

Integration of IGF, FGF, and anti-BMP signals via Smad1 phosphorylation in neural induction

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How do very diverse signaling pathways induce neural differentiation in *Xenopus*? Anti-BMP (Chordin), FGF8, and IGF2 signals are integrated in the embryo via the regulation of Smad1 phosphorylation. Neural induction results from the combined inhibition of BMP receptor serine/threonine kinases and activation of receptor tyrosine kinases that signal through MAPK and phosphorylate Smad1 in the linker region, further inhibiting Smad1 transcriptional activity. This hard-wired molecular mechanism at the level of the Smad1 transcription factor may help explain the opposing activities of IGF, FGF, and BMP signals not only in neural induction, but also in other aspects of vertebrate development.

Received September 18, 2003; revised version accepted October 24, 2003.

A central question in biology is how a small number of cell-cell signaling pathways are integrated during development, leading to the differentiation of many cell types. The induction of the central nervous system (CNS) has been a primary focus of embryological research for many years (Spemann 1938; Harland 2000; Wilson and Edlund 2001; Stern 2002). In *Xenopus*, bone morphogenetic protein (BMP) antagonists such as Noggin, Chordin, and Follistatin inhibit signaling by BMP receptors, causing neural induction through a default pathway (Weinstein and Hemmati-Brivanlou 1999; De Robertis et al. 2000). However, active signals such as fibroblast growth factors (FGFs) and insulin-like growth factors (IGFs) are also potent neural inducers (Hongo et al. 1999; Hardcastle et al. 2000; Streit et al. 2000; Wilson et al. 2000; Pera et al. 2001; RichardParpaillon et al. 2002). Here we show that Chordin, FGF8, and IGF2 have similar phenotypic effects on CNS development and synergize with each other. In loss-of-function experiments, FGF and IGF signaling are required for neurogenesis by Chordin. Microinjection of phosphorylation-deficient mutants of Smad1 suggests that antagonism of BMP receptor activity is not the sole regulator of neural induction in the embryo. In *Xenopus*,

FGF8 and IGF2 induce phosphorylation of the linker region of Smad1 via mitogen-activated protein kinase (MAPK), which further inhibits Smad1 activity (Kretschmar et al. 1997). The results suggest a common molecular mechanism for the action of diverse neural inducers in vertebrate development, in which multiple signaling inputs lead to inhibition of the BMP pathway through the regulation of Smad phosphorylation.

Results and Discussion

The unexpected finding that *IGF* mRNA can induce neural differentiation (Pera et al. 2001) prompted us to compare the phenotypic effects of various neural inducers. First we compared the effects of the BMP antagonist Chordin (Chd), IGF2, or FGF8 on neural plate formation. Careful titration of microinjected mRNAs revealed that each agent was able to expand the neural plate (Fig. 1A–D). At lower doses, *Chd*, *IGF2*, and *FGF8* induced ectopic sensory neurons, marked by *N-tubulin*, in the epidermis (Fig. 1E–H). Interestingly, genetic studies in zebrafish have shown that differentiation of these lateral-most sensory neurons is controlled by a gradient of BMP signals in the ectoderm (Nguyen et al. 2000).

To determine whether these signaling pathways synergized in vivo, we microinjected recombinant proteins into the blastocoele cavity of the embryo (Fig. 1I–P; Cooke and Smith 1989). Co-injection of Chd and IGF2 proteins cooperated, leading to dorsalized embryos with large cement glands, shortened axes, and expanded dorso-anterior structures (Fig. 1L). A synergism was also observed between Chd and FGF8, resulting in the induction of abundant ectopic neurons in the epidermis (Fig. 1P). Although IGF2 and FGF8 have similar effects, we noted differences as well. IGF2 induces prominent eye and cement gland structures (Pera et al. 2001), whereas FGF8 induces massive neurogenesis in the absence of eye and cement gland differentiation (Fig. 1I–P; Christen and Slack 1997; Hardcastle et al. 2000). Thus, although the effects on neural induction showed strong parallels, FGF8 and IGF2 appear to regulate cell fate by other mechanisms as well. All three signaling pathways were required for neurogenesis (Fig. 1Q–S), as the differentiation of primary neurons was inhibited by *BMP7* mRNA (which counteracts Chd), by an IGF receptor antisense morpholino oligo (IGFR-MO, Richard-Parpaillon et al. 2002), or by *dominant-negative FGF receptor 4a* mRNA (*DN-FGFR4a*; Hongo et al. 1999).

Taken together, the results suggested that strong parallels existed between the effects of the BMP antagonist Chd, IGF2, and FGF8 in neural induction (Fig. 1T). In gain-of-function experiments, similar phenotypes were obtained with the three agents, and the microinjected proteins synergized. In loss-of-function studies, the IGF and FGF pathways were found to be required for neurogenesis. Remarkably, neural induction by *Chd* mRNA in the well established animal cap explant assay also required intact IGF and FGF signaling pathways (Fig. 1U, lanes 3–6). Although Chordin is a very potent neural inducer, it does not work in the absence of IGF or FGF signaling. These intriguing relationships between such different neural inducing pathways prompted us to investigate whether a common molecular explanation could be found.

[**Keywords:** IGF, FGF, BMP, Smad, Chordin, neural induction, *Xenopus*]

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Article and publication are at <http://www.genesdev.org/cgi/doi/10.1101/gad.1153603>.

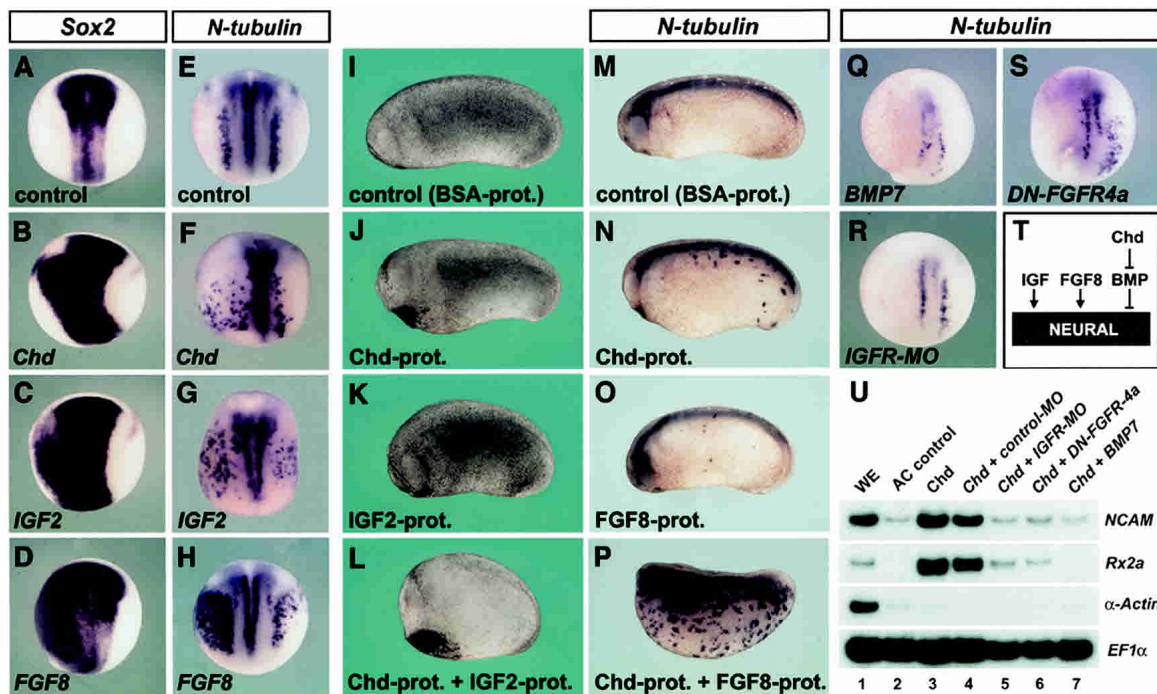


Figure 1. Comparison of neural induction by Chordin, IGF, and FGF8 in *Xenopus* embryos. (A–D) *Sox2* expression: Microinjection of *Chd* (10 pg), *IGF2* (800 pg), or *FGF8* (50 pg) mRNA into a single animal blastomere at the 4–8-cell stage expanded the neural plate on the injected side. (E–H) *N-tubulin* expression: *Chd* (4 pg), *IGF2* (100 pg), or *FGF8* (5 pg) mRNA expanded the lateral-most sensory neurons (Rohon-Beard neurons) into lateral epidermis. (I–P) Embryos injected with protein into the blastocoel at blastula stage. Protein amounts per injection were 2.6 ng Chordin, 2 ng IGF2, and 1.4 ng FGF8, injected together with 40 ng BSA. (I–L) Unstained tail bud-stage embryos. (M–P) In situ hybridization for *N-tubulin*. (Q–S) Loss of *N-tubulin*-positive neurons after a single animal injection of 250 pg *BMP7* mRNA, 16 ng *IGFR-MO*, or 500 pg *DN-FGFR4a* mRNA at the 4-cell stage. (T) Summary of signals involved in neural induction. (U) Animal cap explants from embryos injected with *Chd* mRNA (4 pg/blastomere) alone or in combination with control-MO (16 ng), *IGFR-MO* (16 ng), *DN-FGFR4a* mRNA (500 pg), or *BMP7* mRNA (250 pg). Frequency of embryos with the indicated phenotypes was: B, 34/35; C, 27/32; D, 16/19; F, 14/22; G, 37/51; H, 117/120; Q, 26/30; R, 41/47; S, 34/38; (U) 10 explants per lane (tail bud stage), three independent experiments.

An important effector of BMP signals is the transcription factor Smad1, which becomes phosphorylated at three conserved carboxy-terminal serine residues upon activation by the BMP receptor (BMPR) serine/threonine kinase (Massagué and Chen 2000). In pioneering work, Kretzschmar et al. (1997) showed that Smad1 also undergoes phosphorylation by MAPK in the central linker region (Fig. 2B). Whereas phosphorylation by BMPR promotes nuclear translocation and transcriptional activity of Smad1, phosphorylation by MAPK in the linker region has the opposite effect, causing cytoplasmic localization and inhibition of transcriptional activity (Kretzschmar et al. 1997; Massagué and Chen 2000). These opposing effects were discovered in tissue culture cell lines treated with epidermal growth factor (EGF) or hepatocyte growth factor (HGF), which signal through receptor tyrosine kinases (RTKs) and activate the extracellular signal-regulated kinase (Erk)/MAPK pathway (Kretzschmar et al. 1997). However, the relevance of this MAPK phosphorylation to physiological processes remained to be determined.

Whether the Kretzschmar et al. (1997) paradigm functions in vivo is an important question in cell–cell signaling. This is because these interactions between the EGF and BMP pathways would be hard-wired at the level of Smad1 transcription factor activity. A more flexible way of regulating transcription factor activity is for different pathways to act on separate transcription factors that interact through independent binding sites in individual

target gene promoters. MAPK sites have also been identified in the linker region of Smad2 (Kretzschmar et al. 1999), and a role for these sites in the loss of competence for mesoderm induction by Activin has been reported in *Xenopus* (Grimm and Gurdon 2002). However, the signaling pathway responsible for this phosphorylation has not been identified (Grimm and Gurdon 2002).

The potent neural inducing activity of FGF8 and IGF2 allowed us to investigate in vivo how these signaling pathways interact with the BMP pathway. FGF and IGF signal through RTKs that can activate the Erk/MAPK pathway (Blume-Jensen and Hunter 2001). Using the approach of Kretzschmar et al. (1997), we compared the phenotypic effects of Smad1 constructs encoding wild-type (WT) or phosphorylation-insensitive mutant proteins in which the BMPR or MAPK target serines were substituted by alanine residues (Fig. 2B). Microinjection of *WT-Smad1* mRNA into the animal pole of *Xenopus* embryos at the four-cell stage resulted in an unexpectedly mild ventralization phenotype, with slightly reduced head structures, a modest increase in ventral mesoderm marked by *Sizzled* expression (Fig. 2D,G; Collavin and Kirschner 2003), and a small decrease in CNS neurons marked by *N-tubulin* (Fig. 2E,H). The linker mutant *LM-Smad1* (Fig. 2B) has point mutations in the four MAPK phosphorylation sites (designated 4SP/AP in Kretzschmar et al. 1997). Microinjection of *LM-Smad1* mRNA resulted in strongly ventralized phenotypes; embryos lacked head structures and most of the CNS (Fig. 2K),

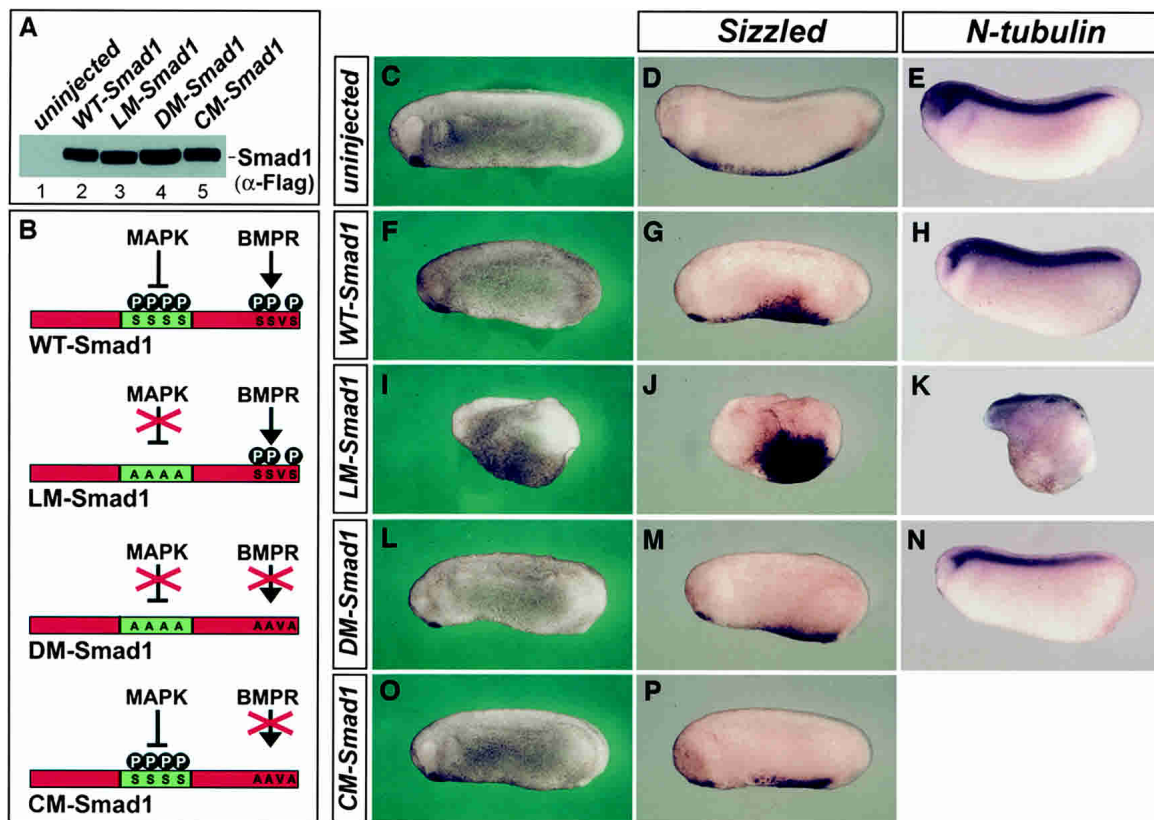


Figure 2. Endogenous embryonic MAPK signals inhibit Smad1 activity in the *Xenopus* embryo. Wild type (WT), linker mutant (LM), double mutant (DM), or carboxy-terminal mutant (CM) *Smad1* mRNAs were injected into each blastomere at the 4-cell stage (250 pg per injection). (A) Western blot showing similar levels of expression of Smad1 proteins at gastrula stage. (B) Diagram of Smad1 mutant proteins. (C–P) Uninjected and injected tail bud-stage embryos, unlabeled or after in situ hybridization with the ventral mesoderm marker *Sizzled* or the neuronal marker *N-tubulin*. Note that *LM-Smad1* (I,J,K) is very active in inhibiting CNS differentiation and expanding ventral tissue. A minimum of 30 embryos were used per injected sample, 10 independent experiments.

and had an expanded ventral mesodermal domain (Fig. 2J). These differences in activity were not caused by differences in levels of Smad1 protein expression (Fig. 2A). This strong Smad1 ventralizing (pro-BMP) activity required both inactivation of the MAPK phosphorylation sites and active phosphorylation by BMPR. This can be inferred from the mild phenotype of the double mutant DM-Smad1, in which both the MAPK and the BMPR phosphorylation sites were inactivated (Fig. 2L–N). A construct having mutations in the carboxy-terminal sites only, CM-Smad1, had weak, if any, effects (Fig. 2O,P). The striking difference between the effects of *WT-Smad1* and *LM-Smad1* mRNA (Fig. 2, cf. H and K) indicates that endogenous MAPK signals are able to antagonize Smad1 activity in the developing *Xenopus* embryo.

The observation that MAPK phosphorylation can inhibit WT-Smad1 activity in vivo was further analyzed in a variety of neural induction assays (Fig. 3). The MAPK-insensitive LM-Smad1 was a much stronger inhibitor of neural plate (*Sox2*) and neuronal (*N-tubulin*) markers (Fig. 3C,G) than either WT-Smad1 (Fig. 3B,F) or DM-Smad1 (Fig. 3D,H). *LM-Smad1* inhibited the ectopic neural induction caused by microinjection of *FGF8* or *IGF2* mRNAs in a cell-autonomous way (Fig. 3L,P), whereas *WT-Smad1* mRNA had little or no effect (Fig. 3K,O). In cells co-injected with *WT-Smad1* together with *FGF8* or *IGF2*, MAPK levels should be high, preventing the anti-neural effects of Smad1 shown in Figure 3K,O, which

become evident only when MAPK phosphorylation is prevented in the LM-Smad1 mutant (Fig. 3L,P). In animal cap explants, injected *Chd*, *FGF8*, and *IGF2* mRNAs had remarkably similar effects on gene expression (Fig. 3Q). They caused induction of anterior (*Otx2*, *Six3*) but not posterior (*Krox20*, *HoxB9*) neural markers, and suppressed epidermal genes (*Cytokeratin*, *Msx1*, *Vent2*) in the absence of mesoderm (α -Actin, α -Globin) induction (Fig. 3Q, cf. lanes 2,3,6,9). We note that although other FGFs induce the formation of mesoderm, which posteriorizes neural tissue (Lamb and Harland 1995), FGF8 suppresses mesoderm (Hardcastle et al. 2000), leading to the differentiation of predominantly anterior neural tissue in animal cap explants. Neural induction by *Chd*, *FGF8*, and *IGF2* in animal cap explants was inhibited, and epidermal differentiation promoted, by co-injection of *LM-Smad1* (Fig. 3Q, lanes 5,8,11). Overexpression of *WT-Smad1* also inhibited neural differentiation (Fig. 3Q, lanes 4,7,10), but to a lesser degree. Taken together, these experiments indicate that phosphorylation by MAPK in the linker region of Smad1 promotes neural induction. When this phosphorylation is prevented, Smad1 becomes a potent antineural agent.

To test whether FGF8 and IGF induce phosphorylation of Smad1 in vivo, we used a gel mobility shift assay at low bisacrylamide concentrations to separate phosphorylated proteins (Chesnel et al. 1997). In NIH3T3 cells, addition of FGF8 protein for 10 min led to phosphoryla-

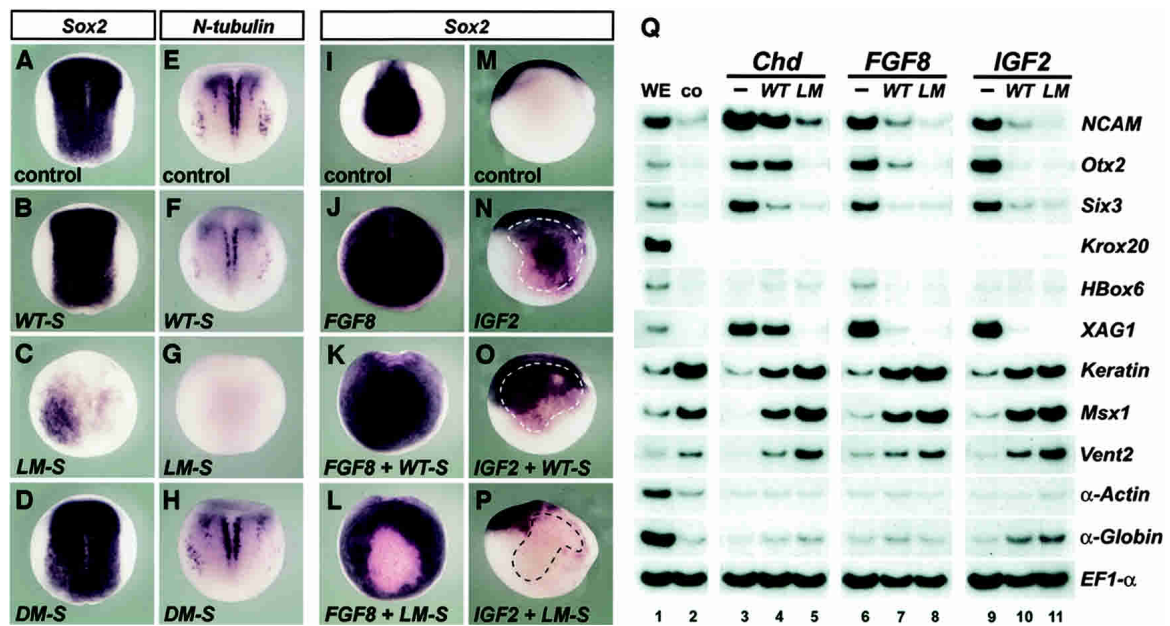


Figure 3. The MAPK phosphorylation mutant LM-Smad1 inhibits endogenous CNS formation and neural induction by FGF8 or IGF2 signals. (A–H) Neurula-stage embryos in dorsal view, uninjected (control) or injected four times into the animal pole with WT-Smad1, LM-Smad1, or DM-Smad1 mRNA. (I–L) FGF8-injected embryos in anterior view. (M–P) IGF2-injected embryos in lateral view. Embryos were injected at the 8-cell stage into a single animal blastomere together with nuclear *lacZ* mRNA as lineage tracer and stained for Sox2. Stippled lines mark the *lacZ*-positive injection sites. Note that LM-Smad1, but not WT-Smad1, blocks neural induction by FGF8 or IGF2 mRNA. (Q) RT-PCR analysis of stage 26 ectodermal explants from embryos injected at the 8-cell stage with the indicated mRNAs. WE, whole embryo; Co, uninjected animal cap explants used as controls. The amounts of injected mRNAs per blastomere were 250 pg *Smad1*, 4 pg *Chd*, 100 pg FGF8, 800 pg IGF2, and 200 pg nuclear *lacZ*. Endogenous neural marker expression was inhibited in: A, 0/72; B, 0/46; C, 58/58; D, 0/35; E, 0/44; F, 37/78; G, 67/68; H, 9/23 embryos. Ectopic Sox2 expression was inhibited in: J, 0/23; K, 0/34; L, 24/32; N, 0/69; O, 0/53; P, 91/109 embryos. In (Q) one whole embryo or 10 animal caps were used per lane, two independent experiments.

tion of endogenous Erk1 and Erk2 and of transfected WT-Smad1 (Fig. 4A, lanes 1–4). The shift in Smad1 mobility was dependent on phosphorylation by MAPK, as it was not detected in LM-Smad1 (Fig. 4A, lanes 5,6). These phosphorylations required the MEK/Erk pathway, because the MEK inhibitor U0126 (Promega) blocked both Erk1/2 and Smad1 phosphorylation (Fig. 4B, lanes 3,4). Incorporation of radioactive orthophosphate into Smad1 proteins in mRNA-injected *Xenopus* embryonic cells confirmed that the linker MAPK sites are indeed phosphorylated (Fig. 4C). In developing *Xenopus* embryos at the blastula stage, microinjection of FGF8 protein into the blastocoel caused a mobility shift of Flag-tagged WT-Smad1, but not of LM-Smad1 (Fig. 4D, lanes 3,5). When IGF2 protein was tested in a similar manner, significant Erk2 phosphorylation in embryos was not detected (data not shown), perhaps owing to low levels of IGF receptor at blastula (Richard-Parpaillon et al. 2002). We therefore turned to *Xenopus* oocytes, which have a robust MAPK response to Insulin and IGF signals (Chesnel et al. 1997). Addition of IGF2 protein to the culture medium of oocytes previously microinjected with WT-Smad1 mRNA induced phosphorylation of the linker region of WT-Smad1 (Fig. 4E). We conclude from these experiments that FGF8 and IGF2 can induce phosphorylation of Smad1 via the MEK/Erk MAPK pathway in vivo. Endogenous MAPK signals were also able to phosphorylate the Smad1 linker region. As shown in Figure 4F, WT-Smad1 but not LM-Smad1 was phosphorylated in early gastrula (stage 10.5) and late gastrula (stage 12.5) *Xenopus* embryos. The onset of this linker Smad1 phosphorylation by MAPK correlated well with the detection

of activated phospho-Erk (Fig. 4F), suggesting that endogenous Erk signaling may cause the linker phosphorylation of Smad1 in the *Xenopus* gastrula.

The results presented here suggest a unified molecular mechanism that can explain neural induction in *Xenopus* by multiple signals through inhibition of the BMP pathway at the level of Smad1 phosphorylation (Fig. 5). BMP antagonists such as Chordin and Noggin prevent the phosphorylation of carboxy-terminal serines in Smad1, so that Smad1 is less active in inhibiting neural differentiation (Weinstein and Hemmati-Brivanlou 1999; De Robertis et al. 2000). FGF8 and IGF2 trigger phosphorylation of the Smad1 linker region by MAPK (the present study), further inhibiting Smad1 transcriptional activity (Kretzschmar et al. 1997) and thus promoting neurogenesis. This molecular mechanism may also help explain neural induction by HGF (Streit et al. 1995, 1997), which can induce Smad1 linker phosphorylation in cultured cells (Kretzschmar et al. 1997). Linker phosphorylation of Smad2/3 has also been shown to negatively control TGF- β action (Kretzschmar et al. 1999; Grimm and Gurdon 2002). It is not known whether Smad2/3 phosphorylation has a role in neural induction, but we note that Smad2 is not active (that is, not phosphorylated at the carboxy-terminus) in *Xenopus* ectoderm (Schohl and Fagotto 2002). Thus, MAPK activity in ectoderm may not affect neural induction via Smad2/3, but rather decrease the competence for mesoderm induction by TGF- β signals (Grimm and Gurdon 2002).

The finding that microinjected WT-Smad1 is unable to antagonize neural development in the presence of endogenous levels of MAPK signals (Figs. 2H, 3B) has impor-

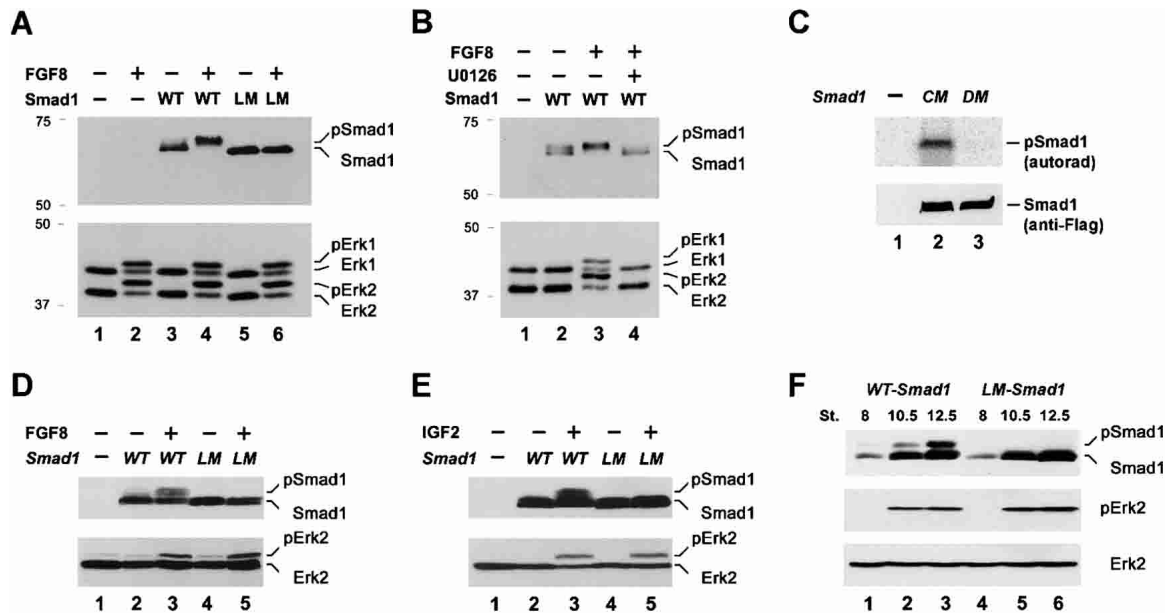


Figure 4. FGF8 and IGF2 induce linker phosphorylation of Smad1 via MAPK in vivo. Proteins in cell lysates were analyzed by Western blot with antibodies against Flag (for Smad1) and Erk1/2. (A) FGF8 shifts the electrophoretic mobility of WT-Smad1 (lane 4), but not of LM-Smad1 (lane 6). NIH3T3 cells were transfected with Flag-tagged Smad1 constructs, serum-starved for 6 h, and recombinant mouse FGF8b protein (100 ng/mL) added for 10 min. (B) Pre-incubation of NIH3T3 cells with the MEK1 inhibitor U0126 (20 μ M) 1 h before the addition of FGF8 suppresses the phosphorylation of Smad1 and Erk1/2 (lane 4). (C) *In vivo* ³²P incorporation into the linker MAPK sites of Smad1 in *Xenopus*. Animal caps from uninjected embryos, injected with CM-Smad1 or DM-Smad1 were explanted at late blastula stage and incubated with 2 mCi/mL ³²P orthophosphate for 45 min. (D) *Xenopus* embryos injected first with 400 pg Smad1 mRNA into each animal blastomere at the 8-cell stage, then with 40 nl FGF8 protein (1.4 ng) into the blastocoele at stage 8 and lysed 30 min later. FGF8 induces phosphorylation of Erk2 and WT-Smad1, but not of LM-Smad1 (lanes 3,5). Erk1 is not expressed in the early *Xenopus* embryo (Chesnel et al. 1997). (E) *Xenopus* oocytes at stages V–VI were injected with 4 ng Smad1 mRNA and 12 h later exposed to 18 ng/mL recombinant human IGF2 protein for 14 h. IGF2 induced phosphorylation of WT-Smad1 and Erk2. (F) Endogenous signals phosphorylate Smad1 linker region in *Xenopus* embryos. Equal numbers of Smad1 mRNA-injected embryos were lysed at mid-blastula (stage 8), early gastrula (stage 10.5), and late gastrula (stage 12.5), and protein extracts were analyzed by Western blot with antibodies against Erk, phosphorylated Erk (pErk), and Flag (for Smad1). Note the mobility shift of WT-Smad1 (lanes 2,3) but not LM-Smad1 (lanes 5,6) in samples with high levels of phosphorylated Erk.

tant physiological implications. *In vivo*, Smad1 transcription factor activity results not only from BMP signaling through BMPR as currently thought, but from a combination of high BMPR activity and low RTK/MAPK signaling. In the case of ectodermal differentiation, the antineural effects of Smad1 will manifest themselves in regions of low BMP antagonists, high BMP, and low levels of signals that activate RTKs and act through MAPK. Presumably it takes multiple signals to achieve the low levels of Smad1 activity required to form the neural plate in embryonic ectoderm.

The demonstration that FGF and IGF can induce an inhibitory phosphorylation of Smad1 *in vivo* may also shed light on other aspects of vertebrate development. During organogenesis there are many instances in which the activities of the FGF and BMP pathways have opposing effects. These include FGF4 and BMP2 in the limb bud (Niswander and Martin 1993), FGF10 and BMP4 in lung morphogenesis (Weaver et al. 2000), FGF2 and BMP4 in cranial suture fusion (Warren et al. 2003), and FGF8 and BMP4 in the initiation of tooth development (Thesleff and Mikkola 2002). These developmental processes may also involve signal integration at the level of Smad1 by the molecular mechanism proposed in the present study on neural induction.

Materials and methods

DNA constructs, morpholino oligonucleotides, and synthetic mRNAs
For mRNA injections we subcloned the amino-terminal Flag-tag and

open reading frame of human Smad1 into pCS2, using pCMV5/Flag-Smad1, pCMV5/Flag-Smad1-4SP/AP, and pCMV5/Flag-Smad1-4SP/AP-AAVA (an invaluable gift from J. Massagué, Sloan Kettering Cancer Center, New York) to generate WT-Smad1, LM-Smad1, and DM-Smad1, respectively. The fact that the biochemical properties of these proteins had been fully characterized (Kretzschmar et al. 1997) was essential for the execution of this study. CM-Smad1 was generated by exchanging an EcoRI/XbaI restriction fragment from DM-Smad1 into WT-Smad1. The antisense IGF2 morpholino oligonucleotide (Gene Tools) was described in Richard-Parpaillon et al. (2002). To prepare sense mRNA, pCS2 constructs of WT-Smad1, LM-Smad1, DM-Smad1, CM-Smad1, *chordin*, *xIGF2*, *xFGF8* (gift from J. Slack, University of Bath, UK), *BMP7*, and *DN-IGFR* were linearized with NotI and transcribed with SP6 RNA polymerase. *DN-FGFR4a* mRNA was synthesized from pSP64T (SalI digestion and SP6 transcription, gift from H. Okamoto, AIST Institute, Japan) and *nIacZ* mRNA from pXEX β gal (XbaI digestion and T7 transcription, gift from R. Harland, University of California, Berkeley).

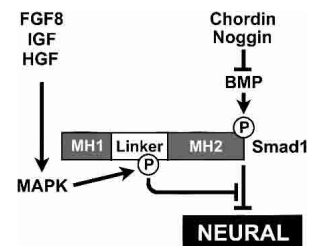


Figure 5. Model of the integration of multiple signaling pathways at the level of Smad1 phosphorylation in neural induction. MH1 and MH2 are evolutionarily conserved Mad-homology domains. Diagram modified from Kretzschmar et al. (1997).

Embryo and oocyte manipulations

In vitro fertilization, embryo and explant culture, lineage tracing, RT-PCR, and in situ hybridization were as reported (Pera et al. 2001). *Xenopus* embryos at the blastula stage were injected into the blastocoel cavity with 40 nl protein solution of 0.1% bovine serum albumin (BSA) alone or together with recombinant mouse Chordin, human IGF2, mouse FGF8b (all from R&D Systems) in 0.1x Barth medium. Manually defolliculated *Xenopus* oocytes (stages V–VI) were incubated with IGF2 protein in 0.1% BSA/OR-2 medium at 16°C as described (Chesnel et al. 1997).

Western blot analysis

Embryo, oocyte, or cell lysates were prepared using RIPA buffer (0.1% NP-40, 20 mM Tris, pH 8.0, 1 mM EDTA, 10% Glycerol, protease inhibitor cocktail [Roche], 1 mM sodium orthovanadate, 1 mM PMSF). Proteins were separated by 12% SDS-PAGE (acrylamide:bisacrylamide = 30:0.18) to resolve phosphorylated forms (Chesnel et al. 1997). Western blots were performed using antibodies against Flag (for Smad1, 1:2000, Sigma) and Erk1/2 (1:1000, Cell Signaling) and phospho-Erk1/2 (1:1000, Sigma). For radioactive labeling of Smad phosphorylation, *Xenopus* embryos were injected with 1 ng *CM-* or *DM-Smad1* mRNA at the four-cell stage and animal caps explanted at stage 9.5. The explants were cultured for 45 min in the presence of 2 mCi/mL [γ - P^{32}] orthophosphate and lysed in RIPA buffer. Smad1 was immunoprecipitated from the lysates and run on an SDS-PAGE gel. The amount of immunoprecipitated Smad1 was quantified by Western blot, and the incorporation of radioactive phosphate was visualized by autoradiography.

Acknowledgments

We thank J. Massagué, H. Okamoto, J. Slack, and R. Harland for gifts of DNA constructs, Y. Sun for advice, Z. Unno for in situ hybridizations, S. L. Zipursky, Y. Sun, G. Weinmaster, K. McLaren, M. Kessel, O. Wessely, and C. Coffinier for comments on the manuscript; A. Cuellar for technical assistance; and A. De Robertis for secretarial help. E.M.P. was an HFSPO fellow. This work was supported by NIH grant HD-21502-17. E.M.D.R. is a Howard Hughes Medical Institute Investigator.

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Note added in proof

While this work was under review, Sater et al. (2003) reported that mutations in the MAPK sites of *Xenopus* Smad1 caused hyperventralizing effects in microinjected embryos. The results of Sater et al. (2003) and those of the present study complement each other, supporting a role for linker phosphorylation of Smad1 in neural induction.

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