BMP regulates vegetal pole induction centres in early *Xenopus* development

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Abstract

***Background*: Bone morphogenetic protein (BMP) plays an important role in mesoderm patterning in *Xenopus*. The ectopic expression of BMP-4 protein hyperventralizes embryos, whereas embryos expressing a BMP-2/4 dominant-negative receptor (DNR) are hyperdorsalized. Mesoderm is initially induced in the marginal zone by cells in the underlying vegetal pole. While much is known about BMP's expression and role in patterning the marginal zone, little is known about its early role in regulating vegetal mesoderm induction centre formation.**

***Results*: The role of BMP in regulating formation of vegetal mesoderm inducing centres during early *Xenopus* development was examined. Ectopic *BMP-4* expression in vegetal pole cells inhibited dorsal mesoderm induction but increased ventral mesoderm induction when recombined with animal cap ectoderm in Nieuwkoop explants. 32-cell embryos injected with *BMP-4* RNA in the most vegetal blastomere tier were not hyperdorsalized by LiCl treatment. The ectopic expression of Smad or Mix.1 proteins in the vegetal pole also inhibited dorsal mesoderm induction in explants and embryos. Expression of the BMP 2/4 DNR in the vegetal pole increased dorsal mesoderm induction and inhibited ventral mesoderm induction in explants and embryos.**

***Conclusions*: These results support a role for BMP signalling in regulating ventral vegetal and dorsal vegetal mesoderm induction centre formation during early *Xenopus* development.**

Introduction

In *Xenopus* embryos, mesoderm forms by a multi-stepped inductive process (reviewed by Kessler & Melton 1994; Heasman 1997). During early development, vegetal pole (VP) cells may exist in two states, the dorsal or ventral (Nieuwkoop 1969; Sudwarti & Nieuwkoop 1971). The dorsal mesoderm, the Spemann organizer, is induced in the subequatorial region (marginal zone) by underlying dorsal VP Nieuwkoop centre cells. Ventral mesoderm is induced in the marginal zone by underlying ventral VP cells. During gastrulation, a dorsalizing signal emanating from Spemann's organizer converts the adjacent lateral mesoderm from ventral to more dorsal fates, such as muscle, heart and pronephros (Dale & Slack 1987; Stewart & Gerhart 1990; Lettice & Slack 1993). Through this series of inductive events, the dorsoventral pattern is established in the mesoderm.

Bone morphogenetic protein 4 (BMP-4) is a member of the TGF-β growth factor family (Lyons *et al.* 1991). Ectopic *BMP-4* expression induces ventral mesoderm in *Xenopus* embryos and explants. Animal cap (AC) ectoderm which has been isolated from *BMP-4* injected embryos expresses a wide range of nonorganizer mesodermal markers (Dale *et al.* 1992; Jones *et al.* 1992; Re'em-Kalma *et al.* 1995). The ectopic expression of *BMP-4* RNA also inhibits dorsal axis formation in whole embryos and activin-induced AC explants; embryos and explants fail to elongate and do not make a detectable notochord or muscle, as determined by histology and molecular markers (Dale *et al.* 1992; Jones *et al.* 1992; Fainsod *et al.* 1994; Schmidt *et al.* 1995). Ectopic *BMP-4* expression inhibits the formation of a functional Spemann organizer, and excess *BMP-4* in the ventral marginal zone inhibits its dorsalization by noggin or a juxtaposed Spemann organizer (Fainsod *et al.* 1994; Re'em-Kalma *et al.*

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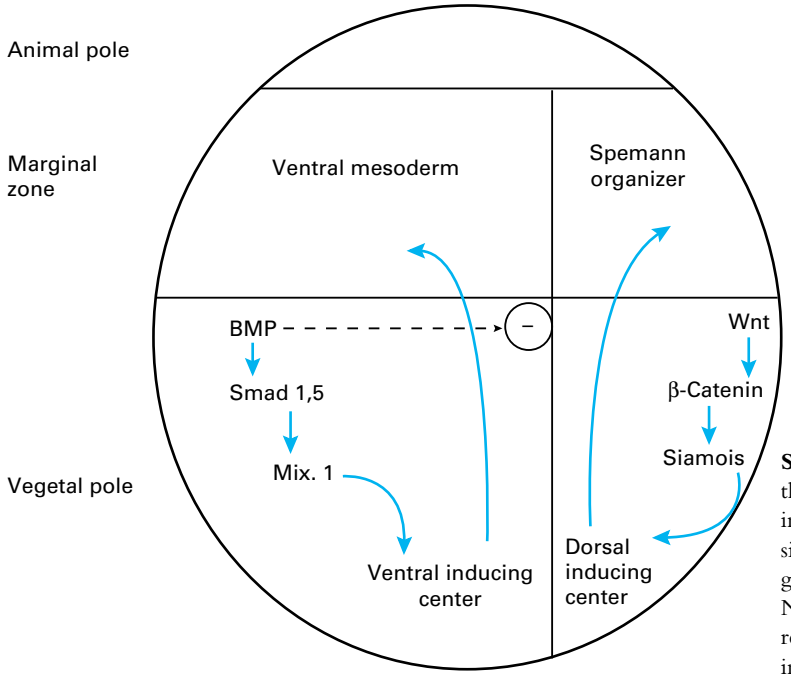

Summary Figure BMP/Smad signalling within the vegetal pole is required for ventral vegetal inducing centre formation. A target of BMP signalling could be the zygotically expressed *Mix.1* gene. BMP activity could inhibit spreading of the Nieuwkoop centre to the ventral vegetal side, thus regulating the ratio of ventral/dorsal mesoderm inducing cells in the vegetal pole.

1995; Jones *et al.* 1996). In *Xenopus* embryos depleted of endogenous BMP-4 activity by the ectopic expression of a *Xenopus BMP 2/4 DNR* or *BMP-4* antisense RNA, ventral mesoderm is dorsalized (Graff *et al.* 1994; Steinbeisser *et al.* 1995). BMP-4 most likely regulates ventral mesoderm formation by activating the expression of the *Xvent-1, Xvent-2*, and *Mix.1* transcription factors in the marginal zone (Gawantka *et al.* 1995; Mead *et al.* 1996; Onichtchouk *et al.* 1996; Papalopulu & Kintner 1996; Schmidt *et al.* 1996). Therefore BMP-4 can induce ventral mesoderm and also perturb dorsal mesoderm formation.

The intracellular signalling molecules, Smads, transduce the signals of TGF-β molecules such as BMP and activin (reviewed in Massague 1996; Heldin *et al.* 1997). The Smad1 and Smad5 proteins are the initial targets of BMP signalling; phosphorylation of cytoplasmic Smad proteins by the BMP receptor causes nuclear translocation, where they presumably activate gene expression (reviewed in Massague 1996; Heldin *et al.* 1997). The ectopic expression of *Xenopus* Smad1 (XSmad1) or murine Smad5 protein in *Xenopus* embryos, activates BMP signalling pathways and hyperventralizes the embryos (Graff *et al.* 1996; Thomsen 1996; Suzuki *et al.* 1997). By ectopically expressing Smad proteins, the regulation of nuclear localization is perturbed and ectopic Smad proteins enter the nucleus and activate target gene transcription (Baker & Harland 1996). Thus, BMP signalling is transduced in an intracellular autonomous manner by the ectopic expression of Smad proteins.

The ectopic expression of the Mix.1 homeobox protein also hyperventralizes embryos in a manner similar to BMP-4, although the phenotype is not as severe as in *BMP-4* injected embryos (Mead *et al.* 1996). *Mix.1* is initially expressed in VP cells at the mid-blastula transition, followed by marginal zone expression (Rosa 1989). *Mix.1* is a downstream target of BMP-4 signalling; its transcription is activated by ectopic *BMP-4* expression in ACs and whole embryos (Re'em-Kalma *et al.* 1995; Mead *et al.* 1996). It has been suggested that *Mix.1* expression may be the final downstream target of BMP signalling, but it has not been shown clearly if Mix.1 ventralization activity is mediated from the marginal zone or the VP (Mead *et al.* 1996). In addition, *Mix.1* is transcriptionally activated by vg1/activin signalling (Rosa 1989). It was suggested that dorsally expressed Mix.1 protein may form inactive heterodimers with the dorsally expressed siamois homeobox protein (Lemaire *et al.* 1995; Mead *et al.* 1996; reviewed in Heasman 1997). Thus, Mix.1 may be at least one of the final downstream targets of the BMP ventralizing pathway.

Previous studies have supported a strong role for BMP-4 signalling in regulating the dorsal/ventral patterning of the mesoderm in the gastrula embryo (Fainsod *et al.* 1994; Graff *et al.* 1994; Re'em-Kalma *et al.* 1995; Jones *et al.* 1996). However, a role for BMP signalling in regulating early VP inducing centre formation has not been determined. The inhibition of Spemann's organizer by BMP-4 could occur by at least

two simultaneous pathways, one in the marginal zone and one in the VP. Ectopic *BMP-4* expression in the dorsal marginal zone could autonomously ventralize cells fated for Spemann's organizer (Re'em-Kalma *et al.* 1995; Jones *et al.* 1996), but on the other hand, ectopic *BMP-4* expression could also modify the formation of vegetal inducing centres.

The ventral vegetal inducing centre is responsible for the early induction of nonorganizer mesoderm in the marginal zone. It is possible that the autonomous BMP signalling pathways in the VP control the formation of the ventral vegetal inducing centre, with BMP regulating the dorsal/ventral inducing centre ratio within the VP. While it is clear that the Wnt/β-catenin pathway suffices to form the dorsal vegetal-inducing Nieuwkoop centre; it is not clear which molecules control the ventral vegetal inducing centre formation (reviewed in Heasman 1997). Since the role of BMP signalling in regulating the ventral vegetal inducing centre formation is unclear, we decided to address this question.

We used recombinant explants formed between vegetal and animal pole cells (Nieuwkoop explants) to address the regulatory role of BMP signalling in VP mesoderm induction centre formation. The ectopic expression of BMP-4, Smad and Mix.1 proteins in VPs reduces dorsal and increases ventral mesoderm induction when they are recombined with normal AC cells. VPs over-expressing the BMP 2/4 DNR induce more dorsal and less ventral mesoderm than control VPs. Similar observations were also seen in embryos injected with these molecules in tier D vegetal blastomeres at the 32-cell stage. These results suggest that, in addition to mesoderm patterning activities of BMP in the marginal zone, maternal BMP activities may also regulate VP mesoderm induction centre formation.

Results

Ectopic *BMP-4* expression inhibits dorsal vegetal but enhances ventral vegetal inducing centre formation

We examined the possible role of BMP-4 signalling on the formation of VP mesoderm induction centres. *BMP-4* RNA (2 ng) was injected vegetally into one-cell *Xenopus* embryos. VPs from injected blastula stage embryos were recombined with an identical stage AC ectoderm from normal embryos (Nieuwkoop explants) and grown until the midgastrula (stage 11) or late neurula (stages 18) stages. RNA was isolated for Northern analysis in order to examine the dorsal and ventral mesoderm marker expression. In the gastrula

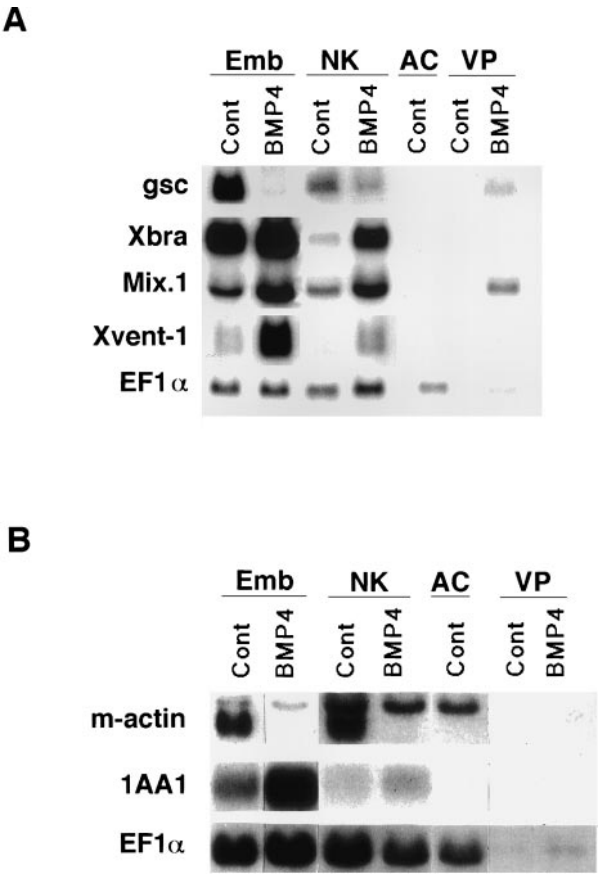

Figure 1 Ectopic expression of *BMP-4* in vegetal poles inhibits dorsal and increases ventral mesoderm induction in gastrula and neurula stage recombinant Nieuwkoop explants. (A) Embryos at the one-cell stage were injected vegetally with 2 ng of *in vitro* transcribed *BMP-4* RNA. Blastula stage VPs removed from injected and uninjected embryos were recombined (Nieuwkoop recombinant explants), with AC ectoderm removed from uninjected embryos at the same stage. Ten recombinant explants were grown until stage 11 and the total RNA was isolated. Total RNA was also isolated from 10 unrecombined VP and AC cultured explants, and pools of five control and *BMP-4* injected embryos. For Northern analysis, all 10 recombinant explants (NK), VPs, ACs, and one embryo (Emb) equivalent of RNA were loaded per well. The filter was sequentially hybridized with the *Xenopus* cDNA probes for *goosecoid* (*gsc*), *Brachyury* (*Xbra*), *Mix.1, Xvent-1* and *EF1α*. *Xbra* was never detected in cultured VP explants (even after long exposures), demonstrating that VP explants were not contaminated with marginal zone cells. (B) Fifteen recombinant explants as described in (A) were grown to stage 18 and total RNA was isolated. For Northern analysis, nine recombinant explant (NK) equivalents of RNA and one embryo (Emb) equivalent of RNA were loaded per well. The filter was sequentially hybridized with the *Xenopus* cDNA probes for *muscle actin* (*m-actin*), *1AA1* and *EF1α*.

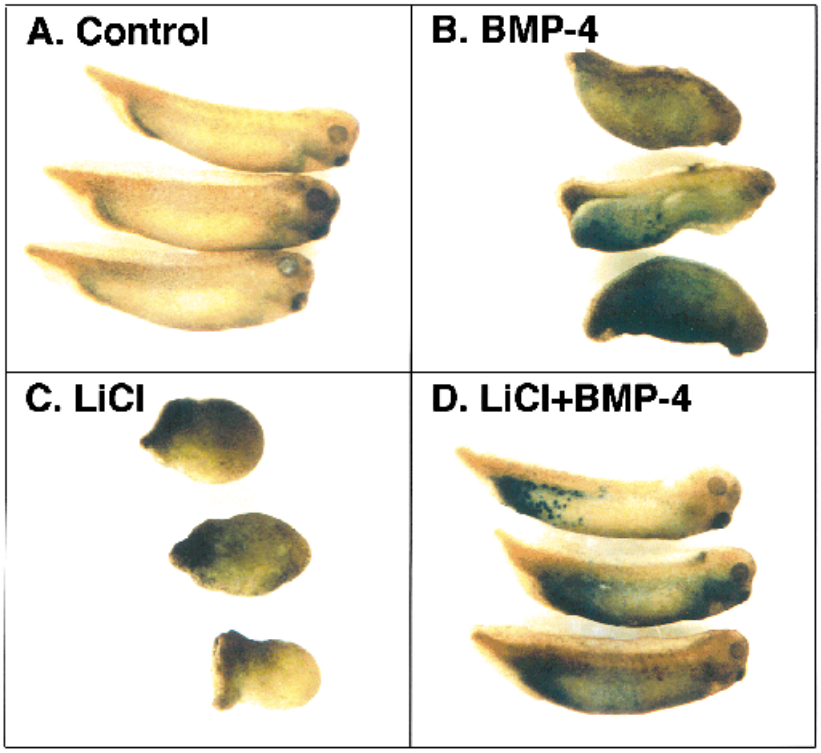

Figure 2 Ectopic expression of *BMP-4* in D tier vegetal blastomeres inhibits hyperdorsalization by LiCl. (A) Three representative stage 35 normal embryos. (B) Three representative *BMP-4* injected (200 pg) embryos. (C) Three representative LiCl-treated embryos. (D) Three representative *BMP-4* injected (200 pg) LiCl-treated embryos.

stage explants injected with *BMP-4* RNA, there was a sharp reduction in the expression of the organizer specific *goosecoid* mRNA (Blumberg *et al.* 1991) in comparison to uninjected control Nieuwkoop explants (Fig. 1A). Expression of the pan-mesodermal *Xbra* (Smith *et al.* 1991) and ventrally expressed *Xvent-1* (Gawantka *et al.* 1995) mRNAs was increased in explants derived from VPs ectopically expressing *BMP-4* RNA, in comparison to controls (Fig. 1A). In addition, the endoderm-mesoderm expressed *Mix.1* gene (Rosa 1989) was also activated in *BMP-4* injected recombinant explants (Fig. 1A). Stimulation of *Mix.1* expression, but not *Xbra* or *Xvent-1* expression, was also observed in *BMP-4* injected VP explants (Fig. 1A). At late neurula stages, *muscle actin* expression was inhibited and the expression of the *1AA1* ventroposterior marker (Greene *et al.* 1993) was stimulated by *BMP-4* injected VPs in Nieuwkoop recombinant explants (Fig. 1B).

We also examined the BMP-4 regulation of vegetal-inducing centres in whole embryos. At the 32-cell stage, embryos were injected into one tier-D vegetal blastomere with 200 pg of *BMP-4* RNA and 200 pg of β-*gal* RNA, which served as a lineage tracer. At the 64–128 cell stage, these embryos (Fig. 2A) were hyperdorsalized by LiCl treatment (Fig. 2C). *BMP-4* injected LiCl-treated embryos (Fig. 2D) had a dorsal-anterior index (DAI) of 5.6 ($n = 9$) in comparison to β-*gal* injected LiCl-treated controls (Fig. 2C) which had a DAI of 9.1 ($n = 21$). β-*gal* staining showed that the *BMP-4* injected cells were restricted to the endoderm cells of VP origin (Fig. 2). The control β-*gal* injected embryos (Fig. 2A) had a DAI of 5.0 ($n = 15$) whereas β-*gal*/*BMP-4* injected embryos (Fig. 2B) had a DAI of 3.0 ($n = 12$). Thus, the ectopic expression of BMP-4 protein in the VP overcomes the hyperdorsalizing effects of LiCl-treatment, and also partially hyperventralizes normal embryos.

Inhibition of BMP activity inhibits ventral vegetal but increases dorsal vegetal inducing centre formation

To investigate the role of endogenous BMP activity within the VP, one-cell embryos were vegetally injected with 0.4 ng of the *BMP 2/4 DNR* RNA, and VPs from injected embryos were recombined with normal AC cells. These explants were grown to late neurula stages in order to measure dorsal and ventral marker expression. In neurula stage explants, *muscle actin* mRNA levels are about sixfold higher than in controls (Fig. 3A); expression of the ventroposterior marker *1AA1* was inhibited about threefold (Fig. 3A). As a control,

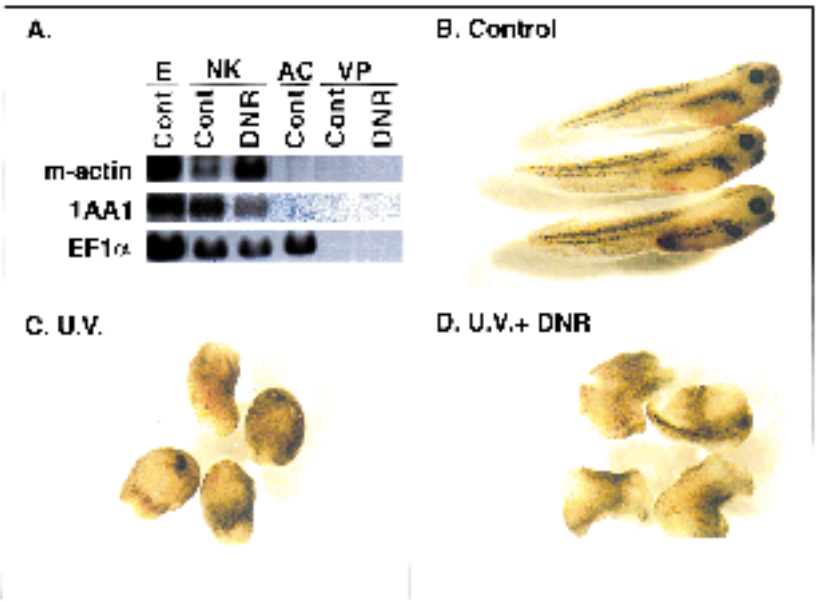

Figure 3 Ectopic expression of *BMP 2/4 DNR* in vegetal poles inhibits ventral and increases dorsal mesoderm induction in neurula stage recombinant Nieuwkoop explants and in embryos. (A) Embryos at the one-cell stage were injected vegetally with 0.4 ng of *in vitro* transcribed *BMP 2/4 DNR* RNA. Blastula stage VPs removed from injected and uninjected embryos were recombined with AC ectoderm removed from uninjected embryos at the same stage. Fifteen recombinant explants were grown until stage 20 and total RNA was isolated. RNA was also isolated from nine unrecombined VP and AC cultured explants. Total RNA was also isolated from a pool of five control embryos. For Northern analysis, nine recombinant (NK), VP, and AC equivalents and one embryo (Emb) equivalent of RNA were loaded per well. The filter was sequentially hybridized with the *Xenopus* cDNA probes for *muscle actin* (*m-actin*), *1AA1*, and *EF1α*. (B) Three representative stage 40 control embryos. (C) Four representative UV-irradiated embryos. (D) Four representative *BMP 2/4 DNR* injected (40 pg) UV-irradiated embryos.

VPs from embryos injected with RNA encoding the *activin DNR* (Hemmati-Brivanlou & Melton 1992) were used in recombinant Nieuwkoop explants. These explants expressed dorsal and ventral markers as did uninjected controls (not shown). In parallel, whole embryos injected with the *activin DNR* expressed reduced levels of *Brachyury* and *muscle actin* mRNAs (not shown), presumably due to an inhibition of receptor activity in the marginal zone (Hemmati-Brivanlou & Melton 1992).

We also examined the effect of eliminating BMP signalling in vegetal inducing centres in whole embryos. Embryos were hyperventralized by UV-irradiation at the one-cell stage. 32-cell stage UV-irradiated embryos were injected into two tier-D vegetal blastomeres with 40 pg of *BMP 2/4 DNR* RNA and 40 pg of β-*gal* RNA which served as a lineage tracer. In this experiment, the DAI of UV-irradiated embryos was about 0.2 ($n = 75$, compare Fig. 3B to 3C). While rescue was not complete (only two out of eight tier-D blastomeres were injected), we consistently saw an increase of at least one DAI unit in embryos injected vegetally with *BMP 2/4 DNR* RNA ($n = 28$, Fig. 3D). Thus, ectopic expression of BMP 2/4 DNR protein in the VP can partially overcome hyperventralization by UV-irradiation.

Ectopic expression of Smad and Mix.1 proteins inhibits dorsal mesoderm induction in Nieuwkoop assays

To examine an autonomous role of BMP signalling in the VP, we ectopically expressed RNA encoding the hyperventralizing XSmad1 (Graff *et al.* 1996; Thomsen 1996) and murine Smad5 proteins (Suzuki *et al.* 1997). *XSmad1* or *Smad5* RNA was vegetally injected into one-cell embryos and VPs were used in Nieuwkoop recombinant explants. As assayed by *muscle actin* expression, *Smad5* was a potent inhibitor of dorsal mesoderm induction in the Nieuwkoop assay, since *muscle actin* expression was fivefold inhibited in comparison to the control explants (Fig. 4A). We found that *XSmad1* was not as potent as *Smad5* in perturbing the VP inducing centres (Fig. 4B), suggesting that the XSmad1/BMP signalling within the VP may be tightly regulated. To further address this point, small amounts of *BMP-4* RNA (250 pg) were co-injected with *XSmad1* RNA to determine if a slight stimulation in BMP-4 signalling could activate the ectopically expressed *XSmad1*. These levels are eightfold lower than the hyperventralizing levels that were injected in the experiments shown in Fig. 1. When co-injected, *BMP-4 and XSmad1* inhibit dorsal mesoderm

induction over fivefold in Nieuwkoop recombinant explants, as assayed by *muscle actin* expression (Fig. 4B); yet neither molecule alone significantly inhibits *muscle actin* expression in separately injected VPs (Fig. 4B). These results suggest that autonomous Smad/BMP signalling pathways in the VP regulate the formation of mesoderm-inducing centres.

To further address an autonomous role for BMP-4 signalling in the VP, we injected embryos with RNA encoding the ventralizing Mix.1 homeodomain protein. VPs expressing ectopic levels of Mix.1 protein inhibited *muscle actin* induction in Nieuwkoop recombinant explants in comparison to controls (Fig. 4C).

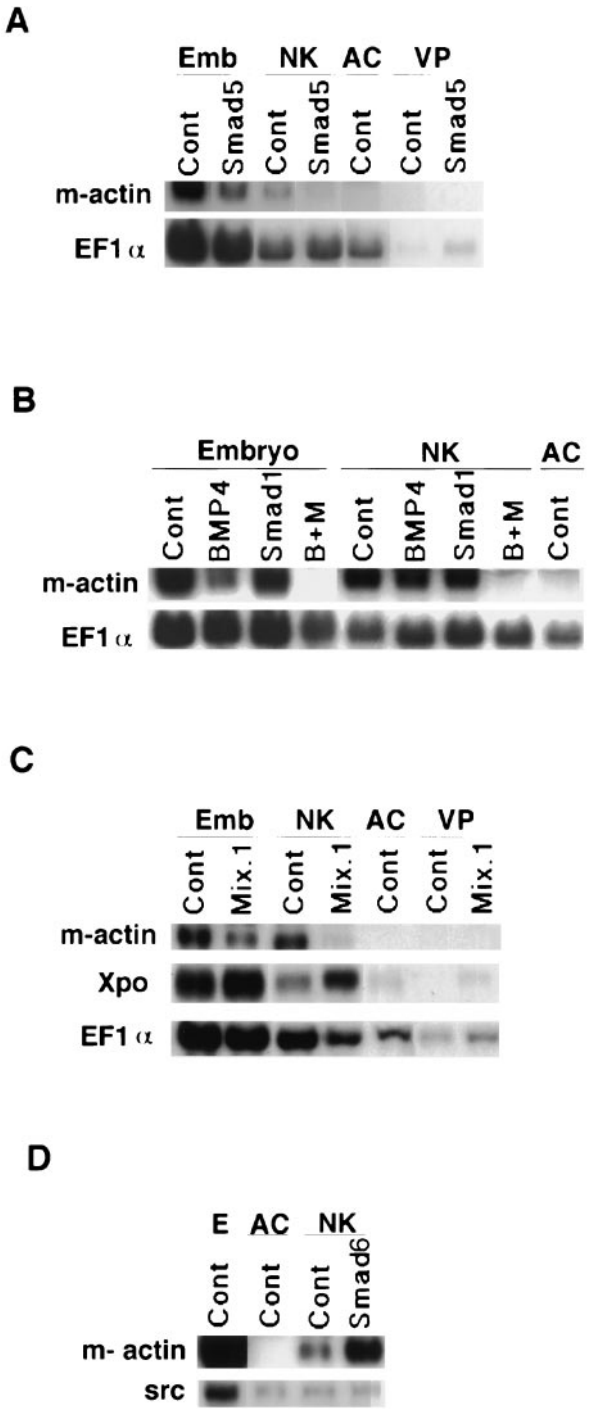

Figure 4 Ectopic expression of Smad and Mix.1 proteins in vegetal poles inhibits dorsal and increases ventral mesoderm induction in recombinant Nieuwkoop explants. (A) Embryos at the one-cell stage were injected vegetally with 2 ng of *in vitro* transcribed *Smad5* RNA. Blastula stage VPs removed from injected and uninjected embryos were recombined with AC ectoderm removed from uninjected embryos at the same stage. Fourteen recombinant explants were grown until stage 20 and total RNA was isolated. Total RNA was also isolated from nine unrecombined VP and AC cultured explants and from pools of five control and *Smad5* injected embryos. For Northern analysis, nine Nieuwkoop explants (NK) equivalents and one embryo (Emb) equivalent of RNA were loaded per well. The filter was sequentially hybridized with the *Xenopus* cDNA probes for *muscle actin* (*m-actin*) and *EF1α*. The DAI of the *Smad5* injected embryos was 1.9 ($n = 18$). (B) Embryos at the one-cell stage were injected vegetally with either 2.5 ng of *in vitro* transcribed *XSmad1* or 0.25 ng of *BMP-4* RNA or both. Nine recombinant explants from each injected group were grown to stage 20 and total RNA was isolated for Northern analysis. Total RNA was also isolated from cultured explants and from pools of five control, *XSmad1*, *BMP-4* and double injected embryos. For Northern analysis, nine AC and Nieuwkoop explants (NK) and one embryo equivalent of RNA were loaded per well. The filter was sequentially hybridized with the *Xenopus* cDNA probes for *muscle actin* (*m-actin*) and *EF1α*. VP explants are not shown, but muscle actin transcripts were never detected in VP explant RNA, even after long exposure times. The DAIs of the *XSmad1* ($n = 41$) and *BMP-4* ($n = 48$) injected embryos were 2.3 and 1.8, respectively. The double injected embryos had a DAI of 0.1 ($n = 36$). Over 5 ng of injected *XSmad1* RNA alone was required to obtain a DAI of < 1. (C) Embryos at the one-cell stage were injected vegetally with 2 ng of *in vitro* transcribed *Mix.1* RNA. Recombinant Nieuwkoop explants as described in (A) were grown to stage 20 and total RNA was isolated for Northern analysis. The filter was sequentially hybridized with the *Xenopus* cDNA probes for *muscle actin* (*m-actin*), *Xpo* and *EF1α*. (D) Embryos at the one-cell stage were injected vegetally with 1.5 ng of *in vitro* transcribed *Smad6* RNA. Recombinant Nieuwkoop explants as described in (A) were grown to stage 20 and total RNA was isolated for Northern analysis. The filter was sequentially hybridized with the *Xenopus* cDNA probes for *muscle actin* (*m-actin*) and *src*. VP explants are not shown, but *muscle actin* transcripts were never detected in VP explant RNA, even after long exposure times.

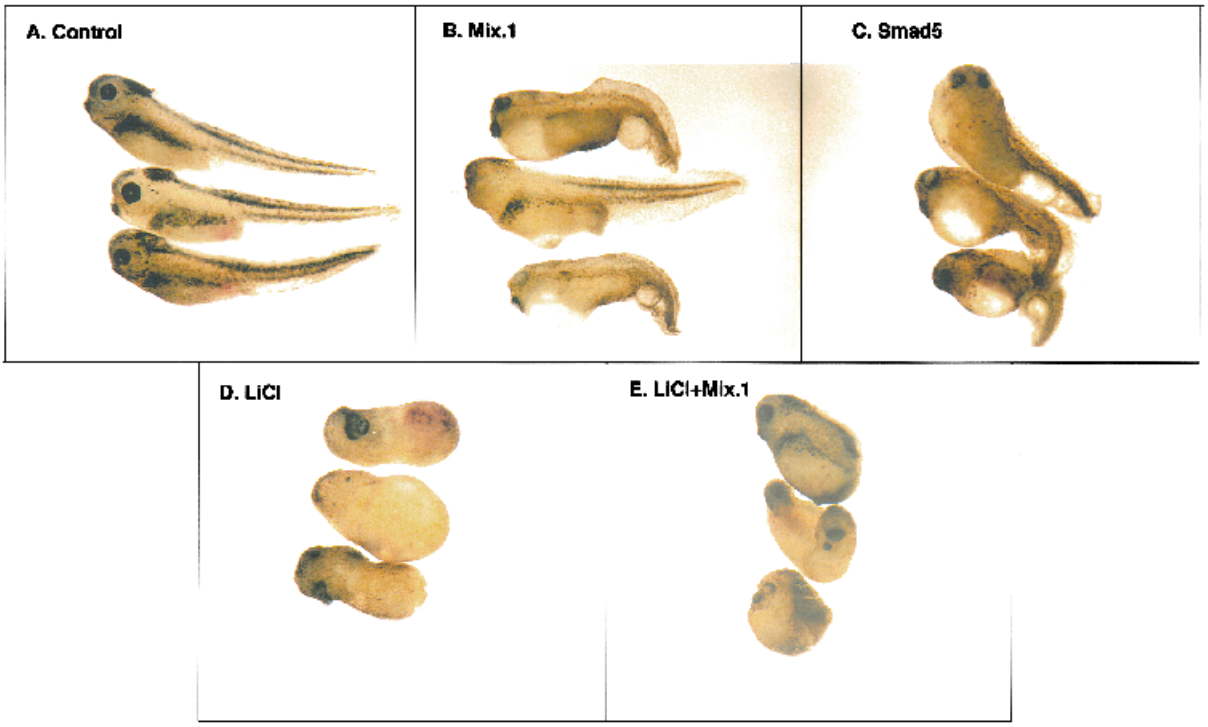

Figure 5 Ectopic expression of *Smad5* or *Mix.1* in D tier vegetal blastomeres ventralizes embryos. (A) Three representative stage 41 control embryos. (B) Three representative *Mix.1* injected (200 pg) embryos. (C) Three representative *Smad5* injected (200 pg) embryos. (D) Three representative LiCl dorsalized, stage 41 embryos. (E) Three representative *Mix.1* injected (200 pg), LiCl dorsalized, stage 41 embryos.

Induction of the ventroposterior marker *Xpo* (Sato & Sargent 1991) is greater in *Mix.1* recombinant Nieuwkoop explants vs. controls (Fig. 4C). Similar *Xpo* expression levels were also observed in Nieuwkoop recombinants in which the VP expressed ectopic levels of *BMP-4* RNA (not shown). These results suggest that dorsal, but not ventral mesoderm induction, is disrupted by ectopic *Mix.1* expression. Since *Mix.1* is an early response gene expressed in the VP at midblastula stages (Rosa 1989), it is a candidate molecule for regulating the ventral inducing centre formation in the VP.

We examined the *Smad5* and *Mix.1* regulation of vegetal inducing centres in whole embryos. At the 32-cell stage, control embryos were injected into two tier-D vegetal blastomeres with either 200 pg of *Smad5* or *Mix.1* RNA and 40 pg of β-*gal* RNA, which served as a lineage tracer. The *Mix.1* and *Smad5* injected embryos (Figs 5B, 5C) both had a DAI of 3.8 ($n = 23$ per injected group) in comparison to β-*gal* injected controls (Fig. 5A), having a DAI of 5.0 ($n = 29$). Ectopic expression of *Mix.1* in two tier-D blastomeres of LiCl-dorsalized embryos lowered the DAI from ≈9 to 8 ($n = 20$ per group, Figs 5D, 5E). Thus, the ectopic expression of Smad5 or Mix.1 proteins in the VP partially hyperventralizes normal embryos.

The Smad6 protein is an antagonist of the BMP/Smad1 signalling pathway; Smad6 binds Smad1 to form an inactive complex (Hata *et al.* 1998). *Smad6* RNA was vegetally injected into one-cell embryos and VPs were used in Nieuwkoop recombinant explants. As assayed by *muscle actin* expression, *Smad6* stimulated dorsal mesoderm induction in the Nieuwkoop assay, since *muscle actin* expression increased by fourfold in comparison to the control explants (Fig. 4D). *Smad6 RNA* was also injected into two tier-D vegetal blastomeres in UV-irradiated hyperventralized embryos. Similar to embryos injected with *BMP 2/4 DNR* RNA (Fig. 3D), ectopic *Smad6* expression also increased the DAI of hyperventralized embryos by at least one DAI unit (not shown). Thus, the inhibition of endogenous Smad1 signalling by Smad6 can alter the inductive potential of the vegetal pole.

Discussion

We have shown that BMP-4 signalling can regulate the formation of vegetal mesoderm induction centres during *Xenopus* development. Ectopic *BMP-4* expression in VP cells inhibits the expression of dorsal

mesoderm markers in recombinant Nieuwkoop explants at gastrula and neurula stages, while levels of ventral markers are increased in these explants. Ectopic *BMP-4* expression in tier D vegetal blastomeres eliminated the dorsalizing effects of LiCl. The ectopic expression of the *BMP 2/4 DNR* in VPs caused an increase in dorsal marker expression and a reduction in ventral marker expression in recombinant Nieuwkoop explants. Ectopic expression of the *BMP 2/4 DNR* in tier D vegetal blastomeres also partially rescued the ventralizing effects of UV-irradiation. These results suggest that BMP signalling may be required for the proper formation of ventral vegetal inducing centres during early *Xenopus* development.

These experiments with ectopic BMP ligand expression cannot unequivocally rule out an inductive role for BMP signalling in the Nieuwkoop induction assay, since BMP secreted from the vegetal pole could alter cell fate in overlying animal pole cells. To examine a strictly cell-autonomous role for BMP signalling in the vegetal pole, we examined the effect of ectopic Smad protein expression in vegetal inducing centre formation. Ectopic *Smad5* expression in the vegetal pole efficiently inhibited the induction of *muscle actin* expression in Nieuwkoop explants. Injection of *Smad5* RNA into tier D vegetal blastomeres ventralized normal embryos and partially inhibited hyperdorsalization by LiCl. Ectopic *XSmad1* alone was fairly inefficient at altering muscle actin induction, but in VPs co-injected with small amounts of *BMP-4* RNA and *XSmad1*, muscle induction was highly inhibited. These results suggest that *XSmad1* and *Smad5* may differ in their efficiency in mediating BMP signalling in the VP. XSmad1 alone may be under tight regulation, since it ventralized only in conjunction with BMP-4 stimulation. Perhaps the heterologous nature of the murine Smad5 protein enables an easier disruption of the BMP signalling pathway in *Xenopus*, or perhaps *Smad5* is a more potent ventralizer of vegetal inducing centres. The Smad 6 protein antagonizes signal transduction by Smad1 (Hata *et al.* 1998). Similar to ectopic expression of the *BMP 2/4 DNR*, ectopic expression of *Smad6* in the VP dorsalized the embryos and recombinant explants. These results support a strong role for cell autonomous BMP-Smad signalling pathways in regulating mesoderm-inducing centre formation in the VP.

Mix.1 was also ectopically expressed in VPs. Similar to the Smads, *Mix.1* also works in a cell autonomous manner. Ectopic *Mix.1* expression reduced dorsal mesoderm marker expression while increasing ventral mesoderm marker expression in Nieuwkoop explants. Ectopic *Mix.1* expression in D tier vegetal blastomeres partially ventralized normal embryos and partially rescued LiCl-dorsalized embryos. *Mix.1* is zygotically expressed in both the VP and the marginal zone at the onset of its transcription at the midblastula transition (Rosa 1989). Previous studies did not identify if the ventralizing activity of Mix.1 protein was a result of expression in the vegetal pole or the marginal zone (Mead *et al.* 1996). Our results show that Mix.1 can ventralize mesoderm by altering vegetal inducing centres. Ectopic BMP expression in isolated VP and AC explants activates *Mix.1* transcription (Fig. 1A; Re'em-Kalma *et al.* 1995; Mead *et al.* 1996). Therefore, BMP signalling may be required for *Mix.1* transcriptional activation in ventral VP regions.

Our data supports the idea that autonomous BMP signalling within the VP regulates vegetal inducing-centre formation (see Summary figure). The BMP-4 and BMP-2 ligands, the BMP 2/4 receptor and the XSmad1 protein, are all maternally expressed (Koster *et al.* 1991; Graff *et al.* 1994; Clement *et al.* 1995; Hemmati-Brivanlou & Thomsen 1995; Graff *et al.* 1996; Thomsen 1996), thus the ventral vegetal inducing centre could be established by maternal components of the BMP pathway. This maternal BMP signalling pathway may activate zygotic *Mix.1* expression in the ventral VP region. Our results suggest a role for the Mix.1 protein in establishing the ventral vegetal inducing centre (Summary figure). *Mix.1* expression could be activated by BMP on the ventral vegetal side and by vg1/activin on the dorsal side. Mix.1 activity could then be inhibited by heterodimer formation with the siamois protein on the dorsal vegetal side (Lemaire *et al.* 1995; Mead *et al.* 1996; Heasman 1997). *Mix.1* activation by BMP/Smad signalling on the ventral vegetal side may be analogous to the activation of *siamois* by maternal Xwnt/β-catenin signalling on the dorsal vegetal side. Thus, as in the case of zygotic *siamois* expression in the Nieuwkoop centre, *Mix.1* can act as a zygotic ventral inducing component in the ventral vegetal pole cells. Models of early mesoderm induction propose a maternal role for vegetal pole inducing centres; this work strongly suggests a role for the combined activities of maternal and zygotic components. It is still unclear if BMP/Smad signalling activates other target proteins in addition to Mix.1, which may specify the ventral vegetal inducing centre. Further experiments will be needed to determine if Mix.1 suffices to make a ventral vegetal inducing centre, or if other maternal or zygotic proteins are required.

Experimental procedures

Xenopus embryos and explants

Ovulation, *in vitro* fertilization, embryo culture and dissections

were carried out as previously described (Re'em-Kalma *et al.* 1995). Embryos were staged according to Nieuwkoop & Faber (1967). Vegetal pole and animal cap explants were removed at stages 8–9 using an eyebrow knife. Recombinant explants grown in 1X Steinberg's medium were juxtaposed to one another with watchmaker's forceps. LiCl treatment of embryos was carried out as previously described (Bonstein *et al.* 1998). UV-irradiation of embryos was carried out as described (Frank & Harland 1991).

RNA injections and lineage tracing

In vitro transcribed full-length RNAs encoding the *Xenopus* BMP-4 (Dale *et al.* 1992), *Xenopus* BMP 2/4 dominant-negative receptor (Graff *et al.* 1994), XSmad1 (Thomsen 1996), Smad5 (Suzuki *et al.* 1997), Smad6 (Hata *et al.* 1998) and Mix.1 (Rosa 1989) proteins were prepared according to Smith & Harland (1991). Different concentrations of RNA (0.25–2.5 ng) were injected into the vegetal hemisphere of one-cell embryos. In each experiment, 15–40 embryos developed until stage 40. At this stage the dorsal-anterior index (DAI) was determined (Kao *et al.* 1988). Embryos at the 32-cell stage were co-injected in one or two tier-D vegetal blastomeres with 20–200 pg of RNA encoding β-galactosidase (β-gal) protein, in addition to 40–200 pg of one of the RNAs described above. β-gal detection was performed as previously described with either blue or red staining reagents (Bonstein *et al.* 1998).

Northern blot analysis

RNA was extracted from embryos and prepared for Northern blot analysis as described (Re'em-Kalma *et al.* 1995). Electrophoresis, probe preparation, filter hybridization and exposure was performed as described (Re'em-Kalma *et al.* 1995). Quantification was performed using a Fuji phosphor-imaging system. *EF1α* or *src* were used as positive standards for comparing the amounts of RNA loaded per well at any given stage.

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References

Baker, J.C. & Harland, R.M. (1996) A novel mesoderm inducer, Madr2, functions in the activin signal transduction pathway. *Genes Dev.* **10**, 1880–1889.

Blumberg, B., Wright, C.V.E., De Robertis, E.M. & Cho, K.W.Y. (1991) Organizer-specific homeobox genes in *Xenopus* laevis embryos. *Science* **253**, 194–196.

Bonstein, L., Elias, S. & Frank, D. (1998) Paraxial-fated mesoderm is required for neural crest induction in *Xenopus* embryos. *Dev. Biol.* **193**, 156–168.

Clement, J.H., Fettes, P., Knochel, S., Lef, J. & Knochel, W. (1995) Bone Morphogenetic protein 2 in the early development of *Xenopus laevis*. *Mech. Dev.* **52**, 357–370.

Dale, L., Howes, G., Price, M.J. & Smith, J.C. (1992) Bone morphogenetic protein-4: a ventralizing factor in early *Xenopus* development. *Development* **115**, 573–585.

Dale, L. & Slack, J.M. (1987) Regional specification within the mesoderm of early embryos of *Xenopus* laevis. *Development* **100**, 279–295.

Fainsod, A., Steinbeisser, H. & De Robertis, E.M. (1994) On the function of *BMP-4* in patterning the marginal zone of the *Xenopus* embryo. *EMBO J.* **13**, 5015–5025.

Frank, D. & Harland, R.M. (1991) Transient expression of XMyoD in non somitic mesoderm of *Xenopus* gastrulae. *Development* **113**, 1387–1393.

Gawantka, V., Delius, H., Hirschfeld, K., Blumenfeld, C. & Niehrs, C. (1995) Antagonizing the Spemann organizer: role of the homeobox gene *Xvent-1*. *EMBO J.* **14**, 6268–6279.

Graff, J.M., Bansal, A. & Melton, D.A. (1996) *Xenopus* Mad proteins transduce distinct subsets of signals for the TGFβ family. *Cell* **85**, 479–487.

Graff, J.M., Thies, R.S., Song, J.J., Celeste, A.J. & Melton, D.A. (1994) Studies of a *Xenopus* BMP receptor suggest that ventral mesoderm-inducing signals override dorsal signals in vivo. *Cell* **79**, 169–179.

Greene, J.M., Otani, H., Good, P.J. & Dawid, I.B. (1993) A novel family of retrotransposon-elements in *Xenopus* laevis with a transcript inducible by two growth factors. *Nucl. Acids Res.* **21**, 2375–2381.

Hata, A., Lagna, G., Massague, J. & Hemmati-Brivanlou, A. (1998) Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. *Genes Dev.* **12**, 186–197.

Heasman, J. (1997) Patterning of the *Xenopus* blastula. *Development* **124**, 4179–4191.

Heldin, C.H., Miyazono, K. & ten Dijke, P. (1997) TGF-β signaling from cell membrane to nucleus through SMAD proteins. *Nature* **390**, 465–471.

Hemmati-Brivanlou, A. & Melton, D. (1992) A truncated activin receptor dominantly inhibits mesoderm induction and formation of axial structures in *Xenopus* embryos. *Nature* **359**, 609–614.

Hemmati-Brivanlou, A. & Thomsen, G. (1995) Ventral mesodermal patterning in *Xenopus* embryos: expression patterns and activity of BMP-2 and *BMP-4*. *Dev. Genet.* **17**, 78–89.

Jones, C.M., Dale, L., Hogan, B.L.M., Wright, C.V.E. & Smith, J.C. (1996) Bone morphogenetic protein-4 (*BMP-4*) acts during gastrula stages to cause ventralization of *Xenopus* embryos. *Development* **122**, 1545–1554.

Jones, C.M., Lyons, K.M., Lapan, P.M., Wright, C.V.E. & Hogan, B.L.M. (1992) DVR-5 (Bone Morphogenetic Protein-4) as a posterior-ventralizing factor in *Xenopus* mesoderm induction. *Development* **115**, 639–647.

Kao, K.R. & Elinson, R.P. (1988) The entire mesodermal mantle behaves as Spemann's organizer in dorsoanterior enhanced *Xenopus* laevis embryos. *Dev. Biol.* **127**, 64–77.

Kessler, D.S. & Melton, D.A. (1994) Vertebrate embryonic induction: mesodermal and neural patterning. *Science* **266**, 596–604.

Koster, M., Plessow, S., Clement, J.H., Lorenz, A., Tiedmann, H. & Knochel, W. (1991) Bone Morphogenetic protein 4

(*BMP-4*), a member of the TGF-β family, in early embryos in *Xenopus laevis*: analysis of mesoderm inducing activity. *Mech. Dev.* **33**, 191–200.

Lemaire, P., Garrett, N. & Gurdon, J.B. (1995) Expression cloning of *siamois*, a *Xenopus* homeobox gene expressed in dorsal-vegetal cells of blastulae and able to induce a complete secondary axis. *Cell* **81**, 85–94.

Lettice, L.A. & Slack, J.M. (1993) Properties of the dorsalizing signal in gastrulae of *Xenopus* laevis. *Development* **117**, 263–271.

Lyons, K.M., Jones, C.M. & Hogan, B.L.M. (1991) The DVR gene family in embryonic development. *Trends Genet.* **7**, 408–412.

Massague, J. (1996) TGFβ signaling: Receptors, transducers, and mad proteins. *Cell* **85**, 947–950.

Mead, P.E., Brivanlou, I.H., Kelley, C.M. & Zon, L.I. (1996) *BMP-4* responsiveness regulation of dorsal-ventral patterning by the homeobox protein *Mix.1. Nature* **382**, 357–360.

Nieuwkoop, P.D. (1969) The formation of mesoderm in urodelan amphibians. I. The induction by the endoderm. Roux's Arch. *Dev. Biol.* **162**, 341–373.

Nieuwkoop, P. & Faber, J. (1967) *Normal Table of Xenopus Laevis (Daudin)*. Amsterdam: North-Holland Publishing Company.

Onichtchouk, D., Gawantka, V., Dosch, R., *et al.* (1996) The *Xvent-2* homeobox gene is part of the *BMP-4* signalling pathway controlling the dorsoventral patterning of *Xenopus* mesoderm. *Development* **122**, 3045–3053.

Papalopulu, N. & Kintner, C. (1996) A *Xenopus* gene, Xbr-1, defines a novel class of homeobox genes and is expressed in the dorsal ciliary margin of the eye. *Dev. Biol.* **174**, 104–114.

Re'em-Kalma, Y., Lamb, T. & Frank, D. (1995) Competition between noggin and bone morphogenetic protein 4 activities may regulate dorsalization during *Xenopus* development. *Proc. Natl. Acad. Sci. USA* **92**, 12141–12145.

Rosa, F.M. (1989) *Mix.1*, a homeobox mRNA inducible by mesoderm inducers, is expressed mostly in the presumptive endodermal cells of *Xenopus* embryos. *Cell* **57**, 965–974.

Sato, S.M. & Sargent, T.D. (1991) Localized and inducible expression of *Xenopus-posterior (Xpo),* a novel gene active in early frog embryos, encoding a protein with a 'CCHC' finger domain. *Development* **112**, 747–753.

Schmidt, J.E., Suzuki, A., Ueno, N. & Kimelman, D. (1995) Localized *BMP-4* mediates dorsal/ventral patterning in the early *Xenopus* embryo. *Dev. Biol.* **196**, 37–50.

Schmidt, J.E., von Dassow, G. & Kimelman, D. (1996) Regulation of dorsal-ventral patterning: the ventralizing effects of the novel *Xenopus* homeobox gene *Vox*. *Development* **122**, 1711–1721.

Smith, W.C. & Harland, R.M. (1991) Injected Xwnt-8 RNA acts early in *Xenopus* embryos to promote formation of a vegetal dorsalizing center. *Cell* **67**, 753–765.

Smith, J.C., Price, B.M.J., Green, J.B.A., Weigel, D. & Herrmann, B.G. (1991) Expression of a *Xenopus* homolog of *Brachyury (T)* is an immediate-early response to mesoderm induction. *Cell* **67**, 79–87.

Steinbeisser, H., Fainsod, A., Niehrs, C., Sasai, Y. & DeRobertis, E.M. (1995) The role of GSC and *BMP-4* in dorsal-ventral patterning of the marginal zone in *Xenopus*: a loss of function study using antisense RNA. *EMBO J.* **21**, 5230–5243.

Stewart, R.M. & Gerhart, J.C. (1990) The anterior extent of dorsal development of the *Xenopus* embryonic axis depends on the quantity of organizer in the late blastula. *Development* **109**, 363–372.

Sudwarti, S. & Nieuwkoop, P.D. (1971) Mesoderm formation in the anuran *Xenopus* laevis (Daudin). *Roux's Arch. Dev. Biol.* **166**, 189–204.

Suzuki, A., Chang, C., Yingling, J.M., Wang, X.F. & Hemmati-Brivanlou, A. (1997) *Smad5* induces ventral fates in *Xenopus* embryo. *Dev. Biol.* **184**, 402–405.

Thomsen, G. (1996) *Xenopus mothers against decapentaplegic* is an embryonic ventralizing agent that acts downstream of the BMP-2/4 receptor. *Development* **122**, 2359–2366.

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