Development 127, 2333-2345 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV4357

The homeobox gene *bozozok* promotes anterior neuroectoderm formation in zebrafish through negative regulation of BMP2/4 and Wnt pathways

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Accepted 20 March; published on WWW 10 May 2000

SUMMARY

The neuroectoderm of the vertebrate gastrula was proposed by Nieuwkoop to be regionalized into forebrain, midbrain, hindbrain and spinal cord by a two-step process. In the activation step, the Spemann gastrula organizer induces neuroectoderm with anterior character, followed by posteriorization by a transforming signal. Recently, simultaneous inhibition of BMP and Wnt signaling was shown to induce head formation in frog embryos. However, how the inhibition of BMP and Wnt signaling pathways specify a properly patterned head, and how they are regulated in vivo, is not understood. Here we demonstrate that the loss of anterior neural fates observed in zebrafish *bozozok* (*boz*) mutants occurs during gastrulation due to a reduction and subsequent posteriorization of neuroectoderm. The neural induction defect was correlated with decreased *chordino* expression and consequent increases in *bmp2b/4* expression, and was suppressed by overexpression of BMP antagonists. Whereas expression of anterior neural markers was restored by ectopic BMP inhibition in early *boz* gastrulae, it was not maintained during later gastrulation. The posteriorization of neuroectoderm in *boz* was correlated with ectopic dorsal *wnt8* expression. Overexpression of a Wnt antagonist rescued formation of the organizer and anterior neural fates in *boz* mutants. We propose that *boz* specifies formation of anterior neuroectoderm by regulating BMP and Wnt pathways in a fashion consistent with Nieuwkoop's two-step neural patterning model. *boz* promotes neural induction by positively regulating organizer-derived *chordino* and limiting the antineuralizing activity of BMP2b/4 morphogens. In addition, by negative regulation of Wnt signaling, *boz* promotes organizer formation and limits posteriorization of neuroectoderm in the late gastrula.

Key words: *bozozok*, Neuroectoderm, Zebrafish, Wnt, BMP2/4, *chordino*

INTRODUCTION

The vertebrate body plan emerges during gastrulation as the three germ layers, mesoderm, endoderm and ectoderm, are induced and patterned along the dorsoventral (DV) and anteroposterior (AP) axes (Harland and Gerhart, 1997). These processes are best understood in amphibia and fish where DV patterning is initiated during the first cleavages by the accumulation of β-catenin in nuclei of dorsovegetal blastomeres of the *Xenopus* embryo and dorsal marginal blastomeres and the underlying yolk syncytial layer (YSL) of the zebrafish embryo (Moon and Kimelman, 1998; Schneider et al., 1996). The homeobox genes *siamois* and *twin* in frog (Laurent et al., 1997; Lemaire et al., 1995), and *bozozok/dharma/nieuwkoid* (*bozozok*) in zebrafish (Fekany et al., 1999; Koos and Ho, 1998; Yamanaka et al., 1998), act downstream of β-catenin as part of the mechanisms establishing the dorsal gastrula (Spemann) organizer (Nieuwkoop, 1973; Spemann, 1938). The dorsalizing activity of the Spemann organizer is common to all vertebrates, involving secretion of factors that antagonize the ventralizing bone morphogenetic proteins (BMPs) and Wnts (reviewed in Harland and Gerhart, 1997; Moon et al., 1998). A resultant gradient of BMP2/4/7 morphogen activity is thought to specify a ventrodorsal progression of cell fates (Nguyen et al., 1998; Wilson et al., 1997). Within the ectoderm, epidermis develops ventrallly where BMP activity is high, whereas neural tissue forms dorsally at lower BMP concentrations (Hemmati-Brivanlou and Melton, 1997; Nikaido et al., 1999).

The neuroectoderm becomes subdivided along the AP axis into forebrain, midbrain, hindbrain and spinal cord. Studies in amphibian embryos led to a two-step model of AP patterning of neuroectoderm. According to this model, anterior neural tissue is induced in the first (activation) step and, subsequently posteriorized by a transformer molecule(s), thereby generating a complete AP succession of neural fates (Nieuwkoop et al., 1952).

The gastrula organizer was originally thought to be responsible for both the activation and transformation steps,

with the BMP antagonists Noggin and Chordin being inducers of anterior neural fates (Lamb et al., 1993; Sasai et al., 1995). However, although perturbations of BMP signaling in live embryos change the balance between neuroectoderm and epidermis specification and affect DV patterning of the neural plate, the global AP neural pattern is not disturbed (Barth et al., 1999; Mullins et al., 1996; Nikaido et al., 1999). Furthermore, organizer transplants do not affect AP pattern but instead induce neuroectoderm whose AP character is dependent on the location of the transplant (Koshida et al., 1998). These studies suggest that pathways and tissues outside the organizer region are involved in specification of AP neural pattern, and/or that this process is completed before the onset of gastrulation.

Gain-of-function analyses and expression of dominant negative mutants of signaling molecules in frog embryos have suggested Wnt, FGF and retinoids as candidates for the posteriorizing transformer signals (Doniach, 1995; Kolm and Sive, 1997; McGrew et al., 1997); however, the identity of the endogenous transformer remains elusive. As demonstrated by transplantation studies in zebrafish and tissue conjugates in frog and chick, such posteriorizing signals could originate from the non-axial dorsolateral mesoderm (Muhr et al., 1997; Woo and Fraser, 1997) and YSL in fish (Koshida et al., 1998).

The significance of BMP and Wnt signaling in AP neural development is underscored by the demonstrations that simultaneous inhibition of these two pathways by co-expression of inhibitors of BMP (tBR, Noggin) and Wnt (dnXWnt8, Dkk-1, FrzB) signaling in frog embryos is sufficient for head development (Glinka et al., 1997, 1998). Furthermore, the multifunctional protein Cerberus, which can bind and antagonize BMP, Wnt and Nodal ligands, is a potent head inducer (Piccolo et al., 1999). In addition, the Wnt antagonist Dkk-1 may be required for head formation, as microinjections of anti-Dkk-1 antibodies result in microcephaly (Glinka et al., 1998). Intriguingly, *cerberus* and *dkk-1* are expressed in the deep endodermal cells of the frog gastrula organizer, consistent with the function of the organizer in head induction. In the mouse, embryological and genetic experiments implicate the extraembryonic anterior visceral endoderm (AVE) in specification of anterior embryonic structures (Beddington and Robertson, 1999; Thomas and Beddington, 1996). Accordingly, a murine homolog of *cerberus*, *cer-1*, is expressed in the AVE (Belo et al., 1997; Shawlot et al., 1998). It remains to be determined, however, by what mechanism inhibition of Wnt and BMP signaling leads to development of a properly patterned head, and how these two pathways are regulated during normal development.

The zebrafish homeobox gene *bozozok* (*boz*) is required at blastula stages for the formation of a complete gastrula organizer and specification of dorsoanterior embryonic structures (Fekany et al., 1999). Here, we demonstrate by gene expression studies and fate-mapping experiments that the forebrain deficiencies in *boz* mutants are due to defects in specific aspects of the dorsal gastrula organizer, resulting in decreased neural induction and increased posteriorization of neuroectoderm. Our studies establish a dual function of the *boz* locus in anterior neuroectoderm development. Through negative regulation of antineuralizing BMP2/4 activity *boz* promotes neural induction. In addition, by negatively regulating the influence of factors such as Wnt8, *boz* promotes organizer formation and limits posteriorization of anterior neuroectoderm at late gastrulation.

MATERIALS AND METHODS

Fish maintenance

Fish and embryos were maintained essentially as described in Solnica-Krezel et al. (1994). For all studies performed here, we used the *m168* allele of *boz* encoding a truncated protein without a homeodomain, which is considered to be a strong/null allele (Fekany et al., 1999; Koos and Ho, 1999).

In situ hybridizations

RNA in situ hybridizations were performed essentially as described in Thisse and Thisse (1998). Processed embryos were mounted in 100% glycerol and photographed using a Zeiss Axiophot.

mRNA injections

mRNA was synthesized using the mMessage mMachine kit (Ambion) from the following linearized templates: chordinS2 (Miller-Bertoglio et al., 1997), nogginA3 (Smith and Harland, 1992) and dnXWnt8 (Hoppler et al., 1996). Embryos were injected through the chorion into the yolk just below the blastoderm at 1- to 8-cell stages using a pneumatic picopump (WPI) as described in Marlow et al. (1998). Embryos were allowed to develop in egg water and manually dechorionated after fixation in 4% paraformaldehyde.

Fate mapping

Embryos were obtained from wild-type parents or from intercrosses of boz^{m168} heterozygotes and/or homozygotes. Embryos were manually dechorionated prior to shield stage in embryo medium (Westerfield, 1996). 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) particles were dissolved in 10 μl dimethyl sulfoxide (DMSO). Embryos were injected at shield stage in the animal pole using a pneumatic picopump (WPI). The fluorescent label was then immediately viewed on a Zeiss Axiophot microscope to ensure correct location of the labeled cells. Any embryos not labeled at the animal pole were discarded. Embryos were allowed to develop until yolk plug closure stage. After overnight fixation in 4% paraformaldehyde, embryos were washed 3× in 0.1 M Tris-HCl, pH 8.2, 0.1% Tween-20. Embryos were incubated in 0.2 mg/ml DAB (3,3′-diamibenzidine, Sigma, St Louis, MO) for 15 minutes and then photoconverted individually in depression slides using a 20× objective on a Zeiss Axiophot microscope. Embryos were washed in PBT, postfixed in Methanol and processed by in situ hybridization as described above.

Morphometric measurements

Measurements of the anterior-to-posterior dimension of the dorsal *wnt8* expression domain were obtained using NIH Image 1.62 from images of wild-type and *boz* embryos following in situ hybridization. All other morphometric measurements were performed essentially as described in Marlow et al. (1998).

RESULTS

Differential effects of the *boz* mutation on neural patterning at early and late gastrulation

Zebrafish *bozozok* (*boz*) mutations result in variable deficiencies of axial mesendoderm as well as forebrain and midbrain tissues at 1 day postfertilization (dpf) (Fekany et al., 1999; Solnica-Krezel et al., 1996). To address when and by what mechanisms anterior defects occur in *boz* mutants, we

analyzed the expression of two *orthodenticle*-related genes, *otx1* and *otx2*, the earliest available markers of prospective forebrain and midbrain (Li et al., 1994). In wild type, both genes are expressed in a broad triangular domain reaching the animal pole of the early to midgastrula (Figs 1A, 6A,B; Li et al., 1994). In *boz* mutant siblings, the *otx1/2* expression domains were either reduced along the mediolateral (ML) and AP axes, with their anterior boundaries not reaching the animal pole, or were absent altogether (Figs 1B, 6E,F). Thus, the anterior neuroectoderm anlage is reduced in *boz* mutants at midgastrulation: the lateral borders are shifted medially and the anterior border is shifted posteriorly.

At later stages of gastrulation, the *otx1/2* expression domains remained reduced mediolaterally in *boz* mutants compared to wild-type siblings (Fig. 1C,D). In contrast to the earlier stages analyzed, the anterior border of the *otx1* expression domain was positioned much closer to the animal pole, being only slightly shifted posteriorly relative to wild-type embryos. The posterior boundary of the expression domain, however, was shifted anteriorly in *boz* mutants (Fig. 1D), compared to their wild-type siblings (Fig. 1C). Based on the above observations, we hypothesized that the differences observed in the expression patterns of anterior neural markers in early and late *boz* mutant gastrulae might reflect distinct roles that *boz* plays in neural induction and patterning.

boz mutants display neural posteriorization

To uncover the nature of the neural patterning defects resulting from the loss of *boz* function, we simultaneously analyzed the expression of several region-specific markers in the early neural plate of wild-type and *boz* mutant embryos. During normal development, the MHB rudiment is already established in the late gastrula, as revealed by the expression of *pax2.1* (Fig. 1E) in the prospective neuroectoderm as two mediolateral stripes flanking the midline (Krauss et al., 1991). In *boz* mutants, these stripes were fused across the midline, reduced mediolaterally, but enlarged along the AP axis (Fig. 1F). Remarkably, the distance from the MHB (*pax2.1* expression domain) and the hindbrain (*krox20* expression domain; Oxtoby and Jowett, 1993) to the anterior boundary of the neural plate (*dlx3* expression domain; Akimenko et al., 1994) was dramatically decreased in strongly affected *boz* mutants (Fig. 1F,H) compared to wild-type siblings (Fig. 1E,G). This apparent shift of the MHB and hindbrain anlagen towards the anterior edge of the neural plate/animal pole suggested that the neuroectoderm is posteriorized in *boz* mutants. These analyses also confirmed that the neural plate was reduced along the ML axis according to the strength of the *boz* mutant phenotype (Fig. 1F,H), compared to wild-type siblings (Fig. 1E,G).

To ask whether the neuroectoderm was posteriorized with respect to the underlying mesoderm, we analyzed the relative positions of ectodermal and mesodermal landmarks in wild-type and *boz* embryos. We simultaneously detected *six3* in the forebrain (Kobayashi et al., 1998) and *pax2.1* in the MHB,

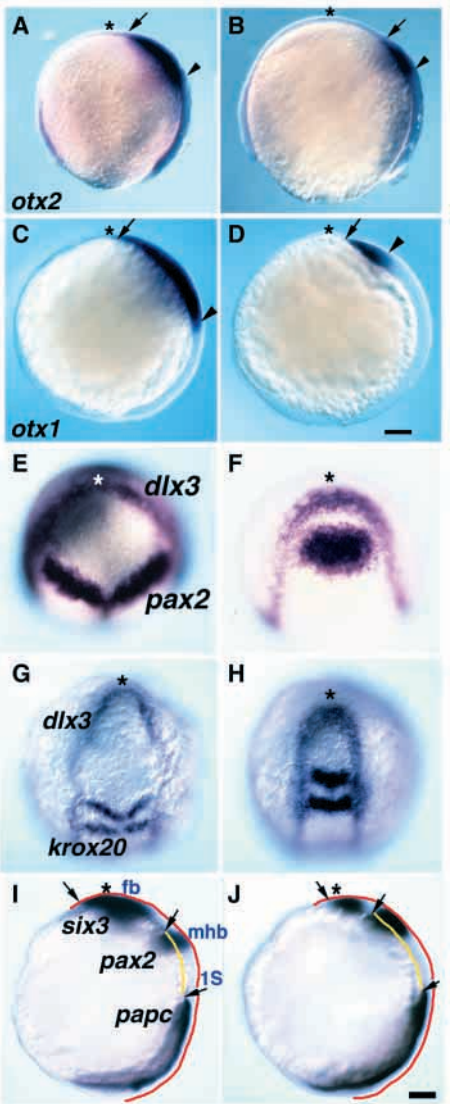


Fig. 1. *boz* mutants exhibit a reduction of the neural anlage and a posteriorization of the neuroectoderm at the end of gastrulation. (A) At midgastrula stage (8hpf), *otx2* expression normally marks the prospective forebrain and midbrain. (B) In *boz* mutant embryos, the *otx2* expression domain is reduced medial-laterally, and its anterior border does not reach the animal pole. (C,D) In the late gastrula, the *otx1* forebrain and midbrain expression domain is severely reduced in *boz* mutants (D), compared to wild type (C). Note that the position of the anterior neuroectoderm border in *boz* recovered significantly compared to the earlier stages of gastrulation (B versus D), being only slightly shifted posteriorly compared to wild type (arrows). But its posterior border (arrowhead) is shifted anteriorly in mutants compared to wild type (C versus D). (E-H) Expression of *dlx3* in the border of the neural plate, *pax2.1* in the prospective midbrain-hindbrain boundary (E,F), and *krox20* in rhombomeres 3 and 5 of the hindbrain (G,H), reveal that the prospective neuroectoderm is reduced mediolaterally in *boz* mutants (F,H), compared to wild type (E,G). (I,J) *six3*, *pax2.1* and *papc* expression in wild type (I) and *boz* (J). The forebrain expression of *six3* is reduced in *boz* mutants compared to wild-type embryos. The posterior borders of the *six3* and *pax2.1* expression domains are shifted anteriorly in *boz* mutants and away from the *papc* expression in presomitic mesoderm (J) compared to wild type (I). The distance between the anterior borders of the *pax2.1* and *papc* expression domains (a yellow line) and the length of the entire embryonic axis (a red line), which were measured as described in the text, is indicated. (E-H) Dorsoanterior views, anterior to the top; (A-D,I,J) lateral views with dorsal to the right; *indicates location of the animal pole. Bar, 100 μm.

together with the presomitic mesoderm marker *papc* (Fig. 1I,J; Yamamoto et al., 1998). This analysis indicated that the posterior border of the *six3* expression domain and the *pax2.1* expression domains were shifted towards the animal pole in *boz* mutants, and away from the anterior edge of *papc* expression in the presomitic mesoderm (Fig. 1J), as compared to wild-type embryos (Fig. 1I). Accordingly, the ratio of the distance between the anterior borders of the *pax2.1* and *papc* expression domains to the total length of the embryo was significantly higher for *boz* mutants (20.6±3.3%; n=10) compared to phenotypically normal siblings (12.9±1.1%; n=6; P<0.001, t-test). This indicated that the prospective MHB boundary within the neuroectoderm is shifted anteriorly with respect to the underlying mesoderm, providing additional evidence that anterior neuroectoderm is posteriorized in *boz* mutants. These studies, however, do not address whether the mesoderm is also posteriorized.

In order to address directly the above hypothesis, we performed fate-mapping experiments. First, we injected a lipophilic dye, DiI, into the animal poles of progeny from *boz*$^{m168/+}$ and/or *boz*$^{m168/m168}$ parents at the onset of gastrulation (5.5 hpf; Fig. 2A; Kimmel et al., 1995). At the end of gastrulation, the DiI-labeled cells were visualized by photoconversion (see Materials and Methods). To analyze neuroectoderm patterning in the labeled embryos, expression of *dlx3*, *pax2.1* and *flh* was detected by whole-mount in situ hybridization. This analysis revealed the position of the labeled cells located at the animal pole during early gastrulation, with respect to the developing neural plate at the end of gastrulation. In wild-type embryos, the labeled cells were detected just ventral to the *dlx3* domain in the prospective non-neural ectoderm (n=6/18), within the anterior *dlx3* expression domain (n=9/18; Fig. 2B), or within the prospective neuroectoderm (3/18). Similarly, in *boz* mutants, which were recognized by reduced/absent notochordal *flh* expression (Talbot et al., 1995), the labeled cells were present just ventral to the *dlx3* expression domain (n=8/15; Fig. 3C), within the anterior *dlx3* expression domain (n=6/15), or within the prospective neuroectoderm (n=1/15). The labeled cells remained at the animal pole in both wild type and *boz* mutants, indicating that abnormal movements of prospective forebrain cells are not involved in the generation of the *boz* phenotype. These fate-mapping experiments confirmed that, in both wild type and *boz* mutants, the anterior boundary of neuroectoderm straddles the animal pole. However, in contrast to wild type, in *boz* mutants, the labeled cells were found more frequently in non-neural ectoderm, indicating a small posterior shift of the neuroectoderm anterior border had occurred in *boz* mutants, consistent with a reduction of neuroectoderm in *boz*. Moreover, the distance between the labeled cells and the *pax2.1* expression domain in the MHB was smaller in *boz* (Fig. 2C) than in wild-type embryos (Fig. 2B), consistent with neuroectoderm posteriorization in *boz*.

Together, these analyses showed that the deficiencies of anterior neural structures, as well as the anterior shift of hindbrain observed in *boz* mutants at 1 dpf (Fekany et al., 1999), can be traced back to defects occurring at gastrulation. Based on the above observations, we concluded that loss of *boz* function leads to both reduction and posteriorization of neuroectoderm.

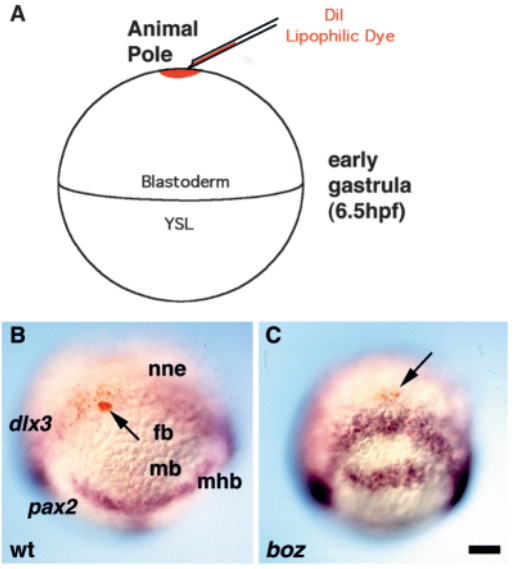


Fig. 2. Fate mapping of prospective forebrain cells in *boz* mutants. (A) Embryos were injected with lipophilic dye (DiI) into the animal pole at the onset of gastrulation, (6-6.5 hpf). (B) At the end of gastrulation (9.5 hpf), a wild-type embryo exhibits DiI-labeled and photoconverted cells (arrow) within and just ventral to the *dlx3* expression domain, demarcating the boundary between non-neural (nne) and neural ectoderm. (C) A *boz* embryo in which labeled cells are detected just ventral to the *dlx3* domain. Note that the distance between the labeled cells and *pax2.1* expression in the mhb is smaller in *boz* than in wild type. (B,C) Dorsoanterior views, anterior to the top. fb, forebrain; mb, midbrain; mhb, midbrain-hindbrain boundary; nne, non-neural ectoderm; YSL, yolk syncytial layer. Bar, 100 μm.

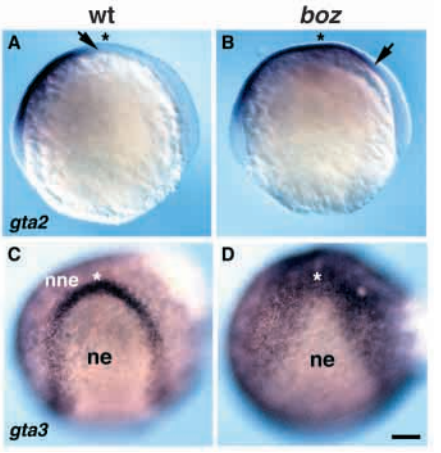


Fig. 3. Non-neural ectoderm is enlarged in *boz* mutant gastrulae. (A) *gta2* is expressed in the non-neural ventroanimal ectoderm of wild-type embryos at midgastrula (8 hpf). (B) The *gta2* expression domain and its anterior border (arrow) is expanded dorsally in *boz* mutants. (C,D) In late gastrulae, non-neural ectoderm, marked by *gta3* expression, is expanded dorsally in *boz* (D) compared to wild type (C). However, the anterior boundary of *gta3* expression, which straddles the animal pole in wild type (C), is positioned closer to the animal pole in *boz* (D) than the *gta2* expression boundary in the early gastrula (B). (A,B) Lateral views with dorsal to the right; (C,D) dorsoanterior views. * indicates the animal pole. ne, neuroectoderm. Bar, 100 μm.

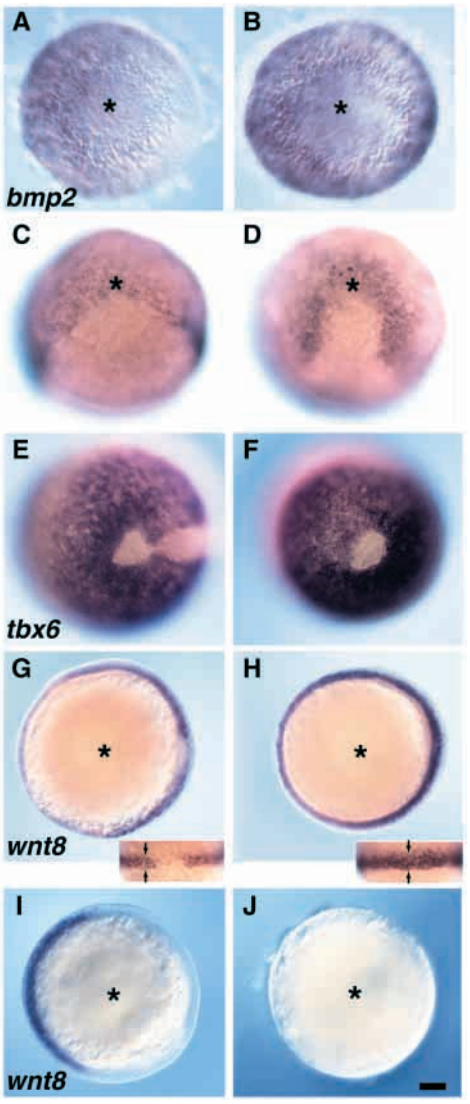


Fig. 4. *boz* is required to limit the expression of *bmp* and other ventrolateral markers. (A,B) At 30% epiboly (4.7 hpf) *bmp2b* is excluded dorsally in wild type (A) but not in *boz* (B). (C)At the end of gastrulation (10 hpf) *bmp2b* is expressed ventrolaterally in the prospective non-neural ectoderm in wild type. (D) In *boz*, this expression is expanded dorsally. (E) *tbx6* is expressed in non-axial mesendoderm of wild-type embryos at the end of gastrulation. (F) In *boz*, *tbx6* expression is not excluded from the dorsal side. (G,H) At midgastrula (8 hpf), expression of *wnt8* in the blastoderm margin is excluded from the dorsal midline of wild type (G), but not *boz* mutants (H), arrows indicate dimension of *wnt8* expression domain measured. (I,J) Ectopic expression of synthetic *boz* RNA in wild-type embryos results in reduced (I) or absent (J) *wnt8* expression. (A,B) Animal views, dorsal to the right; (C,D) dorsoanterior views, animal pole to the top; (E,F) vegetal views, dorsal to the right; *marks the animal pole. Bar, 100 μm.

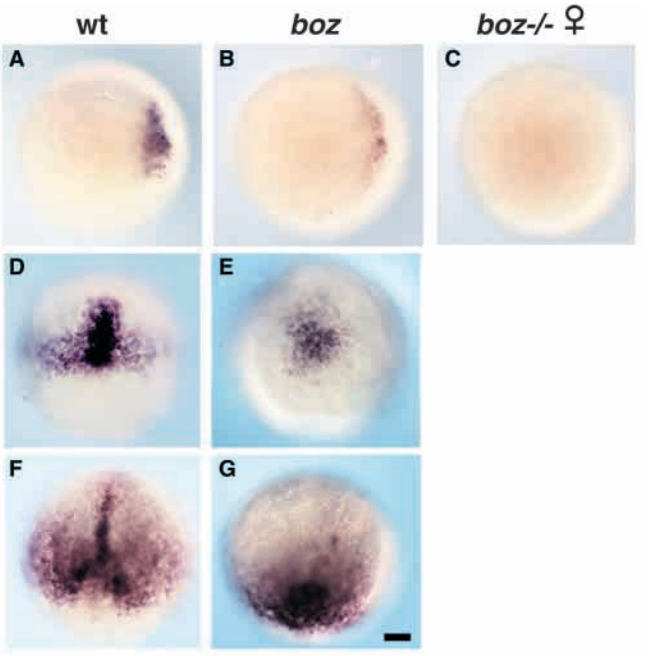


Fig. 5. *boz* is required for normal *din* expression. *din* expression in wild type (A,D,F) and *boz* (B,C,E,G). At oblong stage (3.7 hpf), *din* expression in the dorsal blastoderm margin of wild-type embryos (A) is reduced in *boz* mutant progeny from heterozygous females (B), and absent in *boz* progeny from homozygous females (C). *din* expression continues to be reduced in *boz* at the midgastrula stage (8 hpf) in the lateral domains and is absent in the axial mesoderm (E), compared to wild type (D). By late gastrulation (F,G), *din* expression has recovered in the lateral domains in *boz*, but the axial mesoderm expression remains reduced or absent (G). (A-C) Animal pole views, dorsal to the right; (D-G) dorsal views, animal pole to the top. *boz*–/– indicates embryos that were progeny of homozygous *boz* females. Bar, 100 μm.

Non-neural ectoderm forms in *boz* mutants at the expense of neuroectoderm

If the reduction of anterior neuroectoderm in *boz* mutants resulted from a neural induction defect, *boz* mutants should exhibit enlarged non-neural ectoderm. Indeed, in *boz* mutants, *gta2* expression, which marks the prospective non-neural ectoderm in the ventrolateral half of wild-type gastrulae (Fig. 3A; Detrich et al., 1995), spread into the dorsal and animal pole regions (Fig. 3B) in a fashion complementary to the reduction of anterior neuroectoderm anlage (compare Figs 1B and 3B). At later stages of gastrulation, *gta3* expression marks the ventrally limited non-neural ectoderm anlage (Fig. 3C; Neave et al., 1995) but, in *boz* mutants, the *gta3* expression domain was expanded dorsally (Fig. 3D). Expansion of *gta3* expression in the late *boz* gastrulae complemented the reduced expression of anterior neuroectoderm markers (compare Figs 3D and 1H). Together, these observations indicated that loss of *boz* function results in an increase of non-neural at the expense of neural ectoderm. Consequently, at the end of gastrulation, the portion of ectoderm that develops as neural tissue in *boz* mutant embryos is reduced mediolaterally and slightly shortened along the AP axis.

boz negatively regulates *bmp2b/4* and *wnt8* expression

The above findings are consistent with the notion that loss of anterior neural fates in *boz* mutants is at least partially due to decreased neural induction. BMP2/4/7 act as ventral morphogens to promote epidermal and inhibit neural fates within the ectoderm (Kishimoto et al., 1997; Neave et al., 1997;

Nguyen et al., 1998; Wilson and Hemmati-Brivanlou, 1995). In zebrafish blastulae, *swr* (*bmp2b*) transcripts, which are initially distributed uniformly in the entire blastoderm, become excluded from the dorsal side (Fig. 4A; Kishimoto et al., 1997; Nguyen et al., 1998; Nikaido et al., 1997). In contrast, *boz* mutant siblings exhibited *bmp2b* expression throughout the blastoderm at this stage of development (Fig. 4B), consistent with a recent report (Koos and Ho, 1999). During gastrulation, *bmp2b* transcripts were not observed in the dorsalmost aspect of *boz* mutant gastrulae, including a discrete *bmp2b* expression domain observed in the dorsal margin of wild-type embryos (Nikaido et al., 1997). However, the ventrolateral *bmp2b* expression domain was enlarged in *boz* mutants with respect to wild-type siblings (not shown). At the end of gastrulation, the *bmp2b* expression domain remained enlarged in *boz* mutants, exhibiting a shape similar to that observed for *gta3* (compare Figs 4D and 3D). Therefore, *boz* function is required to limit *bmp2b* expression in the dorsal region of the blastula and gastrula. Loss of *boz* function also resulted in increased expression of the ventrolateral and absence of the dorsal expression domain of the *bmp4* gene during gastrulation (data not shown; Chin et al., 1997; Nikaido et al., 1997).

To determine whether increased *bmp2b/4* expression resulted in ventralization of *boz* gastrulae, we monitored the expression of genes that are normally expressed in discrete dorsoventral domains. While *eve1* expression is detected by 30% epiboly in the ventrolateral marginal blastoderm of wild-type embryos (Joly et al., 1993), *boz* mutants showed expansion of the *eve1* expression domain towards the animal pole and towards the dorsal side (not shown). Expression of a T-box-related gene, *tbx6*, in the blastoderm margin is excluded from the dorsal midline of wild-type gastrulae (Fig. 4E; Hug et al., 1997), but it was detected along the embryonic circumference of *boz* mutants (Fig. 4F). Similarly, zygotic *wnt8* expression in the blastoderm margin of early and mid gastrulae (60% and 80% epiboly) was excluded dorsally in wild-type embryos (Fig. 4G; Kelly et al., 1995), but not in *boz* mutants (Fig. 4H). We measured the anteroposterior dimension of the *wnt8* expression domain and found that it was 30% larger in *boz* compared to wild type (n=10). Therefore, the dorsal margin of *boz* mutant gastrulae is not only deficient in expression of organizer-specific genes (Fekany et al., 1999), but also exhibits ectopic expression of ventrolateral markers.

To further test if *boz* can inhibit *wnt8* expression, we injected synthetic *boz* mRNA at the 1- to 4-cell stage into wild-type embryos and analyzed *wnt8* expression during gastrulation. Indeed, 11% of the *boz* RNA-injected embryos exhibited no *wnt8* expression (Fig. 4J), 58% exhibited a *wnt8* expression domain reduced to only about half the circumference of the margin (Fig. 4I), and 31% exhibited a normal *wnt8* expression domain (n=65). Therefore, *boz* is necessary and sufficient for repression of *wnt8* gene expression in the dorsal region of the gastrula.

During development, the activity and expression of *bmp* genes is inhibited by BMP antagonists such as Chordin and Noggin (Piccolo et al., 1996; Zimmerman et al., 1996). Therefore, we asked whether expression of the *chordino* (*din*) gene, which encodes the zebrafish homolog of *chordin*, was affected by the *boz* mutation (Miller-Bertoglio et al., 1997; Schulte-Merker et al., 1997). In wild-type embryos, *din* expression can be detected soon after mid-blastula transition, in a domain of cells close to the blastoderm margin (Fig. 5A; Miller-Bertoglio et al., 1997). However, in *boz* mutant siblings, *din* expression was confined to a few marginal blastomeres (Fig. 5B). Moreover, *boz* mutant progeny obtained from $boz^{m168/m168}$ homozygous females, lacking both maternal and zygotic wild-type *boz* function, exhibited a complete loss of *din* expression at this stage (Fig. 5C). During gastrulation, *din* expression in *boz* mutants (Fig. 5E) was reduced or absent in cells that will become axial mesoderm and was decreased, compared to wild-type siblings (Fig. 5D), in the bilateral expression domains flanking the midline (Fig. 5E), as recently reported (Koos and Ho, 1999). However, in late *boz* gastrulae, *din* expression was not significantly decreased in the bilateral expression domains (Fig. 5G). Therefore, both maternal and zygotic *boz* functions are essential for *din* expression prior to and during early gastrulation, but might be dispensable for this role in the late gastrula.

BMP inhibition neuralizes *boz* mutants without suppressing the AP patterning defect

If increased BMP signaling was the main defect underlying reduction of neuroectoderm and forebrain in *boz* mutants, overexpression of BMP antagonists should suppress these phenotypes. Therefore, we injected RNA encoding zebrafish Chordin or *Xenopus* Noggin into progeny of $boz^{m168/+}$ (or $boz^{m168/m168}$) parents or into wild-type embryos at the 1- to 4-cell stage (Miller-Bertoglio et al., 1997; Smith and Harland, 1992). At early gastrula stages (60-70% epiboly) in the injected wild-type embryos, the ectodermal *otx1* expression domain was expanded ventrally and often encircled the embryo, but the position of its posterior border was not altered significantly (Fig. 6C), compared to uninjected embryos (Fig. 6A). Similarly, in the injected *boz* mutants, the ectodermal *otx1* expression domain was expanded ventrally and anteriorly, but not posteriorly (Fig. 6G,H), relative to uninjected *boz* embryos (Fig. 6E,F; Table 1). In contrast to *otx1* expression in ectoderm, however, *otx1* expression in axial mesoderm (Fig. 6B,D) was not restored in *boz* mutants dorsalized by *noggin*/*chordin* RNA injections (Fig. 6F,H). These results indicated that the

Table 1. Effect of Chordin and Noggin on anterior neuroectoderm development in *boz* mutants

Parental genotypes	RNA injected	Dosage	Total no. embryos	% Wild type	% with *boz* phenotype*	% with expanded *otx1* expression
$boz^{-/-} \times boz^{-/-}$	none	–	58	32.8	67.2	0
$boz^{-/-} \times boz^{-/-}$	*chordin*	550 pg	164	4.3	4.9	90.8
wt	none	–	72	100	0	0
wt	*noggin*	150 pg	220	3.2	0	96.8
$boz^{+/-} \times boz^{-/-}$	none	–	12	50	50	0
$boz^{+/-} \times boz^{-/-}$	*noggin*	150 pg	22	0	0	100

*Scored as reduced *otx1* neuroectodermal expression domain at 60-70% epiboly.

reduction of anterior neuroectoderm in early *boz* gastrulae likely results from a BMP-dependent neural induction defect.

When *noggin* or *chordin* RNA-injected embryos were analyzed at the late gastrula stage, both wild-type and *boz* embryos exhibited elongated shapes typical of dorsalized mutants (Mullins et al., 1996; Solnica-Krezel et al., 1996). Furthermore, in both wild-type (Fig. 6K,L) and *boz* embryos (Fig. 6O,P) ectoderm was neuralized, as revealed by circumferential expression of *six3* and *pax2.1* genes, in the prospective forebrain and MHB anlagen, respectively (Hammerschmidt et al., 1996b; Miller-Bertoglio et al., 1997; Neave et al., 1997). In striking contrast to the early gastrula stage, the *six3* forebrain expression domain remained reduced along the AP axis in dorsalized late *boz* mutant gastrulae (Fig. 6O,P). Furthermore, no midline gap was observed in the *pax2.1* expression domain, which was shifted anteriorly (Fig. 6O) compared to wild type (Fig. 6K), as observed for uninjected *boz* mutants (Fig. 6M). These observations indicated that, at the end of gastrulation, inhibition of BMP signaling neuralized ectoderm in *boz* mutants, without suppressing deficiencies of anterior neural fates. Based on the observation that expression of anterior neural markers induced by BMP inhibition in early *boz* gastrulae was not maintained in the neuralized ectoderm of *boz* mutants during late gastrulation, we concluded that *boz* influences AP neural patterning independent of neural induction.

Inhibition of Wnt signaling suppresses the deficit of anterior neural fates in *boz*

Secreted Wnt signaling molecules have been proposed to act as posteriorizing factors during neural patterning in *Xenopus* embryos based on their ability to suppress anterior and induce posterior neural fates in gain-of-function experiments (McGrew et al., 1995, 1997). Conversely, a dominant negative form of *Xenopus* Wnt8 (*dnXWnt8*), a specific inhibitor of class I axis-inducing Wnt molecules, causes enlarged heads when overexpressed (Glinka et al., 1997; Hoppler et al., 1996). These data, combined with the observed ectopic expression of *wnt8* in *boz* mutants (Fig. 4I,J), suggested the transformation of anterior into more posterior neural fates in *boz* may result from ectopic Wnt signaling.

We therefore investigated whether dnXWnt8 could suppress any aspects of the *boz* mutant phenotype. Expression of the organizer-specific gene *gsc* is dramatically reduced in *boz* mutant gastrulae compared to wild-type siblings (Fig. 7B versus 7A; Fekany et al., 1999). However, 37% of the $boz^{m168/+}$ progeny injected with synthetic *dnXwnt8* RNA at the 1- to 4-cell stage, exhibited laterally expanded *gsc* expression at the shield stage and the fraction of embryos with reduced *gsc* expression declined compared to uninjected controls (Fig. 7C; Table 2). In addition, injections of *dnXwnt8* RNA suppressed the deficit of *din* expression in *boz* mutant gastrulae (Table 2; data not shown). Therefore, inhibition of Wnt signaling can suppress several aspects of organizer formation in *boz* mutants. Ectopic expression of dnXWnt8 also suppressed the deficit of *otx1* expression in *boz* mutants at midgastrulation (70% epiboly), similarly to inhibition of BMP signaling (Table 2; data not shown). Furthermore, ectopic expression of dnXWnt8 also resulted in a posterior expansion of the *six3* expression domain in both wild-type (Fig. 7E) and *boz* embryos (Fig. 7G), and restored notochordal *flh* expression in *boz* mutants (Table 3). To study further the effects of dnXWnt8 on the penetrance and expressivity of the *boz* phenotype, we analyzed the morphology of *dnXwnt8* RNA-injected progeny of $boz^{m168/+}$ parents at 1 dpf for the degree of head and notochord deficiencies (Fekany et al., 1999). This analysis revealed that ectopic expression of dnXWnt8 decreased the penetrance of the *boz* mutant phenotype (22%; n=166), compared to uninjected siblings (39%; n=136). In addition, the mutants observed in the injected group had less severe anterior defects, or exhibited normal head morphology, as well as less extensive notochord deficiencies (phenotypic classes IV and V) than their uninjected control siblings (classes II and III; Fig. 7H). Therefore, inhibition of Wnt signaling, unlike inhibition of BMP signaling, is able to suppress the anterior and notochord defects caused by the *boz* mutation.

Table 2. Effect of Wnt signaling inhibition on organizer formation

Parental genotypes	RNA (pg)	Marker analyzed	Total no. of embryos	% with boz phenotype	% with expanded expression
$boz^{-/+} \times boz^{-/-}$	none	*gsc*	22	50	0
	600-1800		57	19	37
	none	*din*	38	29	0
	600-1800		43	16	35
	none	*otx1*	22	36	0
	600-1800		27	0	81

Table 3. Effect of Wnt signaling inhibition on forebrain and notochord development in *boz*

Parental genotypes	RNA	Total no.of embryos§	% with *boz* phenotype	% mutants without notochord*	% mutants with reduced forebrain anlage‡
$boz^{-/+}$ x $boz^{-/-}$	none	343	48.4	57.8	93.4
	dnwnt8 200-400 pg	364	20.9	38.0	50.0

*Scored as complete absence of *flh* notochordal expression domain.
‡Scored as a reduction of *six3* expression domain.
§Represents an average of multiple experiments.

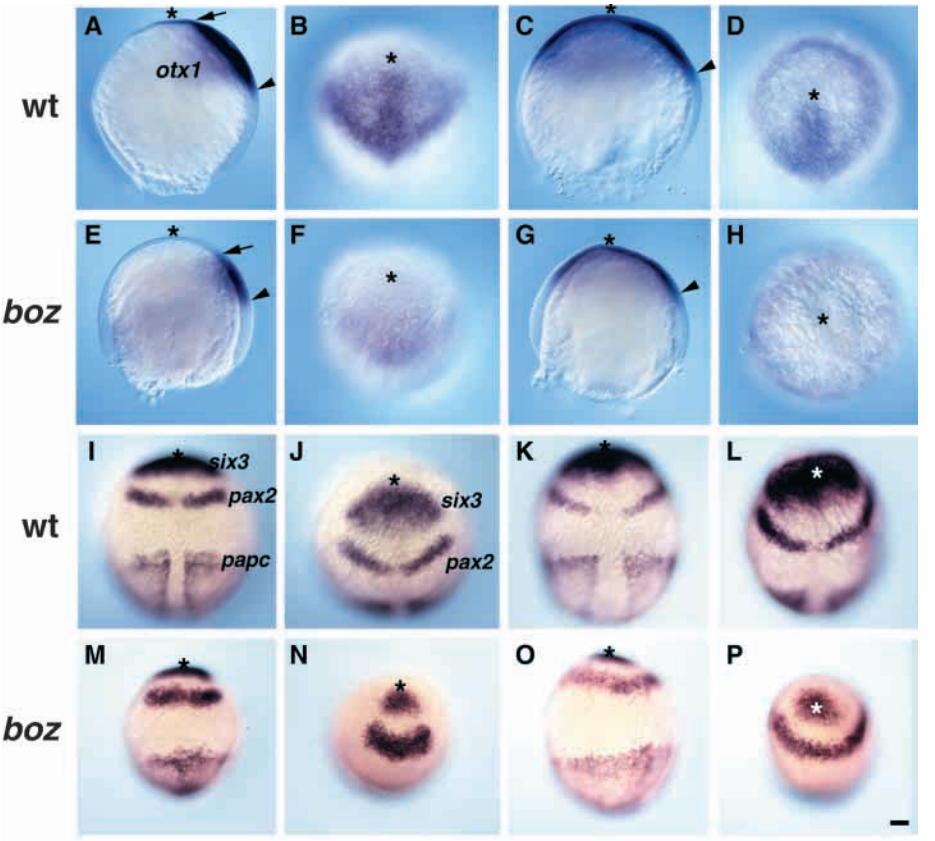


Fig. 6. Inhibition of BMP signaling suppresses the neural induction but not AP patterning defect in *boz*. (A-H) *otx1* expression in control and *din* RNA-injected embryos at 80% epiboly. (A,B) *otx1* is expressed in the prospective forebrain, midbrain and axial mesoderm of wild-type gastrulae. (E,F) In *boz*, the neuroectodermal *otx1* expression is reduced, while the axial mesoderm expression is absent. In *din* RNA-injected embryos, *otx1* ectodermal expression spreads around the embryonic circumference in both wild type (C) and *boz* mutants (G), but the mesodermal *otx1* expression remains absent in *boz* embryos (H). Note that the posterior borders of *otx1* expression (arrowheads) are not altered by din overexpression in both wild type and *boz*. (I-P) Expression of *six3* in the forebrain anlage, *pax2.1* in the MHB, and *papc* in paraxial mesoderm, in control and *din* RNA-injected embryos at the end of gastrulation (10 hpf). In *boz* mutants, the *six3* expression domain is reduced, the *pax2.1* expression domain is reduced mediolaterally and shifted anteriorly, and the *pax2.1* and *papc* expression domains are fused in the midline (M,N), compared to wild type (I,J). Overexpression of *din* causes radial expansion of the *six3*, *pax2.1* and *papc* expression domains in wild-type (K,L) and *boz* embryos (O,P). In injected *boz* embryos, the *pax2.1* and *papc* expression domains remain fused in the midline, *six3* is still reduced along the AP axis and *pax2.1* remains shifted anteriorly (O,P). (A,C,E,G) Lateral views, dorsal to the right; (B,D,F,H,J,L,N,P) dorsoanterior views, anterior to the top; (I,K,M,O) dorsal views, anterior to the top; *indicates the animal pole. Bar, 100 μm.

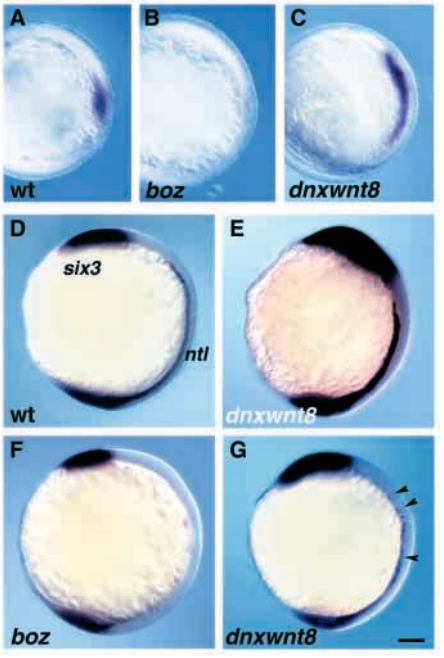
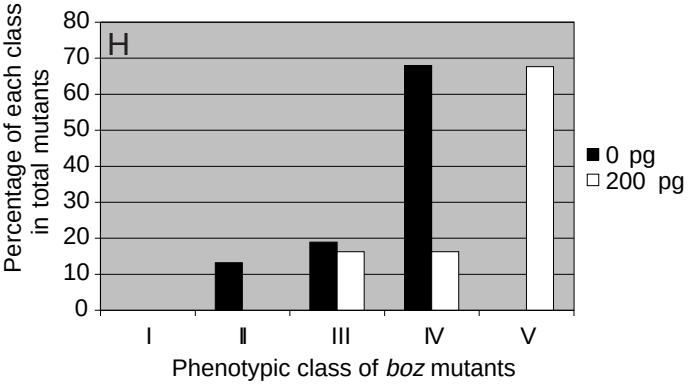


Fig. 7. Inhibition of Wnt signaling suppresses the loss of organizer and anterior neural fates in *boz*. Uninjected control wild-type (A,D) and *boz* embryos (B,F) and embryos injected with synthetic *dnXWnt8* RNA (C,E,G). In wild-type embryos (A), *gsc* is expressed dorsally, while expression is reduced in *boz* mutants (B). Embryos injected with *dnXwnt8* RNA exhibit laterally expanded *gsc* expression (C). Wild-type, uninjected embryos express *six3* in the forebrain anlage, and *ntl* in notochord precursors and the tailbud (D). *boz*, uninjected embryos have reduced *six3* expression and do not express *ntl* in the notochord (F). Wild-type embryos injected with *dnXWnt8* RNA exhibit enlarged *six3* and *ntl* expression domains (E). Similarly, *boz* embryos also exhibit an enlarged *six3* expression domain and incomplete *ntl* expression in the notochord (arrowheads, G). The expressivity of the *boz* phenotype analyzed at 1dpf in the injected group was also reduced compared to the control group (H). Class I *boz* mutants are the most severe with no notochord and forebrain. Class V mutants are the least severe *boz* mutants exhibiting only a small break in notochord. (A-C) Anterior views, dorsal to the right; (D-G) lateral views, dorsal to the right. Bar, 100 μm.



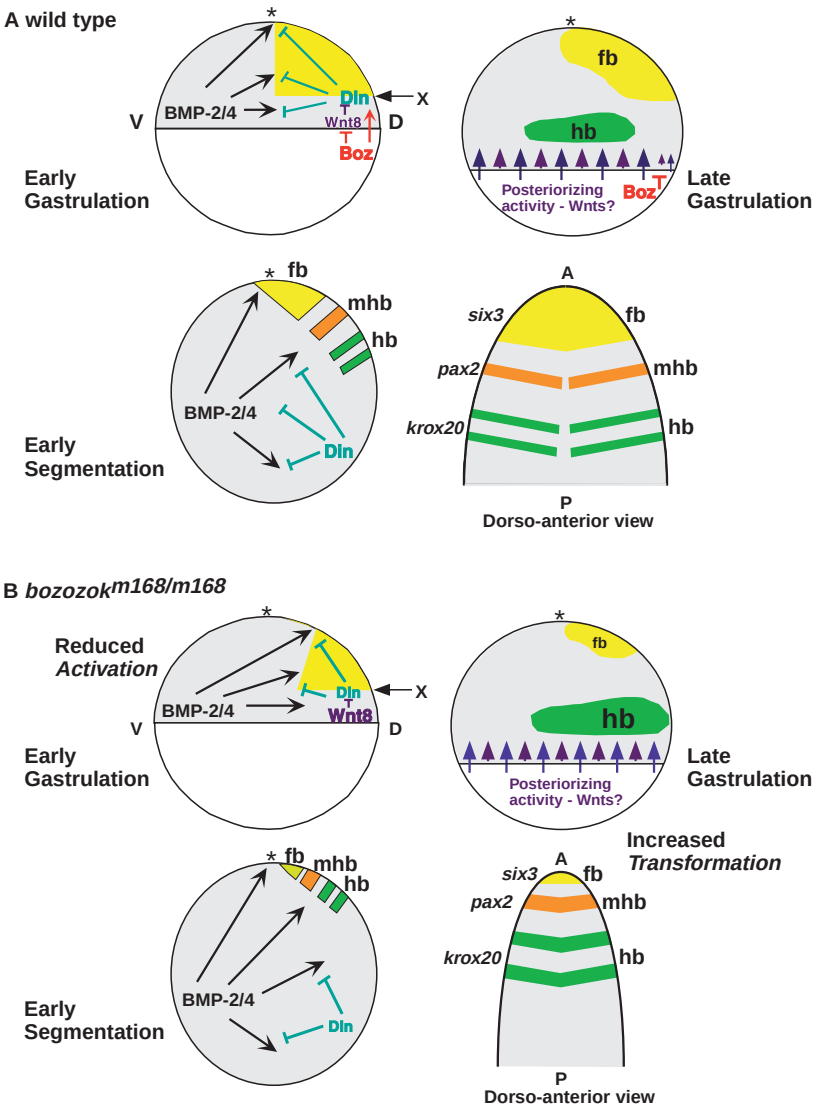


Fig. 8. The homeodomain protein Bozozok specifies anterior neural fates by promoting neural induction ('activation step') and limiting posteriorization of neuroectoderm ('transformation step'). (A) Induction and patterning of neuroectoderm during normal development. *boz* inhibits the Wnt pathway aiding formation of the gastrula organizer. In the early gastrula, *boz* positively regulates the expression of *din* and other BMP antagonists dorsally, which in turn inhibit BMP signaling, and induce anterior neuroectoderm with appropriate anterior and lateral boundaries. The posterior boundary of this initially anterior neuroectoderm is determined by an unknown, BMP and *boz*-independent pathway (X). In the late gastrula, *boz* dorsally inhibits the posteriorizing activity of Wnt and/or other factors while in the lateral blastoderm margin these posteriorizing factors transform some of the anterior neuroectoderm into more posterior neural fates. This results in specification of appropriately sized neuroectoderm along the AP and ML axes and a normal progression of forebrain, mhb and hindbrain anlagen in the neural plate at the early segmentation stage. (B) In *boz* mutants, Wnt activity is not inhibited and the organizer does not form properly. *din* expression is decreased and consequently *bmp2b/4* expression is increased, resulting in dorsal expansion of the prospective non-neural ectoderm, with a corresponding reduction of neural ectoderm along the mediolateral and AP axes. Note that, in the early gastrula, the posterior border of neuroectoderm is located normally in *boz*. In the late gastrula, *boz* mutants exhibit dorsally expanded *wnt8* expression and ectopic posteriorizing activity. At early segmentation stages, *din* and *bmp2/4* expression is less affected in *boz* mutants, resulting in neuroectoderm severely reduced along the ML axis, but its anterior border being only slightly shifted posteriorly. Due to ectopic posteriorization, however, there is a loss of anterior neural fates and anterior expansion of more posterior neural fates. (A,B) Lateral views, dorsal to the right, except where noted. fb, forebrain; mhb, midbrain-hindbrain boundary; hb, hindbrain.

The failure of BMP antagonists to suppress the neural patterning defects in *boz* mutants could be due to persistent ectopic expression of *wnt8* in *boz* mutants in which BMP signaling is inhibited. To test this possibility, we analyzed *wnt8* expression at mid-gastrulation (60% epiboly) in wild-type and *boz* embryos after overexpression of *noggin* mRNA at the 1- to 4-cell stage. Indeed, the *wnt8* expression domain was still expanded to cover the dorsal margin in *boz* gastrulae (data not shown). Hence, inhibition of Wnt signaling can influence the BMP pathway in *boz* mutants, but not vice versa.

DISCUSSION

An intriguing aspect of the *boz* mutant phenotype revealed by this work is the dynamic nature of the anterior neuroectoderm deficiency, which results from a combination of a BMP-dependent neural induction defect and Wnt-dependent posteriorization of neuroectoderm during gastrulation (Fig. 8). These studies provide genetic evidence that inhibition of BMP and Wnt signaling is required for vertebrate head development and suggest how spatiotemporal regulation of these two pathways in vivo contributes to AP neural patterning.

boz promotes neural induction by limiting BMP activity

The role of BMP2/4/7 molecules and their antagonists Chordin and Noggin in determining cell fate choice between epidermis and neural ectoderm has been well established (reviewed in Harland and Gerhart, 1997; Sasai and De Robertis, 1997). Our results strongly suggest that the reduction of neuroectoderm in *boz* mutants is due to loss/reduction of *din* expression and consequent increases in BMP2b/4 activity and expression. Accordingly, overexpression of Chordin or Noggin was able to completely neuralize *boz* mutant ectoderm. Since elimination of *din* function results in increased *bmp4* expression (Hammerschmidt et al., 1996b), the reduction of *din* expression in *boz* mutants could alone underlie the increased expression of *bmp2b* and *bmp4* genes. However, it remains to be determined whether *boz* also regulates expression of other BMP antagonists like Noggin (Lamb et al., 1993; Miller-Bertoglio et al., 1999), or negatively regulates the BMP

pathway in any other way. The finding that the reduction of *din* expression and the associated increase in *bmp* expression became less severe in late *boz* mutant gastrulae suggests that factors other than Boz regulate these genes late in gastrulation.

Posteriorization of neuroectoderm in *boz* mutants

Several observations indicated that a BMP-independent posteriorization of neuroectoderm contributes to the loss of anterior structures in *boz* mutants. At early segmentation stages in *boz* mutants, the MHB and hindbrain anlagen were shifted toward the anterior border of reduced neuroectoderm with respect to the underlying mesoderm. Similarly, the fate-mapping studies indicated the midbrain-hindbrain boundary was shifted closer to the anterior border of the neural plate and that prospective forebrain cells located at the animal pole of the gastrula instead contributed to non-neural ectoderm or to more posterior neural fates. In addition, inhibition of BMP signaling neuralized *boz* mutant embryos at the end of gastrulation, but it failed to restore the full expression of forebrain markers. Altogether, these results are consistent with the transformation in *boz* mutants of the anteriormost neuroectoderm, like forebrain and midbrain, to non-neural ectoderm and to more posterior neural fates, such as the MHB and hindbrain.

Inhibition of Wnt signaling by *boz* is required for organizer formation and development of anterior neural fates

Our studies indicated that repression of Wnt signaling by *boz* is required for dorsal organizer formation and to limit posteriorization of neuroectoderm. *boz* mutants exhibited ectopic *wnt8* expression in the dorsal blastoderm margin and overexpression of *boz* RNA in wild-type embryos led to downregulation of *wnt8* expression. Importantly, inhibition of Wnt signaling was able to suppress the organizer defects, as well as axial mesoderm and anterior neuroectoderm deficiencies in *boz* mutants. Upregulation of *din* expression in *boz* mutants by overexpression of dnXwn8 suggests that *boz* can regulate the BMP pathway indirectly via inhibition of Wnt signaling. Overexpression studies in *Xenopus* have shown that Wnt8 negatively regulates axial mesoderm and promotes paraxial mesoderm formation (Hoppler and Moon, 1998). Furthermore, ectopic expression of the Wnt inhibitor ECD8 in ventral blastomeres results in secondary axis formation (Itoh and Sokol, 1999). Similarly, interference with the β-catenin pathway during gastrulation results in secondary axis formation in zebrafish embryos (Pelegri and Maischein, 1998). These studies suggest that inhibition of Wnt signaling during mid/late gastrulation might be important for organizer formation in fish and frog embryos. Our studies establish *boz* as a key negative regulator of Wnt signaling in this process in zebrafish (Fig. 8). Therefore, during organizer formation in zebrafish, the Wnt/β-catenin pathway activates expression of *boz* (Koos and Ho, 1998; Yamanaka et al., 1998), which then inhibits *wnt8* expression and Wnt activity to promote formation of the gastrula organizer and development of axial mesoderm and anterior neuroectoderm.

By what mechanism does ectopic expression of dnXwnt8 suppress the AP patterning defects in *boz*? One possibility is that inhibition of Wnt signaling simply rescues an organizer activity, which in turn suppresses excessive posteriorization of neuroectoderm by Wnt-independent mechanisms. It is noteworthy that loss of zygotic functions of two *nodal*-related genes, *cyclops* (cyc) and *squint* (*sqt*) (Feldman et al., 1998), or elimination of maternal and zygotic function of the *one-eyed pinhead* (*oep*) gene that is essential for Nodal signaling (Gritsman et al., 1999), results in a defective organizer and cyclopia, but not anterior truncations. Therefore, it is rather unlikely that the observed reduced organizer-specific expression of *cyc* in *boz* mutant gastrulae (Sampath et al., 1998) is responsible for the AP neural patterning defects described here.

The second possibility is that continued inhibition of Wnt signaling during gastrulation (including organizer-dependent and/or organizer-independent signals) rescues forebrain development in *boz* embryos injected with *dnXwnt8* RNA. In zebrafish gastrulae, the lateral but not dorsal blastoderm margin exerts a posteriorizing influence upon transplantation into the prospective forebrain region (Woo and Fraser, 1997). Intriguingly, fate mapping of the zebrafish gastrula places the prospective hindbrain territory above the lateral, *wnt8*-expressing marginal region. Conversely, the prospective anterior and ventral neural fates reside in the dorsal midline of the gastrula fate map above the dorsal margin, from which *wnt8* expression disappears in the late gastrula (Kelly et al., 1995; Woo and Fraser, 1995). In *boz* mutants, the embryonic shield does not form and expression of dorsal-specific genes is replaced with expression of ventrolateral-specific genes, like *wnt8* (this work and Fekany et al., 1999). Therefore, we hypothesize that the dorsal blastoderm margin of *boz* mutant gastrulae is transformed into a lateral margin with posteriorizing Wnt activity (Fig. 8). Furthermore, the exclusion of Wnt8 activity from the dorsal midline might be critical for normal development of anterior and ventral neural fates.

The mechanism by which *boz* negatively regulates *wnt*8 expression remains to be elucidated. However, *boz* may indirectly limit Wnt activity, as wild-type *boz* function is required for expression of the zebrafish *dickkopf-1* homolog, *dkk1*, encoding an antagonist of Wnt signaling (Hashimoto et al., 2000). Overexpression of Dkk1 can suppress notochord and forebrain deficiencies in *boz* mutants similarly to the overexpression of dnXWnt8 described here (Hashimoto et al., 2000). In addition, it will be important to study the relationship between *boz* and *hex1*, the zebrafish homeobox gene expressed in the dorsal YSL and capable of inhibiting *wnt8* expression (Ho et al., 1999).

Our results provide support for the Wnt pathway constituting an endogenous component of the transformation process. Such an involvement of Wnt signaling in neural posteriorization has been previously suggested by gain-of-function experiments in which Wnt3a decreased the expression of anterior neural genes (McGrew et al., 1995) and Wnt signaling inhibitors, such as dnXWnt8, Frzb, Dkk1 and Cerberus, induced anterior neuroectoderm markers in frog embryos (Glinka et al., 1998; Leyns et al., 1997; McGrew et al., 1997; Piccolo et al., 1999; Wang et al., 1997). Experiments in *Xenopus* have also implicated other signaling molecules such as FGF and retinoic acid as potential neural posteriorizing factors (Blumberg et al., 1997; Doniach, 1995; Kolm and Sive, 1997). A possible involvement of other FGF molecules and retinoids in the AP patterning defects of *boz* will be worthwhile investigating. Recent elegant transplantation studies have revealed that the

most anterior row (row-1) of the developing neuroectoderm in zebrafish mid/late gastrulae has neural patterning activity (Houart et al., 1998). It remains to be determined whether *boz* affects development of this signaling center. It is noteworthy, however, that removal of row-1 cells leads to ectopic expression of diencephalon-restricted genes, like *sonic hedgehog* (*shh*) (Houart et al., 1998). Conversely, in *boz* mutants, the diencephalic expression of *shh* and *hlx1* is completely eliminated (Solnica-Krezel et al., 1996), thus indicating that *boz* does not alter neural patterning exclusively by affecting row-1 cells.

boz promotes the activation and limits the transformation of neuroectoderm

Several gain-of-function studies in *Xenopus* have demonstrated that head induction occurs either by simultaneous inhibition of BMP and Wnt signaling (Glinka et al., 1997, 1998; Piccolo et al., 1999) or through inhibition of BMP, Wnt and Nodal signaling, mediated by the multifunctional protein Cerberus (Piccolo et al., 1999). Here we have shown that the homeobox gene *boz*, which is required for development of rostral structures, negatively regulates both BMP and Wnt pathways, thus providing genetic evidence that inhibition of BMP and Wnt signaling is involved in anterior neuroectoderm specification in vertebrates. Furthermore, our results revealed that Bozozok regulates these pathways in a fashion consistent with a two-step process of AP neural patterning. Thus, *boz* promotes the first activation step, during which neural tissue with anterior character is induced. Subsequently, *boz* limits the hypothetical transformation of anterior neuroectoderm (Fig. 8).

While BMP inhibitors and neural inducers were considered the activator factors (reviewed in Sasai and De Robertis, 1997), recent reports demonstrated that they do not influence the global AP neural pattern in the embryo (Hammerschmidt et al., 1996a; Kishimoto et al., 1997; Neave et al., 1997; Nguyen et al., 1998). Accordingly, our results here showed that inhibition of BMP signaling enlarged the neuroectoderm in *boz* mutants, but failed to alter the posterior boundary of anterior neural markers both in early and late gastrulae. Based on these observations, we propose that, during the activation step, inhibition of BMP signaling determines the mediolateral and anterior boundaries of anterior neuroectoderm, while an additional BMP and *boz*-independent pathway(s) is involved in determining the posterior border of anterior neuroectoderm induced in the early gastrula (Fig. 8).

Additional embryological and genetic evidence supports the main prediction of Nieuwkoop, that some of the initially induced anterior neuroectoderm is posteriorized in the later stages of gastrulation (Nieuwkoop et al., 1952). When the dorsolateral ectoderm fated to become hindbrain is explanted from early fish or frog gastrulae and cultured in isolation, it expresses forebrain and not hindbrain-specific markers (Grinblat et al., 1999; Kolm and Sive, 1997). Here, we demonstrated that some of the prospective anterior neuroectoderm present in *boz* mutants during early gastrulation was posteriorized in the late gastrula. In contrast to the Nieuwkoop model, in which transforming signals originate from posterior chordamesoderm, our studies of *boz* mutants indicate that chordamesoderm is not required for the transformation step. Rather, formation of the organizer and chordamesoderm might limit the transformation step by excluding the posteriorizing signals from the dorsal midline. Therefore, we hypothesize that three pathways are involved in AP neural patterning in zebrafish. In the early gastrula, (1) the prospective anterior neuroectoderm is specified by BMP/*boz*-dependent neural induction that determines its anterior and lateral boundaries, and (2) a BMP/*boz*-independent pathway that positions the posterior boundary of anterior neuroectoderm. In the late gastrula, (3) a transforming activity, directly or indirectly dependent on Wnt signaling and limited dorsally by *boz*, posteriorizes some of the anterior neuroectoderm, producing the normal AP progression of neural structures.

We thank C. Wright, B. Appel, R. Moon and members of our laboratories for critical discussions and comments on the manuscript. We acknowledge B. Heher for fish care and technical support. cDNA clones were kindly provided by our colleagues C. and B. Thisse, D. Grunwald, T. Hirano, M. Mishina, T. Jovett, R. Moon, I. Dawid, N. Ueno, S. Amacher, C. Kimmel, S. Jolly, E. Weinberg, M. Ekker, M. Akimenko, and L. Zon. This work was supported by a NIH training grant (KFL) and a grant from the March of Dimes Birth Defects Foundation #FY99-0480 to LSK, who is a Pew Scholar.

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