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 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 ventrally 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 transforming 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 (Bedington and Robertson, 1999; Thomas and Bedington, 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 dorsal anterior 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 Thissen and Thissen (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 bozm168 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¢-diaminobenzidine, 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. 1E,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. 1E,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. 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. 1E,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. 1E,H), compared to wild-type siblings (Fig. 1E,G).

![Image](https://example.com/image.png)

*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 mediolaterally, 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; 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 bozm168/+ and/or bozm168/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.
Homeoprotein Bozozok inhibits BMP and Wnt pathways

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 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 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 chordin (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 bozm168/+ parents (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.

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

<table>
<thead>
<tr>
<th>Parental genotypes</th>
<th>RNA injected</th>
<th>Dosage</th>
<th>Total no. embryos</th>
<th>% Wild type</th>
<th>% with boz phenotype*</th>
<th>% with expanded otx1 expression</th>
</tr>
</thead>
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<tr>
<td>boz&lt;sup&gt;-/-&lt;/sup&gt; x boz&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>none</td>
<td>–</td>
<td>58</td>
<td>32.8</td>
<td>67.2</td>
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<td>chordin</td>
<td>550 pg</td>
<td>164</td>
<td>4.3</td>
<td>49</td>
<td>90.8</td>
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<td>–</td>
<td>72</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>wt x noggin</td>
<td>150 pg</td>
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<td>3.2</td>
<td>0</td>
<td>96.8</td>
<td></td>
</tr>
<tr>
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<td>none</td>
<td>–</td>
<td>50</td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>boz&lt;sup&gt;-/-&lt;/sup&gt; x boz&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>noggin</td>
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*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 dorsIALIZED 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 neutralized, 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 dorsIALIZED 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 un.injected boz mutants (Fig. 6M). These observations indicated that, at the end of gastrulation, inhibition of BMP signaling neutralized 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 neutralized 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<sup>m168</sup>/+ 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. 7F) 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<sup>m168</sup>/+ 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. 7I). Therefore, inhibition of Wnt signaling, unlike inhibition of BMP signaling, is able to suppress the anterior and notochord defects caused by the boz mutation.

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**Table 2. Effect of Wnt signaling inhibition on organizer formation**

<table>
<thead>
<tr>
<th>Parental genotypes</th>
<th>RNA (pg)</th>
<th>Marker analyzed</th>
<th>Total no. of embryos</th>
<th>% with boz phenotype</th>
<th>% with expanded expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>boz&lt;sup&gt;+/+&lt;/sup&gt;x boz&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>none</td>
<td>gsc</td>
<td>22</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>600-1800</td>
<td>57</td>
<td>19</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td></td>
<td>none</td>
<td>din</td>
<td>38</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>600-1800</td>
<td>43</td>
<td>16</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td></td>
<td>none</td>
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<td>22</td>
<td>36</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>600-1800</td>
<td>27</td>
<td>0</td>
<td>81</td>
<td></td>
</tr>
</tbody>
</table>

---

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

<table>
<thead>
<tr>
<th>Parental genotypes</th>
<th>RNA</th>
<th>Total no of embryos</th>
<th>% with boz phenotype</th>
<th>% mutants without notochord*</th>
<th>% mutants with reduced forebrain anlage‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>boz&lt;sup&gt;+/+&lt;/sup&gt;x boz&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>none</td>
<td>343</td>
<td>48.4</td>
<td>57.8</td>
<td>93.4</td>
</tr>
<tr>
<td></td>
<td>dnwnt8</td>
<td>200-400 pg</td>
<td>364</td>
<td>20.9</td>
<td>38.0</td>
</tr>
</tbody>
</table>

*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 midlaterally 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 neutralized 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 dnXwnt8 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 wnt8 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; Leys 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, J. David, 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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