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Smad10 Is Required for Formation of the Frog Nervous System

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Summary

Before the nervous system establishes its complex array of cell types and connections, multipotent cells are instructed to adopt a neural fate and an anterior-posterior pattern is established. In this report, we show that Smad10, a member of the Smad family of intracellular transducers of TGFβ signaling, is required for formation of the nervous system. In addition, two types of molecules proposed as key to neural induction and patterning, bone morphogenetic protein (BMP) antagonists and fibroblast growth factor (FGF), require Smad10 for these activities. These data suggest that Smad10 may be a central mediator of the development of the frog nervous system.

Introduction

Over 75 years ago, Spemann and Mangold first defined neural induction in the amphibian embryo (Hamburger, 1988; Spemann, 1938). They demonstrated that a small piece of dorsal mesoderm, transplanted to the ventral side of a host embryo, instructs host epidermal tissues to change fate and form a complete nervous system (Harland and Gerhart, 1997; Sasai and De Robertis, 1997; Spemann, 1938). In addition, the transplant repatterns ventral tissues to more dorsal fates such as heart and kidney (Harland and Gerhart, 1997). The region responsible for these activities is termed the Spemann organizer and its homolog in the mouse and chick is termed the node (Harland and Gerhart, 1997; Sasai and De Robertis, 1997).

In the last decade, a host of neural-inducing, organizer-secreted molecules was uncovered. One group of molecules includes noggin, chordin, follistatin, Xnr3, Cerberus, and Gremlin, all of which induce anterior neural tissue by blocking epidermal-inducing BMP signals (Bouwmeester et al., 1996; Hansen et al., 1997; Harland and Gerhart, 1997; Hemmati-Brivanlou et al., 1994; Hemmati-Brivanlou and Melton, 1997; Hsu et al., 1998; Lamb et al., 1993; Piccolo et al., 1996; Sasai et al., 1995; Sasai and De Robertis, 1997; Zimmerman et al., 1996). Such BMP antagonism is required for normal forebrain development, as revealed by compound mutant mice with disruptions in both noggin and chordin (Bachiller et al., 2000). Recent evidence also points to a key role for FGF, which signals via a receptor tyrosine kinase (RTK) cascade (Chevet et al., 1999; Demo et al., 1994; Ribisi et al., 2000). In *Xenopus*, FGF is secreted by the organizer, induces neural tissue of posterior character, and is required for formation of the spinal cord (Cox and Hemmati-Brivanlou, 1995; Holowacz and Sokol, 1999; Kengaku and Okamato, 1995; Lamb and Harland, 1995; Ribisi et al., 2000). In chicks, FGF is required for neural induction (Streit et al., 2000).

Smads transduce TGFβ superfamily (including BMP) signals and can function as transcription factors (Piek et al., 1999). The Smad family can be divided into three categories: receptor-regulated Smads (R-Smads; Smad1, Smad2, Smad3, Smad5, and Smad8), common Smads (co-Smads; Smad4), and antagonistic Smads (Smad6 and Smad7) (Heldin et al., 1997). R-Smads function downstream of specific subsets of TGFβ ligand-receptor complexes to transduce their signal into the nucleus. This process is thought to depend on phosphorylation of R-Smads on a cluster of serines at their carboxyl terminus, by a TGFβ superfamily serine kinase receptor complex (Derynck and Zhang, 1996; Heldin et al., 1997; Hill, 1999; Massague, 1996; Piek et al., 1999; Wrana and Attisano, 1996; Zhang et al., 1996). After phosphorylation, the Smads dissociate from the receptors and translocate from the cytosol to the nucleus where they bind DNA and regulate gene expression. The co-Smad, Smad4, associates with R-Smads and is not thought to be restricted to any specific TGFβ signaling pathway. Smad10, also termed XSmad4β, is a recently described Smad that is most structurally similar to Smad4 (∼65% identity). Several groups have characterized Smad10 (XSmad4β) through gain-of-function studies (Howell et al., 1999; LeSueur and Graff, 1999; Masuyama et al., 1999). Although the studies were all done in *Xenopus*, different developmental roles were proposed for Smad10 including formation of anterior and posterior neural tissue, induction of mesoderm, and patterning of mesoderm. In part, the different results may be due to differences in experimental approaches and to the potential artifacts inherent in sufficiency tests. To determine the endogenous roles of Smad10, we undertook loss-of-function approaches.

Results

Smad10 and Smad4 Are Distinct

First, we attempted to determine whether the two related Smads (4 and 10) have distinct functional properties and we tried to identify domains or interacting partners that might contribute to any potential differences in activity. Animal cap cells are normally fated to become epidermal tissue but can be converted to endoderm, different types of mesoderm, or neural tissue, depending upon which signal is transduced. To assess Smad4 and Smad10 function, we microinjected mRNA encoding them and analyzed the resultant animal cap explants for expression of molecular markers (Figure 1A). Smad10 did not induce mesodermal markers but did induce the expression of NCAM, a marker of neural tissue. In contrast,

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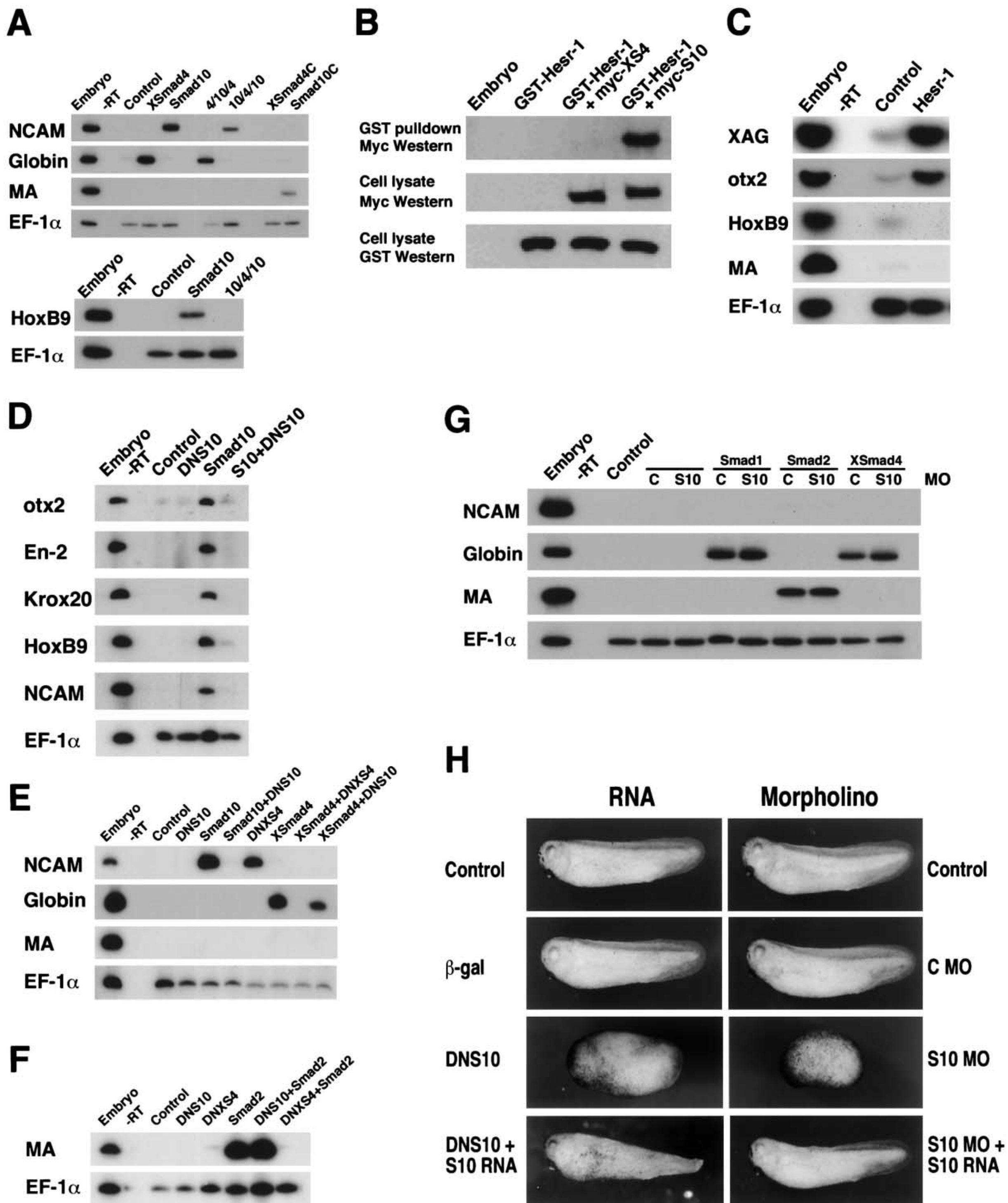

Figure 1. Smad10 and Smad4 Are Distinct

(A) Synthetic mRNA encoding Smad4, Smad10, Smad4/10/4, Smad10/4/10, Smad4-C, and Smad10-C (4 ng) was injected into one-cell embryos, and animal caps were analyzed for expression of molecular markers by RT-PCR. The lane marked Embryo contains total RNA from whole embryos; −RT is identical to Embryo except that reverse transcriptase (RT) was omitted; EF-1α, a ubiquitously expressed message, is a loading control (Krieg et al., 1989).

(B) mRNA encoding GST-Hesr-1 (6 ng), myc-Smad10 (1 ng), and myc-Smad4 (1 ng) were injected into one-cell embryos and a GST pull-down assay was performed.

(C) Animal caps expressing Hesr-1 (1 ng) were evaluated by RT-PCR.

(D) Synthetic mRNA encoding DNS10 (4 ng) or Smad10 (S10, 4 ng) was injected into one-cell embryos alone or together and animal caps were analyzed by RT-PCR.

Smad4 induced the expression of the ventral mesodermal marker globin, but not muscle actin, a marker of dorsal mesoderm, nor NCAM. Next, we determined whether these marked functional differences were also detectable in the carboxyl MH2 region, which contains the activation domain. Of note, when this domain is expressed for the R-Smads, all induce expression of muscle actin, most losing wild-type specificity (Fortuno et al., 2001). Smad10C also induced muscle actin expression; however, Smad4C did not (Figure 1A).

We extended the structural comparison through chimeric analyses. Smads are thought to contain three distinct regions, the amino MH1 domain, the middle linker domain, and the carboxyl MH2 domain; so, we made chimeras swapping all three domains between Smad10 and Smad4 (Figure 1A). Smad4/10/4 was active and induced globin expression similar to wild-type Smad4. The Smad10/4/10 chimera was also active, and like Smad10, it induced NCAM. No other chimera reproducibly induced gene expression; however, Smad4/4/10 and Smad4/10/10 displayed morphological changes. These data suggested that the specificity was contained in the MH1 and MH2 domains. However, unlike wild-type Smad10, Smad10/4/10 caps had prominent cement glands, which indicate anterior neural fates (not shown). Consistent with that, wild-type Smad10 induced the posterior spinal cord marker HoxB9, while Smad10/4/10 did not (Figure 1A). In sum, these data support the notion that the differences of primary structure between Smad4 and Smad10 account for their functional uniqueness and suggest that, in addition to the MH2 domain, the linker and MH1 domains might contain specificity.

One possible explanation for the different transcriptional responses is that Smad4 and Smad10 associate with distinct subsets of transcription factors. No transcription factors had yet been identified that bound to Smad10. So, we performed a yeast two-hybrid screen with Smad10 as bait. One of four positives was Hesr-1 (for hairy and enhancer of split related-1), a member of a family of transcription factors that, in some organisms, controls cell fate decisions including neural development (Fisher and Caudy, 1998; Kokubo et al., 1999). In GST pull-downs, Smad10, but not Smad4, bound Hesr-1 (Figure 1B), although at higher doses the specificity was reduced. In the animal cap assay, Hesr-1 induced the expression of the anterior neural markers XAG and otx2, but not the posterior neural marker HoxB9 or the mesodermal marker M. actin (Figure 1C; Bradley et al., 1993; Hemmati-Brivanlou et al., 1991; Lamb and Harland, 1995; Wright et al., 1990). Thus, Smad10 may act in concert with Hesr-1 to form anterior neural tissue.

Smad1, Smad2, and Smad4 Do Not Require Smad10 for Function

To determine the endogenous role of Smad10 in *Xenopus*, we turned to two distinct tests of necessity: dominant inhibitory forms of Smad10 and morpholino oligonucleotides to block Smad10 translation. In one approach, we attempted to generate an inhibitory form of Smad10 (DNS10) and fused a nuclear localization signal and the engrailed transcriptional repressor domain (EnR) to the MH1 domain and linker of Smad10 (Jaynes and O'Farrell, 1991; Piek et al., 1999). To determine whether DNS10 would inhibit Smad10 activity, we assessed its function in the animal cap assay. In this assay, Smad10 induced the expression of the general neural marker NCAM as well as markers of the forebrain (otx2), midbrain/hindbrain junction (En-2), hindbrain (krox20), and spinal cord (HoxB9) (Bradley et al., 1993; Hemmati-Brivanlou et al., 1991; Lamb and Harland, 1995; Wright et al., 1990; Figure 1D). In contrast, when expressed alone, DNS10 had no detectable activity (Figure 1D). Yet, DNS10 diminished the ability of Smad10 to induce the expression of neural markers (Figure 1D), indicating that DNS10 might block Smad10 activity in whole embryos. Of note, constructs of Smad10 that were identical to DNS10, except for the EnR domain, did not block Smad10 activity (not shown), suggesting that DNS10 might function by blocking Smad10-dependent transcription.

To further address specificity, we examined whether DNS10 would alter the activity of other Smads. We also generated an equivalent construct with *Xenopus* Smad4α (DNXS4) as a specificity control. As Smad4 is a common mediator of Smad activity, DNXS4 should block the function of many Smads. As expected of a molecule that should block Smad1 and, thus, BMP activity, DNXS4 induced NCAM expression (Figure 1E). This is quite distinct from DNS10, which blocked NCAM expression (Figure 1D).

Next, we analyzed the activity of XSmad4 in the presence of either DNS10 or DNXS4. XSmad4 induced the ventral mesodermal marker globin; DNXS4 eliminated this induction, but DNS10 did not (Figure 1E). DNS10 did diminish globin expression, possibly secondary to effects on the Smad complex or nonspecifically via increased RNA concentration. We also tested whether DNS10 or DNXS4 would alter Smad2 function. As described, Smad2 induced muscle actin expression (Graff et al., 1996). While DNXS4 blocked this activity, DNS10 had no effect (Figure 1F). Of note, when the DNXS4- or DNS10-injected embryos were allowed to develop without any further manipulations, they displayed markedly different phenotypes. The phenotype of the DNS10

(E) Animal caps expressing DNS10 (4 ng), Smad10 (4 ng), DNS10 with Smad10, DNXS4 (500 pg), XSmad4 (4 ng), XSmad4 with DNXS4, and XSmad4 with DNS10 were analyzed by RT-PCR.

(F) DNS10 (4 ng), DNXS4 (500 pg), Smad2 (1 ng), Smad2 with DNS10, or Smad2 with DNXS4 were injected for the animal cap assay and analyzed by RT-PCR.

(G) Animal caps expressing Smad1 (4 ng), Smad2 (1 ng), or Smad4 (4 ng) with or without Smad10 MO (5 ng) were assessed by RT-PCR.

(H) Two-cell embryos were injected into the animal pole of both blastomeres with β-galactosidase mRNA (β-gal, 4 ng/cell), DNS10 mRNA (4 ng/cell), control MO (C MO, 5 ng/cell), or with morpholino directed against Smad10 (S10 MO, 5 ng/cell). For the rescue experiments, DNS10 mRNA (4 ng/cell) or Smad10 MO (S10 MO, 35 ng/cell) was coinjected with Smad10 mRNA. The phenotypes were highly penetrant ($>$80%) in multiple experiments (n $>$ 5).

embryos will be described in detail below. The DNXS4-injections produced animals that resembled "bubble embryos" (not shown), indicative of a lack of mesoderm (Hemmati-Brivanlou and Melton, 1992), which supports the idea that Smad4 is the key co-Smad for mesoderm induction.

Given the potential problems with dominant inhibitory constructs, we also attempted a separate and distinct approach to study loss of Smad10 function in embryos. To that end, we synthesized an antisense, morpholino-modified oligonucleotide (morpholinos or MO) targeted to Smad10 (Smad10 MO). Morpholinos block translation with high specificity; this has been demonstrated in vitro, in tissue culture, in zebrafish, and in *Xenopus* (Heasman et al., 2000; Nasevicius and Ekker, 2000; Summerton, 1999). In both *Xenopus* and zebrafish, morpholinos have recapitulated known loss-of-function and mutant phenotypes (Heasman et al., 2000; Nasevicius and Ekker, 2000). Smad10 had been proposed to function in a complex with Smad1, Smad2, and Smad4. However, Smad10 MO, which is active (see below), did not inhibit the ability of Smad1, Smad2, or Smad4 to induce mesoderm in the animal cap assay (Figure 1G). Taken together, these data suggest that we have two approaches, DNS10 and Smad10 MO, to specifically examine the endogenous role of Smad10.

Smad10 Is Required for Normal Development

To assess the endogenous role of Smad10, we inhibited its function by injecting either Smad10 MO or mRNA encoding DNS10 into *Xenopus* embryos and allowed them to develop without further perturbations. As controls, we injected β-galactosidase mRNA (β-gal), a nonspecific morpholino designed by Gene Tools (control MO), and a morpholino targeted to casein kinase I epsilon (CKI MO), a component of the Wnt pathway (McKay et al., 2001; Peters et al., 1999). β-gal and control MO had no visible effect on embryogenesis (Figure 1H). Embryos injected with CKI MO were shorter than and bent compared to control embryos, but appeared otherwise normal (not shown), consistent with a blockade of Wnt signaling (McKay et al., 2001; Sokol, 1996). Of note, both DNS10 and Smad10 MO produced a similar and distinct phenotype in which the embryos were shorter and rounder than controls and appeared to lack normal head structures (Figure 1H). To determine whether DNS10 and Smad10 MO were specifically blocking Smad10, we performed rescue experiments with Smad10 mRNA (Heasman et al., 2000; Hudson et al., 1997; Nasevicius and Ekker, 2000). Of note, Smad10 mRNA, which lacks the 5′ UTR to which most of the morpholino was designed, rescued both the Smad10 MO-dependent and the DNS10 mRNA-dependent phenotypes (Figure 1H).

Xenopus embryos exhibit a reflex movement when touched (Nieuwkoop and Faber, 1967). Although greater than 97% of uninjected and control MO-injected embryos had a normal reflex, only 17% of Smad10 MO-injected embryos exhibited a reflex. Again, Smad10 mRNA rescued the Smad10 MO phenotype, restoring reflex to 93% of embryos. Results with β-galactosidase (no change in % with reflex) and DNS10 mRNA ($<$20% with reflex) were similar to those obtained with the morpholinos, which is consistent with the idea that the effects are specific.

Smad10 Is Required for Formation of the Nervous System

To further study the phenotype produced by loss of Smad10 activity, we analyzed histological sections and found that the notochord, somites (mesodermal derivatives), and the neural tube were normal in control MO-injected embryos (Figure 2A). We observed similar results with β-galactosidase-injected embryos (not shown). Strikingly, while the notochord and somites were still present, the neural tube was eliminated in Smad10 MO- and DNS10-injected embryos (Figure 2A). These data suggest that Smad10 is required for formation of the neural tube but not mesodermal structures. These results were confirmed by in situ hybridizations (Figure 2B) and RT-PCR (Figure 2C) for NRP-1 or NCAM (general neural markers; Lamb and Harland, 1995) and muscle actin (dorsal mesoderm; Mohun et al., 1984). We also coinjected the Smad10 morpholino with Smad10 and found that Smad10 rescued neural induction in Smad10 MO embryos (Figure 2C).

Next, we examined regionally specific neural markers. In situ hybridizations revealed that decreasing Smad10 activity eliminated forebrain (otx2), midbrain/hindbrain (En-2), and spinal cord (HoxB9) formation (Figure 3A). We also noted that melanocytes, a neural crest derivative (Anderson, 1997; Nieuwkoop and Faber, 1967), were absent in the majority of Smad10 MO-injected embryos (Figure 3B). Five percent (2 of 37) of Smad10 MO embryos had melanocytes, compared to 91% (102 of 112) of control MO embryos. Thus, Smad10 activity is required for formation of the nervous system, and at least some of the neural crest.

To further characterize the status of paraxial mesoderm and early neural tissue in embryos with reduced Smad10 activity, we performed whole-mount in situ hybridization on early neurula embryos (Figure 3C). In embryos injected with Smad10 MO, the domain of expression of MyoD, a marker of somites (Hopwood et al., 1989), was slightly reduced when compared with control, uninjected embryos. We also examined the expression of X-ngnr-1b, a proneural gene (Ma et al., 1996), and found that it was undetectable in Smad10 MO embryos (data not shown), just like the late neural markers (Figures 2 and 3). Next, we analyzed the expression of Sox-2, an early, pan-neural marker (Mizuseki et al., 1998). Sox-2 levels were significantly reduced in Smad10 MO embryos compared to controls. The concomitant decrease in the size of both the early somitic mesoderm and neural plate is consistent with the recent report from Harland's group (Mariani et al., 2001). Of note, Sox-2 is the first example of a gene that was expressed in control neural tissue and was still detectable in Smad10-depleted embryos. One possible explanation was that the initial phase of Sox-2 expression is autonomous of the organizer. To evaluate that notion, we examined the expression of Sox-2 in ectodermal explants that were isolated at stage 8.5, prior to formation of the organizer. We found that Sox-2 is present in this tissue, which is destined to an epidermal fate, at stages 8.5, 12.5, and 15 (Figure 3D).

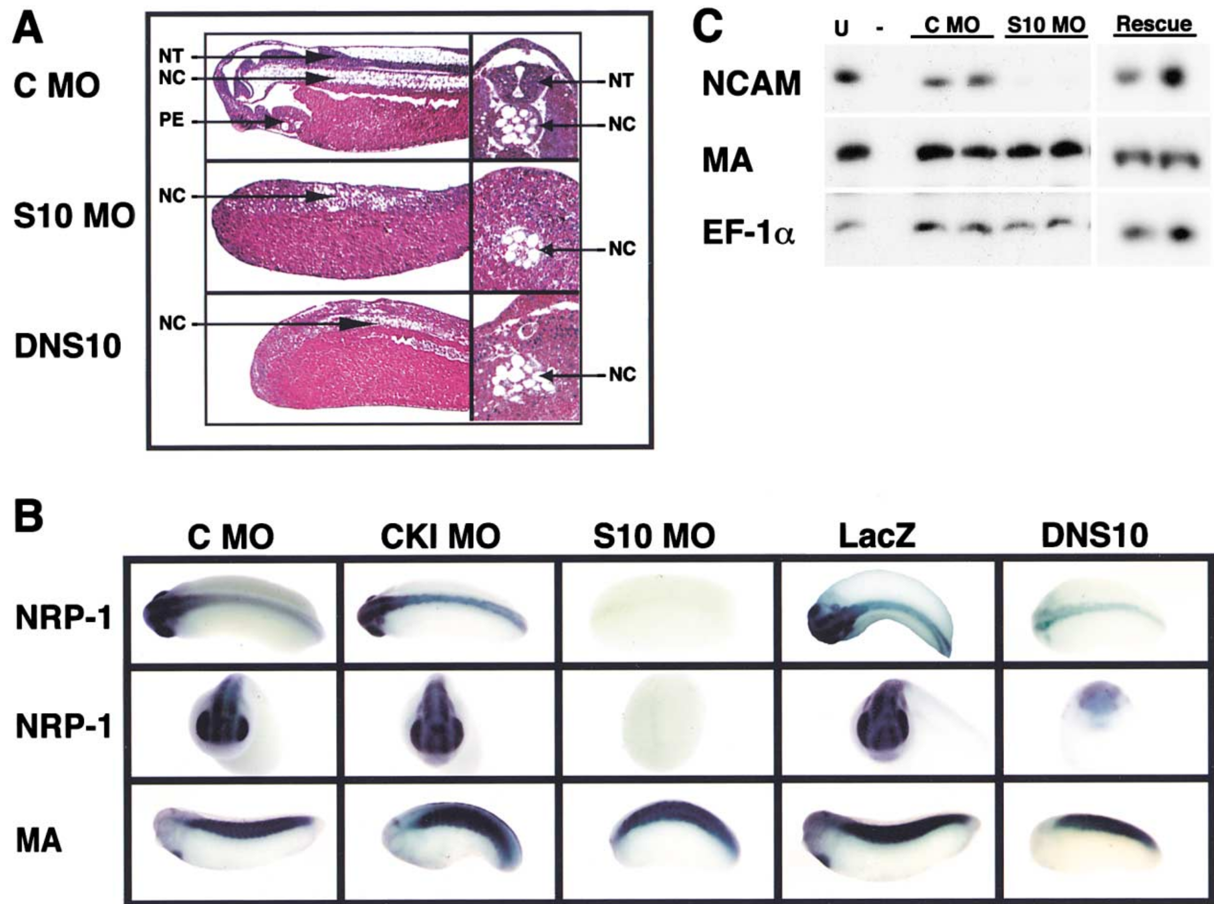

Figure 2. Smad10 Is Required for Formation of the Nervous System

(A) Control MO (C MO, 5 ng/cell), Smad10 MO (S10 MO, 5 ng/cell), or DNS10 mRNA (4 ng/cell) were injected into the animal pole of both blastomeres of two-cell embryos. At stage 35, embryos were processed for histology and hematoxylin and eosin staining. NC, notochord; NT, neural tube; PE, pharyngeal endoderm.

(B) Two-cell embryos were injected with 5 ng of morpholino or 4 ng of mRNA into the animal pole of each blastomere and then analyzed by whole-mount in situ hybridization for expression of the general neural marker NRP-1 (Knecht et al., 1995) and the mesodermal marker muscle actin (MA; Mohun et al., 1984).

(C) Animal caps from embryos injected with control MO, Smad10 MO, and Smad10 MO with Smad10 mRNA (1 ng/cell, rescue) were analyzed by RT-PCR. U, uninjected; −, no RT. The phenotypes were highly penetrant (>80%) in multiple experiments (n > 5).

To determine the fate of those cells that normally would become neural, we injected morpholinos (control or Smad10), and then at the 32-cell stage, injected β-galactosidase mRNA as a lineage tracer into the B1 cells, whose descendents most frequently populate the nervous system (Dale and Slack, 1987). We allowed the embryos to develop and examined the location of the β-galactosidase-expressing cells. Seventy-six percent of control MO embryos expressed β-galactosidase in the nervous system (48 of 63; Figure 3E). In Smad10-depleted embryos, the descendants of the B1 cells were primarily (>75%) found in the outer surface of the embryo (Figure 3E). Thus, it appears that in the absence of Smad10 activity, cells normally predisposed to become neural might adopt an epidermal fate. However, a previous study reducing Sox-2 activity found cells fated to become neural were in a similar location to those lacking Smad10, yet they did not appear to be epidermal cells (Kishi et al., 2000).

Smad10 Is Required for Cardiogenesis and Nephrogenesis

The organizer is important not only in formation of the nervous system, but is also required for heart and kidney development (Harland and Gerhart, 1997). Sufficiency tests had revealed that Smad10 mimics multiple Spemann organizer functions, including neural induction and dorsalization (LeSueur and Graff, 1999). As necessity tests had shown the importance of Smad10 in neural development, Smad10 might also be important in other organizer activities. In support of that, the in situ hybridizations with muscle actin showed that, while the Smad10 morpholino did not affect formation of somitic muscle, it appeared to block heart development (Figures 2B and 4A), which requires the organizer (Harland and Gerhart, 1997). Next, we analyzed the expression of NKX2.5, a marker of heart specification (Tonissen et al., 1994), in morpholino-injected embryos. While control MO had no effect, Smad10 MO reduced the expression

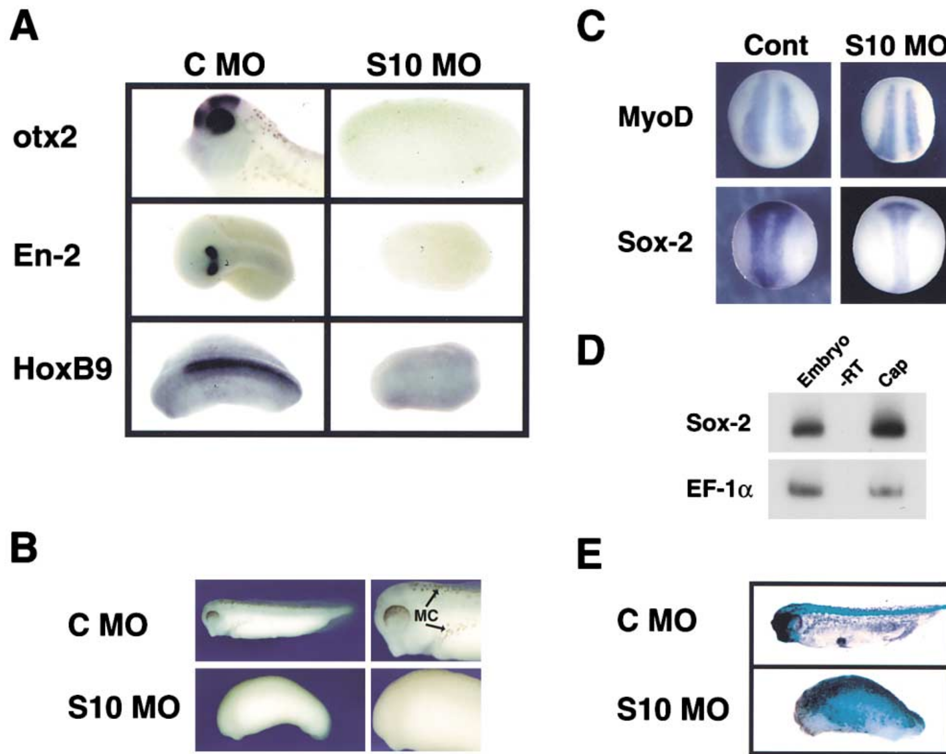

Figure 3. Smad10 Is Required for Expression of Anterior and Posterior Neural Markers and for Formation of Melanocytes

(A) Embryos were injected and analyzed by in situ hybridization as in Figure 2B.

(B) Albino embryos (albino mother, pigmented father) were examined for the appearance of melanocytes (MC; dark cells visible in the C MO embryo).

(C) Embryos were injected and analyzed at early neurula by in situ hybridization as in Figure 2B, except that 12.5 ng of morpholino was injected per cell.

(D) Animal caps were explanted at stage 8.5, prior to organizer signaling, and Sox-2 expression was analyzed by RT-PCR.

(E) Two-cell embryos were injected with morpholinos and, at the 32-cell stage, β-galactosidase mRNA was injected into the B1 blastomere. At stage 27, the embryos were stained with X-gal (blue).

of NKX2.5 (Figure 4B). We also examined nephrogenesis through analysis of WT-1, a kidney-specific marker (Carroll and Vize, 1996), and found that Smad10 MO greatly inhibited WT-1 expression (Figure 4B). Thus, Smad10 seems to be required for heart and kidney formation.

The necessity of Smad10 for multiple organizer-dependent activities raised the possibility that Smad10 might be required for formation of the organizer. However, in situ hybridization (Figures 4C) and RT-PCR (Figure 4D) results showed that Smad10 MO had no effect on expression of the organizer-specific markers noggin and goosecoid, or a more general mesodermal marker, brachyury. Likewise, the formation of the dorsal blastopore lip, the site of organizer invagination (Harland and Gerhart, 1997), appeared temporally and spatially normal in S10 MO embryos (Figures 4C and 4E). In addition, in situ hybridization (Figure 4E) and RT-PCR (Figure 4F) showed no effect of the S10 MO on the expression of markers of anterior/pharyngeal endoderm, Xhex and Cerberus (Zorn et al., 1999), or markers of general endoderm, Xsox17 (Hudson et al., 1997) and endodermin.

Neural Induction by FGF Requires Smad10

Two separate classes of organizer-derived secreted molecules—FGFs and BMP antagonists—are thought to be key to neural induction and anterior-posterior patterning. In *Xenopus*, FGFs induce posterior neural tissue, and FGFs are required for neural induction in the chick and spinal cord formation in the frog (Cox and Hemmati-Brivanlou, 1995; Holowacz and Sokol, 1999; Kengaku and Okamato, 1995; Lamb and Harland, 1995; Ribisi et al., 2000; Streit et al., 2000). As Smad10 is necessary for formation of neural structures, including the spinal cord (Figures 2 and 3), it followed that FGF might require Smad10 for its neuralizing properties. To test this notion, we injected embryos with control MO, Smad10 MO, DNS10 mRNA, or β-galactosidase mRNA and explanted animal caps. During gastrulation, we cultured the caps in the presence of FGF under conditions that induce formation of neural tissue (Lamb and Harland, 1995) and then analyzed the caps for expression of neural markers. In situ hybridizations showed that FGF induced expression of the spinal cord marker HoxB9 in uninjected animal caps and in animal caps injected with control morpholino or β-galactosidase mRNA (Figures 5A and 5B). Of note, both Smad10 MO and DNS10 blocked induction of HoxB9 by FGF (Figures 5A and 5B). These results were confirmed by RT-PCR analysis (Figure 5C). FGF, when added during blastula

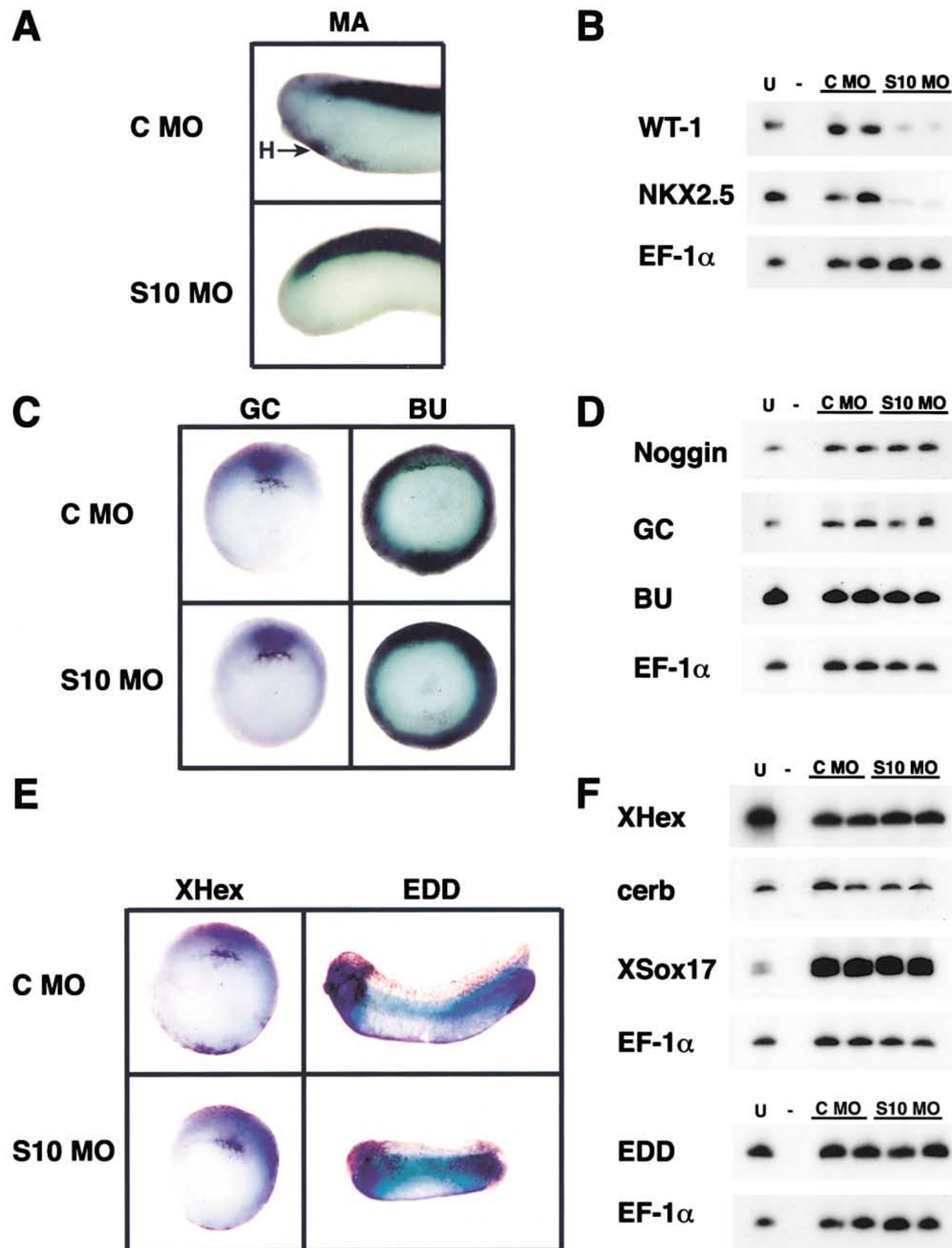

Figure 4. Smad10 Is Required for Formation of Organizer-Induced Tissues

(A) Embryos were injected and analyzed by in situ hybridization as in Figure 2B. H, heart.

(B) Two-cell embryos were injected with 5 ng morpholino into both animal poles and analyzed by RT-PCR at stage 33. WT-1 marks the kidney; NKX2.5 marks the heart.

(C and D) Both animal poles of two-cell embryos were injected with morpholino and at stage 10.25 the embryos were processed for in situ hybridizations (C) or RT-PCR (D). GC, goosecoid; BU, brachyury.

(E and F) Embryos were injected as in (C) and analyzed by in situ hybridization (E) with probes for XHex (stage 10.25) and endodermin (EDD, stage 27) or analyzed by RT-PCR (F) for the endodermal markers XHex (stage 10.25), Cerberus (cerb, stage 10.25), Xsox17 (both α and β isoforms, stage 10.25), and EDD (stage 27).

stages, induces formation of mesoderm (Lamb and Harland, 1995). Decreasing Smad10 activity did not alter the FGF-dependent induction of mesoderm, while XFD (Amaya et al., 1991), the dominant-negative FGF receptor, did (Figure 5D). Thus, Smad10 does not block all FGF activities, but rather is required specifically for neural induction by FGF.

Neural Induction by Noggin Requires Smad10

Many of the secreted neural inducers identified through gain-of-function studies act by inhibiting BMP signaling (Harland and Gerhart, 1997; Sasai and De Robertis, 1997). Unlike the FGFs, BMP antagonists induce anterior neural tissue. As Smad10 is necessary for anterior as well as posterior neural development, we tested the idea

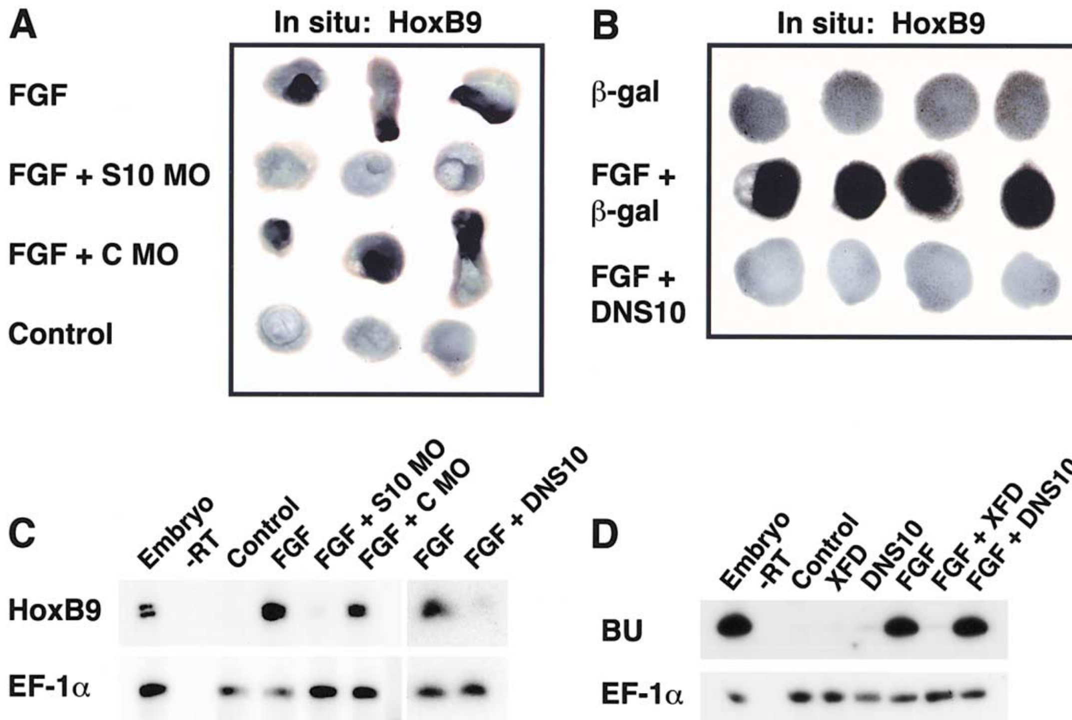

Figure 5. Smad10 Is Required for FGF-Mediated Neural Induction

(A) One-cell embryos were uninjected or injected with Smad10 or control MO and then animal caps were treated with FGF protein at the gastrula stage. These caps, and uninjected/untreated caps (Control), were analyzed at stage 27 by whole-mount in situ hybridization.

(B) β-gal-injected, β-gal-injected/FGF-treated (FGF + β-gal), and DNS10-injected/FGF-treated animal caps were analyzed by whole-mount in situ hybridization.

(C) Animal caps treated as in (A) and (B) were analyzed by RT-PCR.

(D) Uninjected, dominant-negative FGF receptor-injected (XFD, 1 ng), and DNS10-injected animal caps were cultured during the blastula stage in the absence or presence of FGF. These caps were then analyzed by RT-PCR at stage 11.

that Smad10 might be required for neural induction by noggin, a prototypical BMP antagonist. To that end, we injected noggin mRNA alone, with Smad10 MO, or with control MO, and evaluated the expression of neural markers. As assessed by in situ hybridization, noggin induced the expression of NRP-1 (Figure 6A). This expression was not altered by control MOs (Figure 6A). In contrast, Smad10 MO blocked noggin-dependent neural induction (Figure 6A). These results were confirmed by RT-PCR analysis (Figure 6B). Thus, Smad10 activity is necessary for noggin-mediated neural induction.

Smad10 Contains ERK Consensus Phosphorylation Sites, which Are Required for Posterior Neuralization

Smads are central components of TGFβ superfamily signaling and Smads are phosphorylated by TGFβ serine/threonine kinase receptors at their carboxy-terminal domain; this phosphorylation is thought to activate Smads and control their biological function (Derynck and Zhang, 1996; Heldin et al., 1997; Hill, 1999; Massague, 1996; Piek et al., 1999; Wrana and Attisano, 1996; Zhang et al., 1996). Erk kinases also phosphorylate Smads, but in their linker region. This phosphorylation alters the subcellular localization of Smads and often abolishes their activity, although one report suggested the opposite (de Caestecker et al., 1998; Kretzschmar et al., 1997, 1999). Smad10 contains three canonical PX(S/T)P Erk phosphorylation sites within its linker region, which raised the possibility that Smad10 activity might be influenced by a receptor tyrosine kinase pathway (Hill and Treisman, 1995). To test the biological consequences of such potential phosphorylation, we generated a Smad10 construct, Smad10-PXAPx3, that encoded alanine substitutions at each of the three PX(S/T)P phosphorylation sites in Smad10. Then, we injected animal caps with mRNAs encoding wild-type Smad10 and Smad10-PXAPx3 and analyzed neural induction. Both the wild-type and the PXAPx3 mutant induced the expression of NCAM and otx2 (anterior neural; Figure 7A). Although the wild-type Smad10 induced the expression of the spinal cord marker HoxB9, the PXAPx3 mutant did not (Figure 7A). One possible explanation for the lack of HoxB9 expression was that the mutations altered the stability of Smad10. However, the wild-type and PXAPx3 Smad10 protein levels appeared equal at several different stages (not shown). Of note, another form of Smad10 in which the PX(S/T)P Erk phosphorylation sites were mutated to acidic residues activated expression of HoxB9, as well as otx2, like wild-type Smad10.

Although several explanations exist for the loss of spinal cord formation detected when the PX(S/T)P sites were mutated, a parsimonious possibility is that an Erk-dependent phosphorylation of Smad10 was abolished. To explore this option, we performed in vitro phosphorylation assays with recombinant forms of Smad10 and

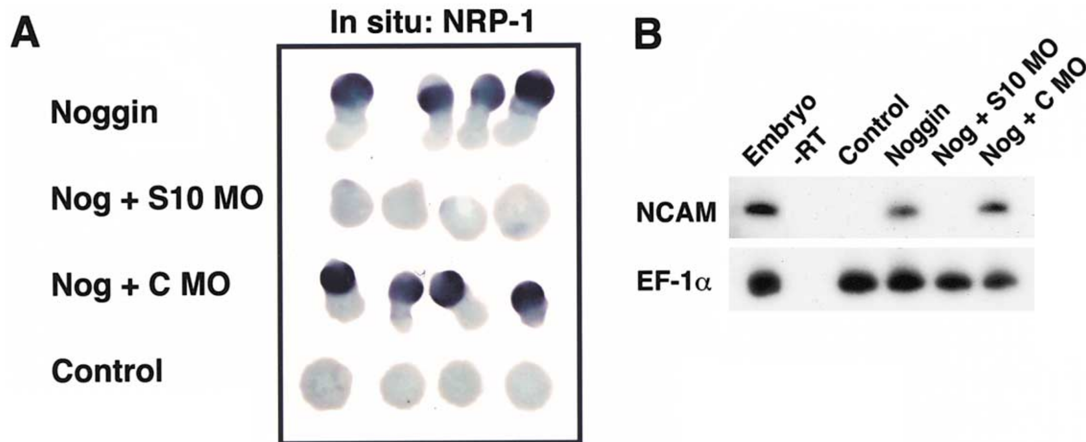

Figure 6. Smad10 Is Required for Noggin-Mediated Neural Induction

(A) One-cell embryos were injected with noggin mRNA (Nog, 250 pg) alone, with Smad10 MO, or with control MO. Animal caps from these as well as uninjected embryos (control) were explanted, cultured, and analyzed by whole-mount in situ hybridization.

(B) Animal caps treated identically to those described in (A) were processed for RT-PCR analysis.

activated Erk2 proteins and found that Erk2 directly phosphorylated Smad10 (Figure 7B). In addition, in vitro phosphorylation assays confirmed that the PXAPx3 mutations diminished Erk phosphorylation of Smad10 (Figure 7C). Thus, phosphorylation of Smad10 at the PX(S/T)P sites might be critical for formation of the spinal cord.

Discussion

Smad10 must be present and able to act for the *Xenopus* nervous system to form. A full range of anterior-posterior neural markers, as well as a neural crest derivative, was lost when Smad10 function was diminished. The observation that Smad10 is required for the neuralizing activity of two important classes of secreted factors (BMP antagonists and FGF) further suggests that Smad10 might act as a key component in the formation of both anterior and posterior neural tissue. Since Smad10 is also required for expression of heart and kidney markers, and formation of these tissues requires organizer activity, these results raise the possibility that Smad10 might be a mediator of several organizer-derived functions. However, the defects in cardiogenesis and nephrogenesis observed when Smad10 activity was reduced might also be secondary to Smad10 functions that are not related to the organizer.

Like many signal transduction cascades, TGFβ signaling is subject to crosstalk regulation by other pathways. For example, the receptor tyrosine kinase (RTK) cascade can modify TGFβ signaling, presumably via Erk-dependent phosphorylation of Smads (de Caestecker et al., 1998; Kretzschmar et al., 1997, 1999; Piek et al., 1999). Typically, this phosphorylation inhibits Smad function by excluding them from the nucleus (Kretzschmar et al., 1999). That is, RTK signaling decreases the amplitude of the TGFβ signal but does not alter its quality. Our data suggest an extension of this idea and imply that an RTK pathway may regulate the function of Smad10 and may do so in a direct biochemical sense. We find that Smad10 contains Erk consensus phosphorylation sites, that Erk2 directly phosphorylates Smad10 in vitro, and that the PX(S/T)P consensus phosphorylation sites on Smad10 are required for this Erk-dependent phosphorylation. Of note, when the Erk consensus sites are mutated to alanine, Smad10 remains functional and generates anterior neural fates; however, the mutant no longer produces posterior neural fates. This suggests that the nonphosphorylated and phosphorylated forms of Smad10 might interact with different subsets of transcription factors to generate distinct cell fates. Erks often phosphorylate and activate transcription factors that regulate gene expression (Blenis, 1993; Egan et al., 1993; Hill and Treisman, 1995). Smad10 may be another example of such a transcription factor. Taken together, these data suggest that an RTK signal, rather than a TGFβ signal, might control Smad10's biological function in anterior versus posterior neural development.

If Smad10 transduces an RTK signal, what ligand might activate the cascade? One plausible candidate is FGF. FGFs signal via an RTK pathway that involves phosphorylation and activation of Erks (Ribisi et al., 2000; Umbhauer et al., 1995). This FGF pathway has similar biological functions to Smad10; both induce posterior neural fates. Furthermore, FGF requires Smad10 for this activity. These data, coupled with the in vitro phosphorylation results and the inability of the Smad10-PXAPx3 mutant to induce spinal cord formation, are consistent with the idea that FGF initiates an RTK pathway that leads to activation of Erk, subsequent phosphorylation of Smad10, and induction of posterior neural fates. Additional biochemical experiments will be required to test this hypothesis.

Smad10 is most similar in primary structure to the co-Smad, Smad4, that associates with R-Smads once the R-Smads are phosphorylated by the ligand-activated receptor complex. Both Hill's and Nishida's groups have presented biochemical evidence that Smad10 can act as a co-Smad and termed Smad10 as Smad4β, to highlight the similarities with Smad4 (Howell et al., 1999; Masuyama et al., 1999). The data presented here are consistent with the idea that Smad10 could be a co-Smad, although our studies do not directly address this

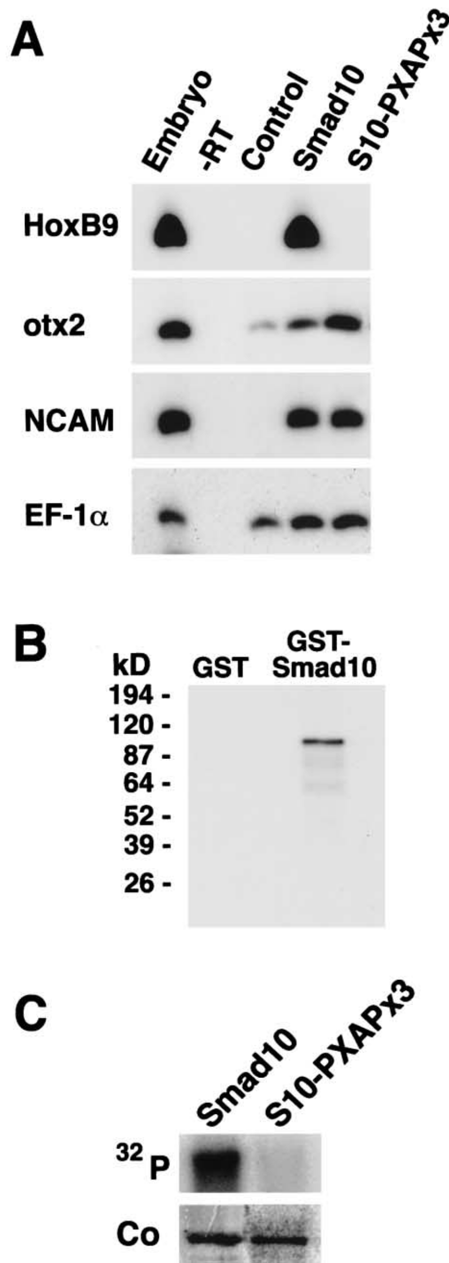

Figure 7. Erk-Mediated Signaling May Regulate Smad10 Activity

(A) Uninjected, Smad10-injected, and Smad10-PXAPx3-injected animal caps were analyzed by RT-PCR.

(B) Purified recombinant GST or GST-Smad10 protein was incubated with purified and activated Erk2 in the presence of [^{32}P]ATP and analyzed with SDS-PAGE and autoradiography.

(C) Recombinant purified His-tag-Smad10 and His-tag-Smad10-PXAPx3 (S10PXAPx3) were tested for phosphorylation by activated Erk in vitro as described in (B). Protein loading was determined by Coomassie (Co) staining.

issue. Many observations suggest that individual potential R-Smad complexes, with either Smad4 or Smad10, might produce different functional consequences. This possibility is supported by the relatively low identity, for Smads, between Smad4 and Smad10 (~65%). Smad1, Smad5, and Smad8 (80%–90% identity) or Smad2 and Smad3 (>90% identity) have a much higher degree of structural conservation. Furthermore, Smad10 and Smad4 have cell biological and biochemical differences, and they display distinct activities in both *Xenopus* embryos and tissue culture cells. These differences support a model in which the two potential co-Smads, 4 and 10, might induce distinct transcriptional responses, even when bound to the identical R-Smad (Howell et al., 1999; Masuyama et al., 1999). These functional differences may, in part, be accounted for by differences in primary structure, which presumably alters the panoply of transcription factors with which the Smads interact. For example, Smad4 induces mesoderm and binds with FAST-1, a transcription factor that is necessary and sufficient for mesoderm formation. In contrast, Smad10 is necessary and sufficient for neural tissue formation and interacts with the neuralizing transcription factor Hesr-1.

Both Hill and Nishida suggested that Smad10 functions in mesoderm formation or patterning (Howell et al., 1999; Masuyama et al., 1999). Although these activities appear distinct from those we have described, the observations were made with different experimental conditions, which may explain the different outcomes. The loss-of-function data presented here support the idea that Smad10 plays a key role in formation of the nervous system and not one in mesoderm induction. Our experiments do not eliminate the possibility that Smad10 also plays a role in mesoderm formation; we targeted the microinjections to neural, and not mesodermal, precursors. In addition, we did not examine ventralization, so we have no data in that regard. Another potential source of complication is the high maternal level of Smad10 mRNA; so, maternal Smad10 protein might also be present. The morpholinos block translation of the Smad10 message and would not affect the maternally contributed Smad10 protein, potentially only decreasing Smad10 protein levels gradually. So early events, such as mesoderm induction, might not be affected, while later processes—neural induction, heart formation, and nephrogenesis—might be significantly altered. The protein inhibitory form of Smad10, DNS10, might overcome this possible limitation. The DNS10 embryos had dramatic perturbations in nervous system formation but mesoderm induction proceeded. However, the DNS10 is less potent than the Smad10 morpholino and might not be able to inhibit mesoderm formation. Another alternative is that Smad4 and Smad10 play redundant roles in mesoderm, but not neural, induction. Further studies, such as with Smad4 morpholinos, should clarify this issue.

The current data indicate that Smad10 is necessary for formation of the frog nervous system and is required by BMP antagonists and FGF signals for neural induction. Many aspects of the molecular mechanisms that control neural development are conserved between vertebrates. BMP antagonists and FGF signaling are thought important in neural formation in frogs, chicks, and mice. Given the requirement of Smad10 in frogs for neural induction by either the BMP inhibitors or FGF and their role in other vertebrates, Smad10 might also be conserved. However, no Smad10 ortholog has yet been identified in chicks or mice. It is possible that Smad10's role is unique to frogs or that this function has been subsumed by other transcription factors such

as Smad4. Of note, functional studies suggest that Smad4 is not the only co-Smad in mice (Sirard et al., 1998). So, it is possible that Smad10 or a related Smad will be discovered in other organisms.

Experimental Procedures

Constructs

For the DNS10 construct, the following elements were fused from amino to carboxyl terminus with SOEing PCR (Horton et al., 1989): a nuclear localization signal (MAPKKKRKVPG), the engrailed repressor domain (EnR, amino acids 1–298; Jaynes and O'Farrell, 1991), and the putative Smad10 DNA binding domain (amino acids 1–365; Piek et al., 1999). DNXS4 was constructed in an identical manner with amino acids 1–319 of XSmad4α substituted for the Smad10 domain. For Smad10-PXAPx3, residues 239 and 253 were changed from serine to alanine (S→A) and residue 289 was changed from threonine to alanine (T→A) by SOEing PCR. The chimeras were generated as described (Fortuno et al., 2001) and were cloned into pCS2+. Both myc-tagged constructs were generated in pCS3+MT. Hesr-1 was identified in a yeast two-hybrid screen as described (Peters et al., 1999), except that Smad10 was the bait. The Hesr-1 open reading frame was cloned into pCS105 for in vitro transcription. To generate the GST-Hesr-1, Hesr-1 was also cloned in-frame into pGEX-4T-3 and then transferred to pCS2+.

Embryological Methods

Plasmids containing constructs for mRNA production were linearized with NotI (Smad10, XSmad4α, myc-Smad10, myc-XSmad4, GST-Hesr-1, β-galactosidase, all the chimeras, Smad10-C, and Smad4-C), AscI (Hesr-1), or SacII (DNS10 and DNS4). mRNA for injection was synthesized as described (Krieg and Melton, 1987). Embryos were obtained, microinjected, cultured, and animal caps were explanted as described (LeSueur and Graff, 1999). RNA extraction and RT-PCR analyses have been described previously (LeSueur and Graff, 1999). Histological sections were processed and stained with hematoxylin and eosin (LeSueur and Graff, 1999). FGF treatment of animal caps was done as described (Lamb et al., 1993; Lamb and Harland, 1995), except that we used a 1:2, rather than a 1:1, mixture of LCMR and CMFM. Caps were treated with 500 ng/ml of recombinant human basic FGF (Promega #G5071). In situ hybridization was performed as described (Knecht et al., 1995; LeSueur and Graff, 1999). For lineage tracing, two-cell embryos were injected with morpholino in both cells. At the 32-cell stage, the B1 blastomere was injected with β-galactosidase mRNA. β-galactosidase expression was assayed with X-gal staining (LeSueur and Graff, 1999). For the GST pull-down assays, one-cell embryos were injected with the indicated mRNAs, blastulae were lysed, and the protein complex was precipitated with glutathione beads and detected by Western blotting as described (Fortuno et al., 2001).

Morpholinos

A standard control morpholino, Smad10 MO (5′-CGCCATCTTTTGC CCTTGTTGTTAC-3′), and CKI MO (5′-TCCCCACTCTCAGCTCCAT GTTTAC-3′) were obtained from Gene Tools, LLC. The Smad10 MO sequence was designed to the 5′ UTR and six bases of the open reading frame. For mRNA rescue of the morpholino effect, synthetic mRNA was made from a plasmid containing only the open reading frame of Smad10 (LeSueur and Graff, 1999) and no UTR, which prevents morpholino targeting (Heasman et al., 2000; Nasevicius and Ekker, 2000; Summerton, 1999). The rescue experiments were done by injecting mRNA and MO together as a mixture.

Phosphorylation Assays

For in vitro phosphorylation assays, purified GST, GST-Smad, or his-tag-Smad fusion proteins were incubated for 15 min at 30°C with 200 ng/ml activated Erk2 (a generous gift from Melanie Cobb) in the presence of [γ-^{32}P]ATP. Reactions were stopped by addition of SDS sample buffer, resolved by SDS-PAGE, and visualized by autoradiography.

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