Xenopus X-box binding protein 1, a leucine zipper transcription factor, is involved in the BMP signaling pathway

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Abstract

We describe a novel basic leucine zipper transcription factor, XXBP-1, which interacts with BMP-4 in a positive feedback loop. It is a maternal factor and is zygotically expressed in the dorsal blastopore lip and ventral ectoderm with the exception of the prospective neural plate during gastrulation. Overexpression of XXBP-1 leads to ventralization of early embryos as described for BMP-4, and inhibits neuralization of dissociated ectoderm. Consistent with mediating BMP signaling, we show that the ectopic expression of XXBP-1 recovers the expression of epidermal keratin and reverses the dorsalization imposed by truncated BMP receptor type I, indicating that it may act downstream of the BMP receptor. Its effects can be partially mimicked by a fusion construct containing the VP16 activator domain and the XXBP-1 DNA-binding domain. In contrast, fusing the DNA-binding domain to the even-skipped repressor domain leads to upregulation of the neural markers NCAM and nrp-1 in animal cap assay. Taken together, the results suggest a role for XXBP-1 in the control of neural differentiation, possibly as an activator.

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Introduction

Embryonic development requires differential temporal and spatial gene expression as well as cell-to-cell interactions, including embryonic induction. In Xenopus, antero-posterior and dorsoventral pattern formation depends in part on the interaction of dorsalizing genes and bone morphogenetic proteins (BMPs)1 (Grunz and Tacke, 1989, 1990; Wilson and Hemmati-Brivanlou, 1995; for review, see Weinstein and Hemmati-Brivanlou, 1997). Disruption of the BMP feedback loop in ectoderm initiates the process of neural induction, resulting in the suppression of epidermal-specific genes and the activation of regulatory and structural genes specific for neural tissues (Weinstein and Hemmati-Brivanlou, 1997; Hemmati-Brivanlou and Melton, 1997). During gastrulation, dorsalizing factors such as noggin (Zimmerman et al., 1996), Xnr3 (Smith et al., 1995), Chordin (Sasai et al., 1995), follistatin (Fainsod et al., 1997), Cerberus (Piccolo et al., 1999), and Dickkopf 1 (Glinka et al., 1998) antagonize extracellular BMPs by preventing their interaction with cognate BMP receptors. In the absence of organizer signals, ectoderm differentiates into epidermis and ventral mesoderm, due in part to the activity of BMPs (Knöchel et al., 1987; Köster et al., 1991; Plessow et al., 1991).

In addition to their role as negative regulators of neural induction, BMPs act as morphogens in dorsoventral mesoderm patterning. At least four mesoderm-related territories can be defined in early gastrula stages: (1) the organizer area, expressing genes such as goosecoid, Xnot, Chordin, and noggin; (2) a dorsolateral territory, expressing genes such as Xmyf5 and Xvent-2; (3) a ventrolateral territory, marked by the expression of Xvent-1 and Xvent-2; and (4) a ventral territory coexpressing Xvent-1, Xvent-2, and sizzled (Dale and Jones, 1999). The expression of these genes is
regulated by BMP-4 in a dose-dependent manner, resulting in three domains of the marginal zone (Dosch et al., 1997). Overexpression of BMP-2, -4, and -7 leads to ventralization of early mesoderm (Dale and Jones, 1999). Blocking BMP-4 signaling in the ventral part of the Xenopus embryo prevents blood formation and induces formation of a secondary dorsal axis (for review, see Graff, 1997).

Besides their involvement in ventral mesoderm formation and neural fate repression, BMPs also play an essential role in limb and tooth development and regulation of apoptosis. The different effects depend on which intracellular cofactors participate in BMP signaling and crosstalk between BMP signaling and other signaling pathways (for review, see von Bubnoff and Cho, 2001). BMPs as well as other members of the transforming growth factor β family utilize the signal transduction cascade mediated by the Smad family of proteins (Massagué, 1996). In Xenopus embryos, Smad1 and Smad5 can mimic many BMP functions and activate BMP-inducible genes (Graff et al., 1996; Suzuki et al., 1997; Henningfeld et al., 2000), while overexpression of Smad8 phenocopies the effect of blocking BMP-4 signaling, leading to the induction of a secondary axis on the ventral side of intact embryos and to direct neural induction in ectodermal explants (Nakayama et al., 1998).

There is abundant evidence that a large number of genes are involved in BMP signaling in different cell types and tissues. In this study, we present data that Xenopus X-box binding protein 1 (XXBP-1), a basic leucine zipper transcription factor, is involved in the BMP signaling pathway. Overexpression of XXBP-1 leads to ventralization of early embryos as does BMP-4. In addition, ectopic XXBP-1 can inhibit neural induction and dorsalization imposed by dominant negative BMP receptor type I (tBMPRI). These results indicate that XXBP-1 mediates epidermal induction as well as inhibition of neural differentiation in the BMP signaling pathway downstream of the BMP receptor. Moreover, we suggest that XXBP-1 functions as a transcription activator involved in the repression of neural fate.

Materials and methods

Embryo manipulations

In vitro fertilization was carried out as previously described (Zhao et al., 2001). Embryos were staged according to Nieuwkoop and Faber, (1975). For UV treatment, fertilized eggs were dejellied thoroughly approximately 20 min after insemination, then immediately transferred to Petri-er petri dishes with a UV-permeable bottom (Heraeus, Hanau, DE) and treated with a UV transilluminator for 45 s. Eggs were left undisturbed at room temperature for 2 h or at least until the first cleavage. Animal caps were dissected from stage 9 embryos in Holtfreter’s solution and cultured in 1 × Barth’s solution until intact embryos from the same batch reached the desired stages. Dorsal and ventral marginal zones (DMZs and VMZs) were isolated at stage 10-10.5. DMZs corresponded to a 60° arc of marginal zone tissue centered at the dorsal lip midline. VMZs were cut analogously from the region opposite the DMZs and then were cultured in 1 × Barth’s solution until the desired stages. The dissociation procedure was described elsewhere in detail (Grunz, 1969; Grunz and Tacke, 1989). Briefly, the animal caps dissected at stage 9 were disaggregated in Ca²⁺/Mg²⁺-free Barth’s solution by gently pipetting with a Pasteur pipette in an agar-coated petri dish. The single cells were kept in Ca²⁺/Mg²⁺-free Barth’s solution for 1 h, followed by normal Barth’s solution for 3 h, then were reaggregated. The reaggregates were harvested for RT-PCR analysis after culture at room temperature overnight.

Constructs

A ZAP Express phage cDNA library constructed from activin-treated ectoderm was screened by using large-scale whole-mount in situ hybridization. XXBP-1 was selected from several interesting clones for further functional study. The coding region was subcloned into pCS2+ (Turner et al., 1994) for microinjection. The DDBJ/EMBL/GenBank Accession No. of XXBP-1 is AF358133.

To construct XXBP1-VP16, the sequence encoding the VP16 activation domain was fused with the sequence encoding the first 126 amino acids of XXBP-1 containing the basic leucine zipper domain (bZIP; see Fig. 6l). The bZIP domain is thought to be responsible for the dimerization and DNA binding in leucine zipper transcription factors (Lewin, 2000). The VP16 activation domain sequence (VP16, 261 bp; Friedman et al., 1988) was amplified from the VPXvent-1 plasmid (Onichtchouk et al., 1998) via PCR using the pfu DNA polymerase and the primers: 5′-GGGCTCTAGATAGTCATTCAAGGGCATTGGT (XbaI site and the stop codon are in boldface). The amplified products were digested with XbaI and gel purified. The bZIP sequence of XXBP-1 (encoding the N-terminal 126 amino acids) was amplified from the pCS2-XXBP-1 plasmid via PCR using pfu DNA polymerase and the primers: 5′-GGGCTCTAGATAGTCATTCAAGGGCATTGGT (XbaI cutting site in boldface) and 5′-GGGCTCTAGATAGTCATTCAAGGGCATTGGT (XbaI site and the stop codon are in boldface). The amplified products were digested with XbaI and gel purified. Both the VP16 and bZIP fragments were cloned into the EcoRI–XbaI-digested pCS2+ to produce XXBP1-VP16. Similarly, the sequence encoding the even-skipped repression domain (Han and Manley, 1993) was fused to the XXBP-1 bZIP sequence to create XXBP1-Eve. The even-skipped repression domain sequence (Eve, 777 bp) was amplified from EveXvent-1 (Onichtchouk et al., 1998) using the primers: 5′-GGGCTCTAGATAGTCATTCAAGGGCATTGGT (XbaI site and the stop codon are in boldface). The amplified products were digested with XbaI and gel purified. Both the VP16 and bZIP fragments were cloned into the EcoRI–XbaI-digested pCS2+ to produce XXBP1-VP16. Similarly, the sequence encoding the even-skipped repression domain (Eve, 777 bp) was amplified from EveXvent-1 (Onichtchouk et al., 1998) using the primers: 5′-GGGCTCTAGATAGTCATTCAAGGGCATTGGT (XbaI site and the stop codon are in boldface). The amplified products were digested with XbaI and gel purified. Both the VP16 and bZIP fragments were cloned into the EcoRI–XbaI-digested pCS2+ to produce XXBP1-VP16. Similarly, the sequence encoding the even-skipped repression domain (Eve, 777 bp) was amplified from EveXvent-1 (Onichtchouk et al., 1998) using the primers: 5′-GGGCTCTAGATAGTCATTCAAGGGCATTGGT (XbaI site and the stop codon are in boldface). The amplified products were digested with XbaI and gel purified. Both the VP16 and bZIP fragments were cloned into the EcoRI–XbaI-digested pCS2+ to produce XXBP1-VP16. Similarly, the sequence encoding the even-skipped repression domain (Eve, 777 bp) was amplified from EveXvent-1 (Onichtchouk et al., 1998) using the primers: 5′-GGGCTCTAGATAGTCATTCAAGGGCATTGGT (XbaI site and the stop codon are in boldface). The amplified products were digested with XbaI and gel purified. Both the VP16 and bZIP fragments were cloned into the EcoRI–XbaI-digested pCS2+ to produce XXBP1-VP16. Similarly, the sequence encoding the even-skipped repression domain (Eve, 777 bp) was amplified from EveXvent-1 (Onichtchouk et al., 1998) using the primers: 5′-GGGCTCTAGATAGTCATTCAAGGGCATTGGT (XbaI site and the stop codon are in boldface). The amplified products were digested with XbaI and gel purified. Both the VP16 and bZIP fragments were clon...
CAGTCTTGTAGGG (XbaI site and the stop codon are in boldface).

The sequence encoding the first 126 amino acids of XXBP-1 containing the bZIP region with a stop codon at the 3’ end was subcloned into pCS2+ to yield truncated XXBP-1. Additionally, this fragment was also cloned into pCS2MTEnR without the stop codon to obtain XBP-EnR with the Drosophila engrailed repressor domain at the carboxyl terminus and an N-terminal myc-tag.

LacZ staining and whole-mount in situ hybridization

LacZ staining was carried out as described previously (Zhao et al., 2001). Whole-mount in situ hybridization was performed as described elsewhere (Harland, 1991; Oschwald et al., 1991).

Microinjection experiments

Capped mRNAs were synthesized from linearized plasmids in vitro by using the Cap Scribe kit (Roche). Dejelled embryos were microinjected in 4% Ficoll 400 dissolved in mids in vitro by using the Cap Scribe kit (Roche). Dejelled microinjection experiments were performed as described elsewhere (Harland, 1991; Oschwald et al., 1991).

Expression pattern of XXBP-1

In whole-mount in situ hybridization (Fig. 2I), XXBP-1 expression was first detectable faintly in the animal half prior to gastrulation. Expression increased particularly in the dorsal blastopore lip at onset of gastrulation (Fig. 2I: D1–D3 for stage 10, E1–E3 for stage 11; Nieuwkoop and Faber, 1975). Additionally, XXBP-1 was expressed to a lesser extent in the entire ventral marginal zone and animal half. As gastrulation progressed, staining was detected as a short dorsal stripe beginning at the blastopore; however, the prospective neural plate was devoid of staining, and strong staining of the ventral area was observed (Fig. 2I: F1–F3 for stage 11.5, G1–G3 for stage 12, H1–H3 for the stage 12.5). At stage 15, XXBP-1 was strongly expressed in the area anterior to the neural plate corresponding to the cement gland primordium (Fig. 2I: I–K). This expression pattern is reminiscent of BMP-4, whose expression is maximal in the cement gland primordium at this stage. To a lesser extent, XXBP-1 was expressed in a stripe starting from the slit-like blastopore at the midline of the neural plate that corresponds to the notochord (Fig. 2I: C and C1). In the late neurula and early tailbud stages, intense staining was observed in the cement gland with two faint extensions to the hatching gland (Fig. 2IL and M). Another area of XXBP-1 expression extended from the cement gland to the ventral side as shown in Fig. 2IM and N. Although there is evidence that human

Results

XXBP-1 is a novel basic leucine zipper transcription factor

The cDNA sequence of the XXBP-1 (GenBank Accession No. AF358133) revealed an open reading frame encoding a polypeptide of 350 amino acids. Between amino acids 85 and 120 of the protein, there is a hypothetical leucine zipper sequence characteristic of basic leucine zipper (bZIP) transcription factors, in which seven residue spacers separate two leucines. A basic region rich in arginine and lysine (amino acid 46–81; Fig. 1) is NH2-terminal to the leucine zipper. The predicted amino acid sequence is most similar to human X-box binding protein 1 (hXBP-1; Accession No. NM_005080, 66% amino acid identity; Liou et al., 1990), rat XBP-1 (Accession No. JG4857, 66% amino acid identity), mouse XBP-1 (Accession No. NP_038870, 66% amino acid identity), and zebrafish Treb5 (Accession No. AY029577, 59% amino acid identity, Liang et al., 2001) as shown in Fig. 1. Although the Xenopus sequence outside the bZIP domain is more divergent as compared with the rat, mouse, human, and zebrafish, it shares high amino acid identity within the bZIP domain (88% amino acid identity to human, 86.7% to mouse, 86.7% to rat, 77.3% to zebrafish). Therefore, it is likely to be the Xenopus homologue and was named Xenopus X-box binding protein 1 (XXBP-1).

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XBP-1 plays an important role in fetal liver development (Reimold et al., 2000), the XXBP-1 expression zone did not correspond to the liver primordium (Fig. 2IIB and B1). In tailbud and tadpole stages, expression was observed in the cement gland, pronephros, ear vesicle, and the upper part of the lens (Fig. 2I: O–R; Fig. 2IID).

The temporal expression of XXBP-1 indicates that XXBP-1 is a maternal factor which is weakly expressed before gastrulation, then upregulated during gastrulation and maintained in the following stages (Fig. 2IIIA). Furthermore, XXBP-1 was ubiquitously expressed in the adult tissues examined with the highest expression levels in liver, lung, and stomach (Fig. 2IIIB).

**Effects of neural inhibitors and neural inducers on the expression of XXBP-1**

The absence of XXBP-1 expression in the prospective neural plate during gastrulation indicates that it may be a negative control factor of neural induction. In respect to both ventralizing and epidermalizing effects on XXBP-1, we investigated the effects of anti-neural factors such as BMP-4, UV irradiation, Msx1, and Dlx3 on the expression of XXBP-1 by whole-mount in situ hybridization. In addition, we used a β-catenin morpholino (MO) as an inhibitor of the Wnt/β-catenin morpholino (MO) pathway to study the expression of XXBP-1 regulated by the Wnt/β-catenin signaling pathway. Both dorsal blastomeres of four-cell-stage embryos were injected with β-catenin MO (8 ng/embryo), BMP-4 (1 ng/embryo), Dlx3 (80 pg/embryo), or Msx1 (80 pg/embryo) mRNA. Whole-mount in situ hybridization with XXBP-1 antisense RNA was performed on the injected embryos at midgastrula (stage 11-12). Overexpression of any of these factors expanded the expression territory of XXBP-1 on the dorsal side of the embryos (Fig. 3I). XXBP-1 was activated in the dorsal as well as ventral marginal zones after injection of BMP-4 mRNA (98%, 42/43 embryos) or β-catenin MO (100%, 23/23 embryos) and in UV-treated embryos (100% 23/23 embryos). XXBP-1 was expressed to a lesser extent after overexpression of either Msx1 (70%, 16/23 embryos) or Dlx3 (100%, 29/29 embryos). The overexpression of Msx1 or Dlx3 expanded the expression of XXBP-1 into the prospective neural plate area. These results indicate that the area where XXBP-1 is expressed can be increased by neural inhibitors. Moreover, XXBP-1 may also be negatively regulated by the Wnt/β-catenin pathway since blockage by the
Fig. 2. Expression of XXBP-1. (I) Spatial expression pattern of XXBP-1. Whole-mount *in situ* hybridization for XXBP-1 at several stages of *Xenopus* early development is shown from different views (dorsal–lateral, dor-lat; vegetal, veg; ventral, ven). (A–C) XXBP-1 is faintly expressed in the animal half at early cleavage stages. (D, E) At the start of gastrulation, XXBP-1 becomes concentrated at the dorsal blastopore lip and is strong in the ventral ectoderm. (F–H) As gastrulation progresses, expression stretches anteriorly from the blastopore, and ventral expression is maintained. (I–K) In the neurula, expression is concentrated in the area anterior to the neural plate (the prospective cement gland) and notochord. Expression also stretches posteriorly along the neural crest in a gradient. (L–N) At stage 19–20, expression is strong in the cement gland, weaker posteriorly in the hatching gland, and in a ventral midline stripe. (O–R) Later stages show expression in the cement gland, pronephros (primarily glomus), ear vesicle, and the upper part of the lens. (II) Histological analysis of XXBP-1 expression. (A) A transverse section through a stage 12 embryo shows expression in the ventral ectoderm (ed) and dorsal blastopore lip (bl, higher magnification in A1). (B) A sagittal section through a stage 18 embryo shows expression in the cement gland (cg) and some ventral mesodermal cells adjacent to ectoderm (arrow, higher magnification in B1). (C) A transverse section through the posterior end of a stage 17 embryo shows expression in the notochord (nc). (C1) Higher magnification of the notochord from (C). (D) A transverse section through a stage 28 embryo shows expression in pronephros (pn). ac, archenteron; bt, blastocoel; en, endoderm; sc, spinal cord; sm, somite. (III) Expression of XXBP-1 in developing embryos and adult tissues. RT-PCR was performed on total RNA extracted from embryos at the different, indicated embryonic stages (A) or adult tissues (B). *Ornithine decarboxylase* (*ODC*) and *histone 4A* (*H4*) were used as loading controls. XXBP-1 is a maternal factor which is weakly expressed before gastrulation, upregulated during gastrulation, and maintained in following stages. XXBP-1 was detected in all adult tissues tested, with higher expression levels in liver, lung, and stomach. br, brain; ey, eye; he, heart; in, intestine; ki, kidney; li, liver; lu, lung; mu, muscle; ne, nerve; ov, ovary; sp, spleen; st, stomach; te, testis; RT-, control without reverse transcriptase.
Embryos injected with $XXBP-1$ (0.2 ng mRNA/embryo) into both dorsal blastomeres at the four-cell stage showed a range of ventralized phenotypes (93%, 81/87 embryos; Fig. 4I), including dorsoanterior index (DAI) 2 to 4 (Kao and Elinson, 1988). The most prominent abnormalities were the suppression of dorsoanterior structures, including the cement gland, eyes, otic vesicles, and in many cases, the entire head. A higher dose of $XXBP-1$ (0.4 ng mRNA/embryo) caused the loss of head structures and axis defects, and was scored as DAI 1 or 2 (100% ventralization, 99/99 embryos). The highest dose of $XXBP-1$ (0.8 ng mRNA/embryo) injected into either both dorsal or all four blastomeres of four-cell-stage embryos resulted in a strong ventralization, including the complete loss of all axial structures, and was classified as DAI of 0 or 1 (93% ventralization with dorsal injection, 38/41 embryos; 100% ventralization with radial injection, 21/21 embryos). Representative phenotypes as well as histological analysis of sections through these embryos are shown in Fig. 4I. Compared with the normal embryo (Fig. 4IF), sections from embryos overexpressing $XXBP-1$ (Fig. 4IB) demonstrated reduction of the notochord and an indiscernible brain or spinal cord (Fig. 4IG). The notochord was either completely absent or severely reduced in embryos injected with the highest amount of $XXBP-1$ RNA (data not shown). The effects of ventral $XXBP-1$ overexpression were dose-dependent, but much less pronounced. Ventral injection of 0.2 ng $XXBP-1$ mRNA per embryo resulted in 12.5% (7/56 embryos) normal, 14% (8/56 embryos) ventralized embryos with a DAI 4-5 and 73% (41/56 embryos) embryos with posterior enlargement (Fig. 5IID). The highest amount of $XXBP-1$ injected ventrally produced microcephaly in 100% of embryos (17/17 embryos).

**Disruption of neural marker genes by overexpression of $XXBP-1$**

The expression pattern of $XXBP-1$ suggests its role as an antagonist of neural induction; moreover, its expression was upregulated by neural antagonists. Next, we asked whether $XXBP-1$ inhibits neural markers. To address this issue, 200 pg $XXBP-1$ mRNA was coinjected with 100 pg $\beta$-galactosidase (LacZ) mRNA as a lineage tracer into a single dorsal blastomere of four-cell-stage embryos. The injected embryos were fixed and stained with X-gal at late neurula stages (stage 18-19). Expression of $XAG2$ (cement gland marker; Aberger et al., 1998), $Otx2$ (anterior neural marker; Blitz et al., 1995), $Rx2A$ (lens marker; Yoshitake et al., 1999), $En2$ (marker for the mid/hindbrain boundary; Hemmati-Brivanlou et al., 1991), $Krox20$ (marker for the r3 and r5; Bradley et al., 1993), $Pax6$ (neural marker; Hollemann et al., 1998), and $Sox3$ (pan neural marker; Penzel et al., 1997) was examined by using whole-mount in situ hybridization (Fig. 4II) in those embryos exhibiting $\beta$-galactosidase activity on only one side of the neural plate region. The overexpression of $XXBP-1$ resulted in the reduction of all

**Overexpression of $XXBP-1$ causes ventralization**

The area of $XXBP-1$ expression overlaps with that of $BMP-4$ and suggests that $XXBP-1$ may play a role in dorsoventral patterning during embryonic development. We used a gain-of-function approach to test this hypothesis.

The image contains a figure (Fig. 3) depicting the effects of neural inhibitors and neural inducers on $XXBP-1$ expression. The text describes various experimental procedures and observations related to the expression and function of $XXBP-1$. It mentions the use of morpholino antisense RNA to disrupt gene expression and the use of RT-PCR to analyze mRNA levels. The text also notes the expression of neural markers such as $BMP-4$, $XXBP-1$, and $Xvent-1$ in embryos and their downregulation upon inhibition or induction. The figure shows whole-mount in situ hybridization results with various antisense RNA probes and sections from embryos overexpressing $XXBP-1$.

**β-catenin MO resulted in an expanded area of $XXBP-1$ expression.**

Next, we investigated the neutralizing effect of neural inducers such as noggin (Zimmerman et al., 1996) and Zic3 (Nakata et al., 1997) on the expression of $XXBP-1$ and $BMP-4$ using the animal cap assay. Either noggin (160 pg/embryo) or Zic3 (160 pg/embryo) was injected into all four blastomeres at the four-cell stage with 160 pg $Dlx1$ or (F) 80 pg $Msx1$ mRNA, and were fixed at midgastrula stages for whole-mount in situ hybridization with $XXBP-1$ antisense RNA. (D) Uninjected embryos were UV irradiated at 302 nm for 45 s. The expression area of $XXBP-1$ was expanded compared with control embryos (E). Whole-mount in situ hybridization and fixation after staining were performed similarly for all embryos to eliminate differences due to overstaining. (II) Gene expression in noggin or Zic3 animal caps. At stage 9, animal caps were dissected from uninjected embryos (AC) or embryos injected into all four blastomeres at the four-cell stage with 160 pg $Zic3$ or 160 pg noggin mRNA. The expression of $BMP-4$, $XXBP-1$, $Xvent-1$, and NCAM was assayed by using RT-PCR from RNA isolated after sibling embryos reached stage 18. ODC served as a loading control. Noggin suppressed $XXBP-1$ and $BMP-4$ expression, but induced NCAM.

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neural markers analyzed: Otx2 (60%, 94/156 embryos), Rtx2A (41%, 47/116 embryos), En2 (49%, 31/63 embryos), Krox20 (71%, 34/48 embryos), Pax6 (50%, 28/56 embryos), and Sox3 (58%, 44/76 embryos). These neural markers were either expressed in a smaller area or the expression level was reduced as seen by decreased staining intensity. In contrast to the neural markers examined, no change of XAG2 expression was observed after injection of the same amount of XXBP-1 mRNA. The inhibition of neural markers suggests that XXBP-1 plays an instructive role in the formation of epidermal tissue. It may act directly or by inducing a secreted molecule, perhaps BMP itself. Alternatively, the perturbation of the neural markers could be a result of direct effects on the mesoderm. In some cases, overexpression of XXBP-1 on one side of the embryo may also have caused improper morphogenetic movements, including defects in gastrulation resulting from injury at the injected side. It cannot be excluded that interference with morphogenetic movements may also have led to the disruption of neural markers. To rule out this possibility, we performed the XXBP-1 overexpression experiments described below in animal caps.

Overexpression of XXBP-1 leads to ventralization of dorsal mesoderm

XXBP-1 is expressed in the dorsal blastopore lip during gastrulation (Fig. 21), suggesting that it may interact with genes affecting dorsoventral patterning during early embryonic development. We therefore studied the influence of XXBP-1 on the dorsal mesodermal marker genes, Chordin (Chd) and goosecoid (gsc), in the dorsal marginal zone (DMZ) using whole-mount in situ hybridization. Embryos were injected with XXBP-1 mRNA (0.1 ng/blastomere) dorsally into the equatorial region at the four-cell stage, and Chd and gsc expression was visualized by using whole-mount in situ hybridization at midgastrula (stages 11-11.5). Overexpression of XXBP-1 led to the suppression of Chd (65%, 37/57 embryos) and gsc (75%, 42/56 embryos), illustrated by their diminished staining in the dorsal blastopore lip (Fig. 4III).

Further evidence for the ventralization of cell fate was provided by RT-PCR analysis of the marker genes involved in dorsoventral patterning. Dorsal marginal zone (DMZ) explants were dissected from embryos radially injected with either low (0.1 ng/blastomere) or high (0.4 ng/blastomere) doses of XXBP-1 mRNA at four-cell stage. The relative expression of various marker genes was examined by RT-PCR in explants cultured until control embryos reached stage 22 (Fig. 4IV). Gsc, a marker for head mesoderm in tailbud embryos, was repressed strongly by XXBP-1. Likewise, Xnot, a marker for notochord, was also suppressed, but to a lesser extent. These results indicate that the formation of both head mesoderm and notochord is suppressed by XXBP-1. Otx2 was downregulated, confirming our in situ hybridization results (Fig. 4IID). Decreased dorsal mesoderm marker expression corresponded to increased ventro-posterior mesodermal marker expression. A dose-dependent upregulation of Xho3, Xvent-1, and BMP-4 was induced by ectopic XXBP-1. Taken together, overexpression of XXBP-1 in the dorsal marginal zone explants led to downregulation of dorsal marker genes, and in parallel, upregulation of ventral marker genes.

XBP-1 prevents neuralization of ectodermal explants induced by disaggregation

XXBP-1 is upregulated by neural inhibitors (Fig. 3I) and downregulated by neural inducers (Fig. 3II), suggesting that it may be involved in epidermal differentiation. It was previously shown that dissociation of ectodermal cells leads to neuralization (Grunz and Tacke, 1989; Godsave and Slack, 1991). We therefore performed a cell dissociation assay to determine whether overexpression of XXBP-1 could inhibit neuralization of disaggregated ectodermal explants. As shown in Fig. 4VA, the disaggregated animal caps (ectodermal cells) were shifted from an epidermal to a neural fate as a result of disaggregation and subsequent reaggregation. In contrast to the intact animal caps, NCAM was upregulated and maintained at a high level after reaggregation. Epidermal keratin was still detected because the outer cell layer of the animal caps was included, which remained partially undissociated. The expression of NCAM was reduced and epidermal keratin was upregulated in explant cells overexpressing either XXBP-1 (0.4 ng/embryo) or BMP-4 (0.8 ng/embryo) compared with cells from the uninjected animal caps. Cell fate change occurred in the absence of mesoderm induction, since the mesodermal markers Xbra and muscle actin were not expressed. Again, these results suggest that XXBP-1 functions as a neural inhibitor in Xenopus ectodermal explants.

It should be mentioned that there was a clear difference in the ability to form reaggregates between normal cells and XXBP-1 overexpressing cells. Large numbers of cells from XXBP-1-injected animal caps did not reaggregate (Fig. 4VD). This was in contrast to control and BMP-4-overexpressing ectodermal cells, which readily formed three-dimensional aggregates (Fig. 4VB and C). These observations indicate that cell adhesion is severely impaired by XXBP-1 overexpression. Only reaggregated cells (indicated by arrow in Fig. 4VD) were harvested for RT-PCR analysis in these experiments.

XXBP-1 reverses dorsalization induced by dominant negative BMP receptor

Both XXBP-1 and BMP-4 are excluded from the prospective neural plate during gastrulation and neurulation based on their expression patterns. Gain-of-function experiments revealed that both XXBP-1 and BMP-4 induced embryo ventralization. Moreover, XXBP-1, like BMP-4, inhibited neuralization of ectodermal explants induced by disaggregation. These similarities imply that the two factors may act
in a common pathway. We tested the relationship between XXBP-1 and the BMP signaling pathway using the animal cap assay and a gain-of-function assay. Either XXBP-1 (0.4 ng XXBP-1 mRNA/embryo), the truncated BMP receptor type I (1.6 ng tBMPRI mRNA/embryo; Suzuki et al., 1994), or a combination of both (1.6 ng tBMPRI and 0.4 ng XXBP-1 mRNA/embryo) was injected into the animal half of each blastomere of the four-cell-stage embryo. Animal caps were dissected from these embryos, and marker gene expression was examined by using RT-PCR analysis (Fig. 5I). The epidermal marker, epidermal keratin, was expressed at the same level in both control and XXBP-1-overexpressing animal caps. In line with previously published data (Suzuki et al., 1994; Sasai et al., 1995), ectopic expression of tBMPRI in ectodermal explants resulted in neural induction, revealed by a strong increase in the expression of the neural markers, Otx2 and NCAM, and decreased expression of epidermal keratin. The expression of epidermal keratin was partially restored accompanied by decreased Otx2 and NCAM expression after tBMPRI and XXBP-1 coinjection. Taken together, the ectodermal cell responded to the combination of tBMPRI and XXBP-1 by adopting a cell fate inclined to epidermis in contrast to the neural fate induced by tBMPRI alone. XXBP-1 attenuated neural induction by tBMPRI in the animal cap assay. Thus, XXBP-1 is likely to be downstream of the BMP receptor.

Gain-of-function experiments also support XXBP-1 action to be downstream of the BMP receptor. Either tBMPRI or XXBP-1 alone or both tBMPRI and XXBP-1 were injected ventrally or radially into four-cell-stage embryos. Ectopic tBMPRI (1.6 ng mRNA per embryo) led to the formation of a secondary embryonic axis when injected ventrally (43%, 23/53 embryos; Fig. 5IIB), and caused dorsalization, indicated by the enlargement of anterior structures, when injected radially (100%, 27/27 embryos; Fig. 5IIE). The dorsalized phenotype was efficiently reversed by coinjection with XXBP-1. Embryos coinjected ventrally with mRNA for both XXBP-1 and tBMPRI (0.2 ng XXBP-1 mRNA and 1.6 ng tBMPRI mRNA per embryo) (Fig. 5IIC) exhibited similar phenotypes to embryos ventrally injected with XXBP-1 mRNA alone (0.2 ng mRNA per embryo; Fig. 5IID). XXBP-1 and tBMPRI coinjected embryos (94.3%, 82/87 embryos) showed a slight repression of anterior axis with a significant posterior enlargement. Additionally, the embryos coinjected radially with XXBP-1 and tBMPRI (0.4 ng XXBP-1 mRNA and 1.6 ng tBMPRI mRNA per embryo) showed a range of ventralized phenotypes signified by the reduced anterior structures (92%, 11/12 embryos; Fig. 5IF and G). In summary, these data support the hypothesis that XXBP-1 is a component of the BMP signaling pathway and acts downstream of the BMP receptor.

**XXBP-1 may act as a transcriptional activator**

Sequence comparison showed that XXBP-1 is a basic leucine zipper transcription factor. This gives rise to the question whether XXBP-1 acts as an activator or repressor of transcription. We synthesized three repressor and activator constructs by fusion of the putative DNA-binding domain of XXBP-1 to the previously characterized sequences encoding the activator or repressor domains of known transcription factors. XXBP1-EnR was fused with the repressor domain from Drosophila engrailed (Jaynes and O’Farrell, 1991), XXBP1-Eve with the repressor domain of even-skipped (Han and Manley, 1993), and XXBP1-VP16 with the activator domain of VP16 (Friedman et al., 1988). A schematic diagram of wild-type XXBP-1 and the fusion constructs is shown in Fig. 6I. Theoretically, the fusion constructs should completely or, at least partially, mimic wild-type XXBP-1 in the overexpression experiments if they have similar actions on transcription as XXBP-1. Moreover, as XXBP-1 could mediate BMP signaling, it was expected that the loss of XXBP-1 function might lead to neural induction in animal cap assays or formation of a secondary embryonic axis in overexpression experiments. In addition, based on the expectation that truncated XXBP-1 may sequester wild-type XXBP-1 and interfere with dimerization, a truncated XXBP-1 construct containing the sequence encoding the basic leucine zipper domain was also created.

Overexpression of high doses of fusion constructs caused apoptosis. The dosages were, therefore, adjusted to avoid this phenotype. Embryos microinjected ventrally with the same dose (0.16 ng mRNA/embryo) of either XXBP1-EnR or truncated XXBP-1 showed spina bifida or a bent axis. Unexpectedly, microinjection of XXBP1-VP16 (0.04 ng mRNA/embryo) produced posterior axis enlargement (96%, 70/73 embryos), a phenotype similar to that of wild-type XXBP-1, although the reason for this is unclear. Ventral overexpression of XXBP1-Eve (0.16 ng mRNA/embryo) produced a secondary axis in 10% of the embryos or primarily gastrulation defects giving rise to spina bifida (81%, 79/98 embryos) (Fig. 6I–F).

Embryos microinjected dorsally with the same amount of mRNA (0.16 ng mRNA/embryo) of XXBP1-Eve, XXBP1-EnR, or truncated XXBP-1 showed either gastrulation defects leading to spina bifida or the loss of anterior structures, which could have also been due to interference with morphogenetic movements. Embryos ectopically overexpressing XXBP1-VP16 (0.04 ng mRNA/embryos) dorsally exhibited either the loss of dorsoanterior structures (46%, 23/50 embryos; Fig. 6IG) as with overexpression of wild-type XXBP-1 or exogastrulation (54%, 27/50 embryos; Fig. 6IH).

If XXBP-1 is a transcriptional activator, the chimeric protein containing the VP16 activator domain should have similar effects to that of XXBP-1. Thus, the XXBP1-Eve construct would function as a dominant negative mutant and would induce neural markers, such as NCAM in contrast to wild-type XXBP-1 in the animal cap assay. Ectodermal explants were dissected from the embryos injected with one of the various constructs or wild-type XXBP-1, and were cultured until control embryos reached stage 27. The relative expression level of XAG1, Otx2, NCAM, nrp-1, Msx1,
Fig. 4. Biological activity of XXBP-1. (I) Phenotypes of XXBP-1-injected embryos. (A–C) Examples of ventralized phenotypes after dorsal injection of 200 pg XXBP-1 mRNA at the four cell stage. (D, E) The ventralized embryos exhibited no anteroposterior or dorsoventral axes, after either dorsal injection of 400 pg XXBP-1 mRNA or radial injection of 800 pg XXBP-1 mRNA. (F–G) A transverse section through the embryo injected dorsally with 200 pg XXBP-1 mRNA shows a reduced notochord (nt) and an indiscernible spinal cord (sc) as compared with the control embryo (F). pn, pronephros; sm, somite. (II) Disruption of neural marker genes in vivo by XXBP-1. A single dorsal blastomere was coinjected with 200 pg XXBP-1 and 100 pg LacZ mRNA at the four cell stage. The embryos were fixed at the late neurula stages and stained for LacZ to identify the injected side, and whole-mount in situ hybridization with antisense probes for the cement gland marker, XAG2, and neural marker genes was carried out. (A–N) The expression pattern in control embryos is shown at the left, and expression after XXBP-1 overexpression on one side is shown at the right. (A–B) XAG2 expression was not changed by XXBP-1 overexpression at this dose. (C–N) XXBP-1 overexpression inhibited the expression of Otx2 (anterior neural marker), Rx2A (lens marker), En2 (marker for the mid/hindbrain boundary), Krox20 (marker for r3 and r5), Pax6 (neural marker), and Sox3 (pan-neural marker). (O, P) Embryos injected only with lacZ mRNA as a control showed no changes in En2 or Sox3 expression. (III) Overexpression of XXBP-1 downregulates the dorsal mesoderm markers, Chordin and goosecoid. Embryos were injected dorsally into the marginal zone with 0.1 ng/blastomere of XXBP-1 mRNA at the four cell stage and fixed at midgastrula for whole-mount in situ hybridization with Chd and gsc antisense RNA. (A, C) Expression in control embryos. (B, D) XXBP-1 overexpression reduced both Chd and gsc expression. (IV) Overexpression of XXBP-1 leads to ventralization of dorsal mesoderm. Dorsal and ventral marker genes were analyzed by using RT-PCR in whole embryos (st. 22 embryo), dorsal or ventral marginal zone explants (DMZ or VMZ, respectively) from un.injected embryos, or embryos radially injected with 0.1 or 0.4 ng XXBP-1 mRNA per blastomere at the four-cell stage. Marginal zones were explanted at the start of gastrulation and cultured until sibling embryos reached stage 22. ODC served as a loading control. RT−, control without reverse transcriptase. (V) XXBP-1 inhibits neural induction in dissociated ectodermal explants. Animal caps were dissected from uninjected embryos (AC) and embryos injected with either 0.4 ng XXBP-1 or 0.8 ng BMP-4 mRNA into all four blastomeres at the four cell stage. The ectodermal cells from the animal caps were dissociated then allowed to reassociate and cultured subsequently for 24 h. (A) RT-PCR was used to analyze the expression of the neural marker, NCAM; ectodermal marker, epidermal keratin (EK); and mesoderm markers, actin and Xbra. BMP-4 was injected as a positive control for inhibition of neural induction. ODC was employed as a loading control. (B–D) At 24 h after reaggregation, the ectodermal cells from XXBP-1 animal caps were not able to reaggregate as well as cells from control or BMP-4 animal caps. The majority of XXBP-1-overexpressing ectodermal cells did not reaggregate. Only reaggregated cells (indicated by arrow) were harvested for RT-PCR analysis. RT−, control without reverse transcriptase.
Fig. 5. **XXBP-1** may be involved in the BMP signaling pathway. (I) **XXBP-1** overexpression reduces neural induction by **tBMPRI**. At stage 9, animal caps were dissected from uninjected control embryos (AC) or embryos injected with either 0.4 ng **XXBP-1**, 1.6 ng **tBMPRI**, or 1.6 ng **tBMPRI** + 0.4 ng **XXBP-1** mRNA into all blastomeres at the four-cell stage, and were cultured until sibling embryos reached stage 28. Neural and epidermal marker genes were analyzed by using RT-PCR with RNA from animal caps or intact embryos (st. 28 embryo). Overexpression of **XXBP-1** attenuated the neural induction by **tBMPRI**. 

**EK**, epidermal keratin; **RT**, control without reverse transcriptase. (II) Microinjection of **XXBP-1** reverses **tBMPRI**-induced dorsalization. Phenotypes were examined after overexpression of either **XXBP-1**, **tBMPRI**, or both. (A) A secondary axis was induced by injection with 1.6 ng **tBMPRI** mRNA ventrally at the four cell stage. (C) Coinjection of 1.6 ng **tBMPRI** and 0.2 ng **XXBP-1** mRNA ventrally at the four cell stage reversed formation of the secondary axis and caused posterior enlargement like injection of 0.2 ng **XXBP-1** mRNA alone (D). Phenotypes of embryos microinjected into four blastomeres at the four cell stage were shown in (E–G). Two cement glands (arrows) were induced by **tBMPRI** alone (1.6 ng of **tBMPRI** mRNA) (E), but coinjection with **XXBP-1** mRNA (1.6 ng **tBMPRI** and 0.4 ng of **XXBP-1**) reversed embryo dorsalization (F, G).

Fig. 6. Overexpression of fusion constructs and truncated **XXBP-1**. (I) Fusion and truncated **XXBP-1** constructed. The amino terminal sequence (amino acids 1–126) of **XXBP-1**, containing the basic leucine zipper DNA binding domain (bZIP), was used to create several fusion constructs (white box). The remaining **XXBP-1** sequence (amino acids 127–350) is indicated by the vertically lined box and was replaced by the VP16 activation domain (shaded, slashed box) to form **XXBP1-VP16**, or by the even-skipped repressor domain (shaded box) to form **XXBP1-Eve**. The amino terminal fragment of **XXBP-1** was also subcloned into pCS2MTEnR, containing the sequence encoding the engrailed repression domain (shaded, hatched box) and the Myc-tag (slashed box) to obtain **XXBP1-EnR**. Truncated **XXBP-1** encodes the amino acids 1–126. (II) Phenotypes of embryos injected with fusion constructs. (A, D) Ventral injection with either 0.16 ng **XXBP1-EnR** mRNA or 0.16 ng truncated **XXBP-1** mRNA produced embryos with spina bifida. (B, C) Ventral injection with 0.16 ng **XXBP1-Eve** mRNA show either a secondary axis (B) or spina bifida (C). (E, F) Ventral injection of either 0.04 ng **XXBP1-VP16** mRNA or 0.2 ng wild-type **XXBP-1** mRNA, respectively, resulted in posterior enlargement. (G, H) Dorsal injection (Dor) of the same amount of **XXBP1-VP16** caused either loss of dorsoanterior structures (G) or exogastrulation (H). Note that **XXBP1-Eve** is able to induce a secondary axis in embryos, but ventral injection with **XXBP1-VP16** induces a similar phenotype to that of wild-type **XXBP-1**, suggesting that **XXBP-1** may be an activator of transcription. (III) Expression of fusion constructs in animal caps indicate **XXBP-1** may be an activator of transcription. Animal caps were dissected from uninjected embryos (AC) or embryos injected with 0.08 ng **XXBP1-VP16**, 0.64 or 0.32 ng **XXBP1-Eve**, 0.32 ng truncated **XXBP-1**, 0.32 ng **XXBP1-EnR**, 1.6 ng **tBMPRI** or 0.32 ng **XXBP-1** mRNA, and cultured until sibling embryos reached stage 27. RT-PCR was used to measure the expression of the marker genes, XAG1, Otx2, NCAM, nrr-1, Msc1, and Epidermal Keratin (EK) in RNA from animal caps or whole embryos (st. 27 embryo). The neural marker genes, NCAM and nrr-1, were induced by **XXBP1-Eve**, but not by **XXBP1-VP16** or wild-type **XXBP-1**, suggesting that **XXBP-1** functions as a transcription activator. ODC was employed as a loading control. **RT**–, control without reverse transcriptase.
and epidermal keratin was investigated by using RT-PCR (Fig. 6III). As expected, XXBP1-Eve induced the neural markers, NCAM, npr-1, and Otx2, in a dose-dependent manner. Overexpression of truncated XXBP-1 resulted in a minor induction of the neural markers, and Otx2 could be slightly induced by XXBP1-EnR. In contrast, the epidermal marker, Msx1, was moderately downregulated by XXBP1-Eve, truncated XXBP-1, XXBP1-EnR, and tBMPRI. The expression of epidermal keratin was also reduced in XXBP1-Eve-injected ectodermal explants. Injection of either XXBP1-VP16 or wild-type XXBP-1 produced similar results with the exception that XXBP1-VP16 slightly up-regulated XAG1. These data support the view that XXBP-1 may function as a transcriptional activator during the embryonic development.

Discussion

In this study, we describe a novel leucine zipper transcription factor, XXBP-1, which is expressed in the ventral ectoderm and dorsal blastopore lip of the Xenopus gastrula. XXBP-1 causes embryo ventralization and may function as an activator downstream of the BMP receptor in the signaling pathway.

Differentially restricted pattern of XXBP-1 expression in Xenopus embryos

The expression pattern of XXBP-1 suggests a function as a negative regulator of neural induction. The results from microinjection experiments and animal cap assays presented in this paper support this view. The fact that XXBP-1 is expressed in the dorsal blastopore lip but induces embryo ventralization is reminiscent of ADMP (anti-dorsalization morphogenetic protein; Moos et al., 1995). In light of the classical view, both XXBP-1 and ADMP are unexpected genes expressed in the Spemann–Mangold organizer area since it was primarily thought of as only the source of dorsalizing signals. The classical genes expressed in the Spemann–Mangold organizer generally act as neutralizing or dorsalizing factors, and induce neutralization and a secondary axis when ectopically expressed. However, overexpression of either XXBP-1 or ADMP caused suppression of dorsal and anterior structures as well as the neural marker, NCAM. Additionally, XXBP-1 suppressed gsc and Chdα, and upregulated BMP-4, Xvent-1, and Xho3, playing an instructive role in epidermal induction. These pieces of evidence support the view that local negative regulatory mechanisms may exist in the Spemann–Mangold organizer to restrain the classical neutralization and dorsalizing activity. The dorsalizing and neutralizing activity as well as negative regulatory mechanisms are simultaneously required in the organizer for the establishment of a precise spatial control in the organizer to direct appropriate execution of complex biological processes during embryonic development. The fact that ADMP is not inhibited by noggin in contrast to XXBP-1 (Dorsch and Niehrs, 2000) suggests that both genes may function through distinct pathways. The expression of XXBP-1, being excluded from the prospective neural plate of the gastrula, and located maximally in the cement gland primordium of the neurula, is similar to that of BMP-4 (Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995; Gammill et al., 2000), indicating that XXBP-1 may be involved in BMP signaling. The expression of Xlim-1 and of XXBP-1 overlaps in the Spemann–Mangold organizer at gastrula stages and in the pronephros at tailbud stages; however, it remains to be shown if an interaction between Xlim-1 (Taira et al., 1994) and XXBP-1 exists.

XXBP-1 may act downstream of the BMP receptor and up-regulate BMP-4 expression

The BMP signaling pathway is implicated in both mesoderm induction and dorsoventral patterning. The binding to BMP-4 by organizer-secreted antagonists such as Chordin, Noggin, Follistatin, and Cerberus is thought to be responsible for the establishment of a morphogen gradient of BMP-4 activity, which specifies different dorsoventral fates in early gastrulae (for reviews, see Dale and Jones, 1999; De Robertis et al., 2000). The experiments presented here provide evidence supporting the hypothesis that XXBP-1 is involved in the BMP pathway. Firstly, the expression pattern of XXBP-1 at the gastrula stage indicates that it may play a negative role in neural induction. In the neurula, XXBP-1 is intensely expressed in the cement gland primordium, as is BMP-4. Secondly, ectopic expression of BMP-4 upregulated XXBP-1 expression in the dorsal marginal zone and vice versa. Thirdly, the overexpression of either XXBP-1 or BMP-4 ventralized the embryo in a dose-dependent manner, resulting in phenotypes ranging from microcephaly to belly pieces. Fourthly, both BMP-4 and XXBP-1 suppressed gsc in DMZ explants and were downregulated by noggin in the animal cap assay. Fifthly, XXBP-1-like BMP-4, inhibited neural induction in dissociated ectodermal cells. Finally, coinjection of XXBP-1 with tBMPRI reversed the dorsalization induced by tBMPRI alone, and directed a proepidermal cell fate in the animal cap assay.

Our results support the hypothesis that XXBP-1 acts downstream of the BMP receptor. Coinjection of XXBP-1 and tBMPRI caused suppression of the neural markers, NCAM and Otx2, and the partial restoration of epidermal keratin expression, reverting the effects of tBMPRI alone. These results indicate that the ectoderm adopted a more epidermal cell fate, and that XXBP-1 was sufficient to attenuate neuralization induced by tBMPRI. Moreover, the dorsalization and formation of a secondary axis after injection of tBMPRI was also reversed by XXBP-1 coinjection, suggesting that XXBP-1 acts downstream of the BMP receptor. Furthermore, BMP-4 was induced in the DMZ by XXBP-1, which may indicate that XXBP-1 is required for the maintenance of BMP-4 expression.
Sequence analysis identified XXBP-1 as a basic leucine zipper transcription factor. This class of transcription factors acts as both activators and repressors of transcription. One strategy to characterize whether a given transcription factor acts as an activator or repressor in a certain system is to construct a dominant-negative mutant (Conlon et al., 1996; Horb and Thomsen, 1997; Onichtchouk et al., 1998; Gómez-Skarmeta et al., 2001). Using this approach, XXBP-1 showed activity suggestive of an activator during BMP signaling by several criteria. When fused with the activator domain of VP16, the XXBP1-VP16 showed similar biological activities to wild-type XXBP-1. Both wild-type XXBP-1 and XXBP1-VP16 injected on the ventral side caused a posterior enlargement of the larval tail area only. In the animal cap assay, both XXBP1-VP16 and XXBP-1 did not induce neural markers. In contrast, when fused with the repressor domain of Eve, the XXBP1-Eve was able to induce a secondary axis when ventrally overexpressed at least in some cases. Moreover, XXBP1-Eve induced the neural markers, NCAM and myr-1, and downregulated Msx1 and epidermal keratin in the animal cap assay. Thus, XXBP1-Eve upregulates genes that would ordinarily be repressed by XXBP-1, supporting that XXBP1-Eve functions as the dominant negative mutant of XXBP-1. Another repressor construct, XXBP1-EnR, did not induce pan-neural markers, or a secondary axis, but caused defects in gastrulation. This conflicting result may be explained by the interference of the myc-tag, directly N-terminal to the XXBP-1 bZIP domain, with DNA binding activity of this fusion construct. It should be mentioned that gastrulation defects were observed in some of the embryos after the overexpression of each construct, which can be interpreted in light of their mechanism of action. A strong transcriptional activator or repressor domain will be active wherever there is an accessible binding site, and not only in cells expressing XXBP-1. Furthermore, the activity of the constructs is not developmentally regulated during embryonic development. This uncoupling from developmental regulation will inevitably lead to pleiotropic effects during embryonic development. The truncated XXBP-1 had a weak effect on neural induction indicated by slightly increased Otx2 and NCAM expression and caused gastrulation defects giving rise to spina bifida. This suggests that XXBP-1 may form homodimers to carry out its function.

**XXBP-1 interacts with the β-catenin/Wnt pathway**

In *Xenopus*, the organizer formation requires both TGF-β and β-catenin signaling (Harland and Gerhart, 1997; Heasman, 1997; Niehrs, 1999, 2000). Signals originating from the organizer are involved in further patterning along both the anteroposterior and dorsoventral axes and in the induction of the central nervous system of the embryo. However, several pieces of evidence suggest that ectoderm on the dorsal side is predisposed to become neural in contrast to ventral ectoderm (London et al., 1988; Sokol and Melton, 1991; Kuo et al., 1998). Several lines of evidence indicate that β-catenin/Wnt signaling participates in this process as well as later in neural plate pattern formation (Wylie et al., 1996; McGrew et al., 1995). The repression of BMP-4 in the dorsal mesoderm and prospective neural plate is mediated by β-catenin/Wnt signaling during gastrulation (Baker et al., 1999). Moreover, β-catenin activates the early expression of secreted BMP antagonists, such as Chordin, in a preorganizer region at the dorsal side of the *Xenopus* blastula (Wessely et al., 2001). In this study, we showed that the expression of XXBP-1 was expanded over the entire DMZ in embryos in which β-catenin/Wnt signaling was blocked by β-catenin MO. This phenotype was mimicked by dorsal overexpression of BMP-4 and UV treatment. These results offer further evidence for the existence of crosstalk between BMP and β-catenin/Wnt signaling. Another group has also reported evidence to support crosstalk between β-catenin/Wnt signaling and Dlx3, another BMP target gene. It was shown that the repression of Dlx3 is mediated by signaling via β-catenin, but is probably not dependent on the induction of the Xnr3 or Chordin by β-catenin (Beanan et al., 2000).

Taken together, our data support a view that XXBP-1 acts downstream of the BMP receptor. Additionally, XXBP-1 negatively regulates the dorsalizing and neuralizing activities of the Spemann–Mangold organizer, and may mediate BMP signaling during epidermal induction and inhibition of neural differentiation.

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