylase complex (HDAC) (Liu et al., 2001). DRAP1 interacts with FAST-1, thereby preventing FAST-Smad2-Smad4 complex from binding to its cognate DNA targets (Iratni et al., 2002). In addition, inhibitory Smads (Smad6 and Smad7) compete with R-Smads for binding to activated type-I receptors and thus inhibit the phosphorylation of R-Smads (Shi and Massague, 2003; ten Dijke and Hill, 2004). Our data show that SRF precludes the association of Smad2 and FAST-1 induced by Activin signal (Fig. 7). This suggests that SRF could function to impede Smad2-FAST complex-mediated transcription in Activin/Nodal signaling. Supporting this, gain-of-function phenotypes of SRF are similar to those of maternal FAST-depleted embryos (Kofron et al., 2004) and can be rescued by coexpression of an activated mutant of FAST-1 (FAST-VP16^A) (Fig. 7). We cannot, however, exclude the possibility that SRF could also affect FAST-independent transcription as FoxH1 depletion in animal caps has no effect on the induction of Nodal target genes in response to Xnr1 or Activin

ligands (Kofron et al., 2004), whereas overexpression of XSRF significantly inhibits the same response (Fig. 2). Given that Smad2 binds to SRF via its MH2 domain, which associates with the general transcriptional co-activators p300 and CAATT-binding factor (CBF) (Pouppnot et al., 1998; Shi and Massague, 2003), SRF may repress the Smad2-mediated transcription of various genes in a cell context-dependent manner by preventing the interaction of the MH2 domain and these co-activators. By contrast, a recent study shows that SRF associates with Smad3 and activates TGF- β 1-dependent transcription during myofibroblast differentiation (Qiu et al., 2003). On the other hand, the general mechanism by which SRF regulates gene transcription is known to involve cooperation with the ternary complex factors (TCF), which are phosphorylated and activated by MAP kinase cascades (Chai and Tarnawski, 2002). In *Xenopus*, SRF was shown, together with the TCF-type Ets protein Elk-1, to regulate the transcription of *Xegr-1*, an organizer-specific gene, downstream of the FGF-initiated MAP kinase pathway (Panitz et al., 1998). Interestingly, TGF- β receptors can activate MAP kinase signaling pathways (Derynck and Zhang, 2003). These activated MAP kinase cascades inhibit or enhance Smad activity by phosphorylating it, depending on the cell signaling context; but, in some cases, they regulate Smad-independent transcription. It will be interesting to examine

whether the TCF- and the Smad-dependent SRF regulation of gene transcription involve distinct signaling cascades or whether both of them could be controlled via MAPK pathways by TGF- β signaling. In addition, it remains to be investigated in more detail how SRF could regulate gene expression in a positive or negative fashion depending on its transcription-factor binding-partners.

In summary, we have found that SRF functions to ensure proper germ-layer specification by inhibiting Activin/Nodal signaling in *Xenopus* early development. SRF may dictate which genes are induced in response to this signaling by regulating the formation of specific complexes between Smad and transcription factors. It will be interesting to examine how the expression and activity of SRF are controlled during germ-layer formation.

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