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# The zebrafish *bozozok* locus encodes Dharma, a homeodomain protein essential for induction of gastrula organizer and dorsoanterior embryonic structures

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## SUMMARY

**The dorsal gastrula organizer plays a fundamental role in establishment of the vertebrate axis. We demonstrate that the zebrafish *bozozok* (*boz*) locus is required at the blastula stages for formation of the embryonic shield, the equivalent of the gastrula organizer and expression of multiple organizer-specific genes. Furthermore, *boz* is essential for specification of dorsoanterior embryonic structures, including notochord, prechordal mesendoderm, floor plate and forebrain. We report that *boz* mutations disrupt the homeobox gene *dharma*. Overexpression of *boz* in the extraembryonic yolk syncytial layer of *boz* mutant embryos is sufficient for normal development of the overlying blastoderm, revealing an involvement of extraembryonic structures in anterior patterning in fish similarly to murine embryos. Epistatic analyses indicate that *boz* acts downstream of β-catenin and upstream to TGF-β signaling or in a parallel pathway. These studies provide genetic evidence for an essential function of a homeodomain protein in β-catenin-mediated induction of the dorsal gastrula organizer and place *boz* at the top of a hierarchy of zygotic genes specifying the dorsal midline of a vertebrate embryo.**

Key Words: Spemann organizer, Nieuwkoop center, Notochord, Prechordal mesendoderm, Dorsal forerunner cells, A-P patterning, *bozozok*, Axis

## INTRODUCTION

A series of inductive events establishes the vertebrate embryonic axis. The dorsal gastrula organizer, or Spemann organizer, is a key component identified as a region on the dorsal side of the amphibian gastrula that induces a secondary axis when transplanted to the ventral side of a host embryo. The donor organizer differentiates into axial tissues including notochord, prechordal plate and ventral neural fates, induces and patterns neural tissue and coordinates gastrulation movements (Spemann, 1938). Structures homologous to the Spemann organizer have been identified in other vertebrates based on similar inductive properties and gene expression patterns, and include the node in mouse and chick, and the embryonic shield in fish (Harland and Gerhart, 1997; Lemaire and Kodjabachian, 1996). Secreted factors like Chordin and Noggin perform some of the inductive functions of the dorsal gastrula organizer by antagonizing ventrally expressed bone morphogenetic proteins-2/4 (BMPs). A gradient of BMP-2/4 activity is instructive in dorsoventral patterning of the germ layers during gastrulation (Neave et al., 1997; Nguyen et al., 1998; Thomsen, 1997).

The genetic hierarchy underlying the establishment of the gastrula organizer is only beginning to be elucidated (Moon and Kimelman, 1998). In frog and fish embryos, microtubule-mediated transport of dorsal determinants from the vegetal pole towards the prospective dorsal blastomeres (Jesuthasan and Strahle, 1997; Rowning et al., 1997) is essential for the establishment of the dorsal blastula organizer, or Nieuwkoop center (Dale and Slack, 1987; Nieuwkoop, 1973). The Nieuwkoop center of the frog embryo acts predominantly in the dorsal vegetal endodermal precursors and induces the organizer via non-autonomous signals in the overlying blastoderm, but might also contribute to the organizer through

self-induction (reviewed in Moon and Kimelman, 1998). A key step in the formation of the Nieuwkoop center is nuclear localization of β-catenin, an effector of the Wnt signaling pathway (Gumbiner, 1995; Heasman et al., 1994; Larabell et al., 1997; Schneider et al., 1996). Dorsally localized β-catenin, in complex with Tcf-like transcription factors, is thought to trigger axis formation by transcriptional activation of zygotic target genes. *Xenopus* Siamois and Twin, related homeodomain transcription factors, may be direct targets of the Tcf/β-catenin complex and could act as intermediaries between the maternal and zygotic programs executing axis formation (Brannon and Kimelmann, 1996; Fan and Sokol, 1997; Kessler, 1997; Laurent et al., 1997; Lemaire et al., 1995).

Formation of the gastrula organizer may require the coordination of the β-catenin, TGF-β and possibly other signaling pathways (Heasman, 1997; Kimelman et al., 1992). The TGF-β superfamily members Activin, Vg1 and Nodal-related proteins, which are often classified as mesoderm-inducing factors (MIFs), have axis-inducing activities (Kessler and Melton, 1995; Smith et al., 1990). The integration of the TGF-β and β-catenin pathways is best seen in the regulation of the organizer-specific expression of *goosecoid* (*gsc*), whose promoter features both β-catenin-binding sites and TGF-β response elements (Crease et al., 1998; Watabe et al., 1995).

In the zebrafish blastula, the functional equivalent of the Nieuwkoop center is likely to be located in the dorsal yolk syncytial layer (YSL) underlying the blastoderm (reviewed in Schier and Talbot, 1998; Solnica-Krezel, 1999). Accordingly, the YSL has mesoderm-inducing and patterning activities (Mizuno et al., 1996) and nuclear accumulation of β-catenin is detected in the dorsal YSL (Schneider et al., 1996). The homeobox gene *dharma* is a likely target of β-catenin. In the zebrafish blastula, *dharma* is expressed predominantly in the dorsal YSL and it can induce gastrula organizer formation when overexpressed in the yolk syncytium (Yamanaka et al., 1998). Furthermore, two *nodal-related* genes, *cyclops* and *squint*, have been implicated in organizer development and function, with *squint* being expressed in the YSL (Erter et al., 1998; Feldman et al., 1998; Rebagliati et al., 1998a,b; Sampath et al., 1998).

Extraembryonic structures have also been implicated in axis formation during murine development. Recent important discoveries indicated that, in the mouse embryo, notwithstanding the central role of the organizer/node in axis formation, the extraembryonic anterior visceral endoderm (AVE) may be responsible for development of anterior embryonic structures. Surgical removal of extraembryonic AVE leads to reduced expression of anterior neural markers (Thomas and Beddington, 1996). Furthermore, chimera experiments have shown the wild-type functions of TGF-β pathway components, Nodal and Smad2, are necessary in the AVE for proper anterior-posterior (AP) patterning (Varlet et al., 1997; Waldrip et al., 1998). It has been proposed that the AVE is an equivalent of dorsoanterior yolky endoderm, a component of the amphibian gastrula organizer. AVE might fulfill a function of a head organizer that is physically distinct from the trunk organizer activity residing in the node (Beddington and Robertson, 1998; Bouwmeester and Leyns, 1997).

To investigate the genetic basis of axis formation, we have analyzed the zebrafish *bozozok (boz)* locus. At 1 day postfertilization (dpf), *boz*$^{m168/m168}$ mutants exhibit a reduction or loss of the main organizer derivatives, notochord and prechordal plate, as well as deficiencies in anterior and ventral CNS structures, suggesting a key role of this gene in axis formation (Driever et al., 1997; Solnica-Krezel et al., 1996). Here we report that *boz* is required at the blastula stages for development of the dorsal gastrula organizer and specification of organizer-derived axial mesodermal fates. Moreover, we show that *boz* encodes the homeodomain protein, Dharma, recently isolated based on its ability to induce axis formation in the zebrafish embryo (Yamanaka et al., 1998). We demonstrate that *boz* function in the YSL is sufficient to induce the organizer and development of anterior structures in the overlying mutant blastoderm. These studies establish *boz* as an essential component of the axis induction pathway in zebrafish, acting presumably at the top of a hierarchy of zygotic genes activated by maternal β-catenin.

## MATERIALS AND METHODS

### Fish maintenance

Fish and embryos were maintained essentially as described in Solnica-Krezel et al. (1994). *boz* mutants were maintained in an AB/India background (Knapik et al., 1996).

### Screen for γ-irradiation-induced *boz* mutations

$F_1$ progeny of γ-irradiation mutagenized males and wild-type females (obtained from M. Halpern, Carnegie Institution of Washington) were crossed with *boz*$^{m168/+}$ females. One out of 250 males repeatedly produced 4% *boz* mutant progeny in such crosses.

### In situ hybridization and immunostaining

RNA in situ hybridizations were performed essentially as described in Oxtoby and Jowett (1993) and double in situ hybridizations as in Marlow et al. (1998). β-catenin was detected with diluted raw antisera (gift from P. Schneider and R. T. Moon) as in Solnica-Krezel and Driever (1994).

### mRNA injections

mRNA was synthesized using Ambion's mMessage mMachine kit. mRNA injections into 1- to 8-cell embryos were performed as described in Marlow et al. (1998). For YSL injections, embryos were dechorionated with Pronase. After YSL formation (1000-cell stage), embryos were coinjected with *boz/dharma* and fluorescent dextran into the yolk just below the blastoderm. After injections, the localization of the RNA was inferred from visualization of the fluorescent dextran using epifluorescence.

### Linkage analysis

5-day-old embryos from *boz*$^{m168/+}$; AB/Tu, were digested in 90 μg/ml protease (Qiagen), 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.05% SDS at 37°C for 48 hours. A *dharma* DNA fragment was amplified with PCR primers Dha39 5′-ATA CTC ACG CAG CTT TTG GG-3′ and Dha 350R 5′-GGT TGC CCT GCA TAG TAA GTC-3′. The PCR conditions were 94°C 2 minutes (1 cycle), then 94°C 30 seconds, 56°C 30 seconds and 72°C 40 seconds (30 cycles), followed by 72°C 7 minutes (1 cycle). Amplified products were digested by *Hae*III and resolved on 1.8% agarose gels.

### Sequence analysis of *boz*$^{m168}$

Oligonucleotides corresponding to *dharma* were used in PCR reactions with genomic DNA from *boz*$^{m168/m168}$ mutant embryos and

from wild-type siblings. Amplified fragments, purified by agarase treatment after electrophoresis in low-melting-point agarose, were sequenced with an ABI 377 automated sequencer. The stop codon in *boz*$^{m168}$ was identified in several independently amplified fragments and confirmed by RFLP analysis.

### Retrospective embryo genotyping

Following in situ hybridization, single embryos were digested in 20 μl of water with 20 μg/ml proteinase K and 5 μl PBT at 55°C overnight. After boiling for 5 minutes, 4 μl of digested solution served as a template in 20 μl PCR reactions. PCR conditions were as above, but with 40 cycles.

## RESULTS

### *boz* is required for the formation of multiple dorsal and anterior tissues

*bozozok*$^{m168}$ (*boz*$^{m168}$) was identified as a recessive, zygotic effect, embryonic lethal mutation that disrupts development of midline embryonic structures with variable penetrance and expressivity (Driever et al., 1997; Solnica-Krezel et al., 1996). We observed an increased severity of the *boz*$^{m168}$ phenotype in an India/AB hybrid genetic background, in which mutant embryos frequently exhibited severe anterior truncations. Five *boz* phenotypic classes (I-V) were defined based on morphological criteria (Fig. 1). Class I mutants are the most severe, with complete loss of notochord, eyes and forebrain, reduction of midbrain and an abnormal neural keel. In these mutants, expression of the forebrain marker *emx1* is reduced or absent. However, *krox20* expression domains in rhombomeres 3 and 5 of the hindbrain are enlarged relative to wild type (Fig. 1E). A similarly variable phenotype is exhibited by a putative allele, *boz*$^{i2}$, described recently (Blagden et al., 1997). Furthermore, in crosses between *boz*$^{m168/+}$ and fish heterozygous for a new γ-irradiation-induced allele, *boz*$^{v9}$, 6% of the progeny ($n$=1,675) displayed a variable *boz*$^{m168}$-like phenotype. *boz*$^{v9}$ segregates like a translocation, presumably leading to a deficiency of the *boz* locus (see below). Notably, the *boz*$^{v9/m168}$ phenotype was not stronger than the *boz*$^{m168/m168}$ phenotype, suggesting that the *boz*$^{m168}$ allele strongly reduces or eliminates *boz*$^{+}$ function (see below).

Due to the decreased penetrance in progeny of older females (K. D. F. and L. S. K., unpublished data), *boz*$^{m168/m168}$ homozygotes are sometimes viable. In crosses of *boz*$^{m168/m168}$ females with wild-type males, 100% of the progeny are phenotypically wild-type *boz*$^{m168/+}$ heterozygotes, indicating that there is not a strict maternal requirement for *boz* function. All experiments described in this manuscript utilized the *boz*$^{m168}$ mutant allele (*boz*) unless indicated otherwise.

### *boz* affects specification of axial tissues

Notochord and prechordal plate are reduced or missing in *boz* mutants by the tailbud stage (Solnica-Krezel et al., 1996). Here, we asked when the loss of these axial structures can first be detected in strongly affected *boz* mutants. At 60% epiboly (7 hours postfertilization, hpf), *ntl* expression in prospective notochord cells above the dorsal blastoderm margin was reduced or absent in *boz* embryos (Fig. 2A,B; Schulte-Merker et al., 1992). Furthermore, expression of *ntl* mRNA in dorsal forerunner cells, located just below the blastoderm margin in wild-type embryos, was not observed in *boz* mutants, consistent with the absence of these cells in *boz* gastrulae (Fig. 2A,B and data not shown). Dorsal forerunner cells give rise to the epithelial lining of Kupffer's vesicle, a transient structure located midventrally in the extending tailbud during somitogenesis (Fig. 2G; Cooper and D'Amico, 1996; Kimmel et al., 1995). Examination of *boz* embryos revealed a lack of a morphologically distinct Kupffer's vesicle (Fig. 2H).

Further analysis at 1 dpf revealed that *type II collagen (col2a1)* expression, normally marking the hypochord and the floor plate of the spinal cord, as well as the nascent notochord in the posterior part of the tail (Yan et al., 1995), was reduced or missing in *boz* mutant embryos (Fig. 2I,J). Therefore, the *boz*$^{m168}$ mutation interferes with formation of multiple axial cell types including notochord, prechordal

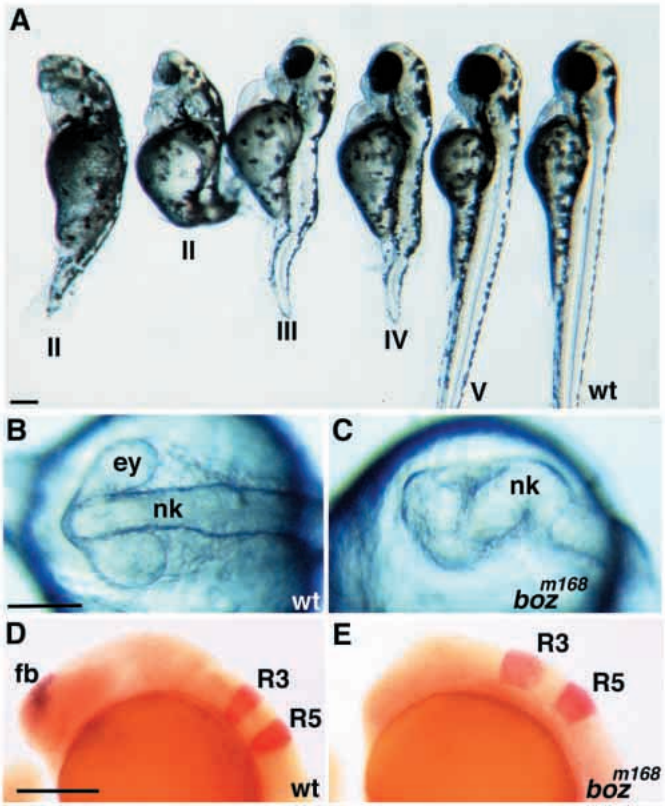


**Fig. 1.** *boz*$^{m168}$ causes deficiencies in dorsal and anterior embryonic structures with variable penetrance and expressivity. (A) *boz* mutant classes. From right to left, lateral views of wild-type, class V, class IV, class III and two class II *boz* larvae (3 dpf). There is a break in the notochord of class V *boz* larva, while the class IV mutant has a gap in notochord, spanning many somites. The class III mutant exhibits a gap in notochord and partially fused eyes, while class II mutants lack notochord completely and display cyclopia. (B,C) The neural keel in class II *boz* mutant embryos is highly abnormal, and the most anterior structures are missing (C), compared to wild type (B) (10-somite stage). (D,E) In wild-type embryos, *emx1* (blue) is expressed in the forebrain (fb) and *krox20* (red) in hindbrain rhombomeres R3 and R5 (D). In *boz*$^{m168}$ class I embryos, *emx1* expression is absent, while the *krox20* expression domains appear enlarged (E) (17 hpf, lateral views). Scale bar, 200 μm.

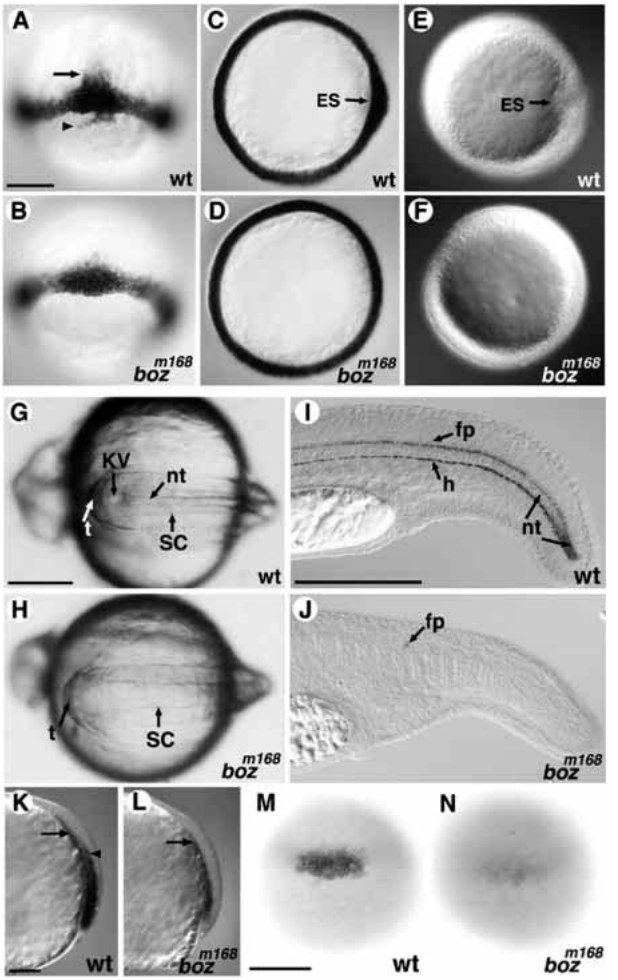


**Fig. 2.** Development of axial tissues is affected by the *boz*$^{m168}$ mutation. (A-D) (60% epiboly) *ntl* expression around the blastoderm margin is similar in wild-type (A) and *boz* embryos (B). *ntl* expression in notochord precursors above the margin (arrow), and in dorsal forerunner cells below the margin (arrowhead) in wild type (A) is reduced/absent in *boz* (B). The thickening of *ntl* expression seen in the animal view on the dorsal side of wild-type embryos (C) is not observed in *boz*$^{m168}$ mutants (D). (E,F) The embryonic shield (ES) forms as a dorsal thickening of the germ ring in wild-type embryos (E), but it does not form in *boz* mutants (F). (G,H) During somitogenesis, Kupffer's vesicle is seen posterior to the notochord in wild-type embryos at 10 somites. (H) Both Kupffer's vesicle and the notochord are absent in *boz*$^{m168}$ mutant siblings. (I,J) *col2a1* expression in floor plate, hypochord, and nascent notochord at 1 dpf in the wild-type tail. All of these expression domains are absent, with exception of a few floor plate cells, in *boz*$^{m168}$ mutants (J). (K,L) *axial* expression at 70% epiboly is normally seen in the endoderm (closest to the yolk, arrow) and dorsal mesoderm (arrowhead in K). In *boz*$^{m168}$ mutant gastrulae, mesodermal but not endodermal *axial* expression is reduced/absent (arrow in L). Lateral views with dorsal to the right. (M,N) Dorsal *flh* expression in wild-type embryos (M) is decreased in *boz*$^{m168}$ embryos at the germ ring stage (N). ES, embryonic shield; KV, Kupffer's vesicle; nt, notochord; sc, spinal cord; t, tailbud; fp, floor plate; h, hypochord. Scale bar, 200 μm.

plate, hypochord, floor plate and dorsal forerunner cells, already during gastrulation, when these cell types are being specified.

### *boz* affects dorsal gastrula organizer formation

Since *boz* mutant embryos displayed deficiencies in organizer-derived axial tissues during gastrulation, we investigated effects of the *boz*$^{m168}$ mutation on organizer development. The zebrafish embryonic shield, an equivalent of the Spemann gastrula organizer, is first observed as a thickening of the germ ring on the dorsal side of wild-type gastrulae at 6 hpf (Kimmel et al., 1995; Fig. 2E). In contrast, strongly affected *boz* gastrulae exhibited a circumferentially uniform germ ring (Fig. 2F). Additionally, a dorsal thickening of the *ntl* expression domain due to accumulation of *ntl*-expressing cells was not observed in *boz* embryos (Fig. 2C,D). Expression of other genes in the embryonic shield was decreased or absent in *boz* mutants, including *sonic hedgehog*, *lim1*, *sek1* and *TARAM-A* (data not shown; Krauss et al., 1993; Renucci et al., 1996; Toyama et al., 1995; Xu et al., 1994). Analysis of *axial* (*HNF-3β* homolog) expression demonstrated that *boz*$^{m168}$ affected only the dorsal mesodermal expression domain of this gene, corresponding to prechordal plate and notochord precursors, while its expression was normal in the presumptive endodermal cells localized close to the YSL (Fig. 2K,L; Strahle et al., 1996).

Notably, at the onset of gastrulation, expression of the notochord-specific *floating head* (*flh*) gene in the dorsal marginal cells of the forming germ ring was very reduced in *boz* embryos (Fig. 2M,N; Talbot et al., 1995). Furthermore, at 40% epiboly (5 hpf), the normal dorsal expression of *gsc* was strongly decreased (Fig. 3A,B; Schulte-Merker et al., 1994; Stachel et al., 1993). Since the embryonic shield does not form in *boz* mutants, and expression of dorsal-specific genes is affected before the shield stage, the *boz*$^{+}$ function must be required at the blastula stages for formation of the complete organizer.

### *boz* acts downstream or parallel to the maternal β-catenin pathway

The dorsal gastrula organizer is thought to be induced by signals regulated by maternal β-catenin, emanating from the Nieuwkoop center at the blastula stage (reviewed in Moon and Kimelman, 1998; Heasman, 1997). Considering the effects of *boz*$^{m168}$ on organizer formation, we carried out experiments designed to determine where *boz* acts with respect to components of this pathway.

Treatment of early zebrafish blastulae with 0.3 M lithium chloride (LiCl) dorsalizes embryos by activating the β-catenin pathway (Hedgepeth et al., 1997; Klein and Melton, 1996). Accordingly, treatment of wild-type embryos with LiCl led to expansion of the dorsal expression domain of *gsc* around the circumference of the gastrula (Stachel et al., 1993; compare Fig. 3A and C). In contrast, when clutches containing *boz* embryos were treated with LiCl a distinct class of embryos (<25%) was observed, expressing very low levels of *gsc* around the margin (Fig. 3D). Therefore, LiCl treatment can only induce ectopic *gsc* in *boz* mutants at low levels, similar to those found in untreated mutant embryos (compare Fig. 3B and D). In another experiment, embryos treated with LiCl were dorsalized, displaying, during early segmentation, laterally expanded somites and ectopic notochords. LiCl-treated *boz* embryos also had slightly expanded somites, but they still lacked notochord (data not shown). Among 1,068 progeny of *boz* heterozygotes treated with LiCl, 84% exhibited ectopic

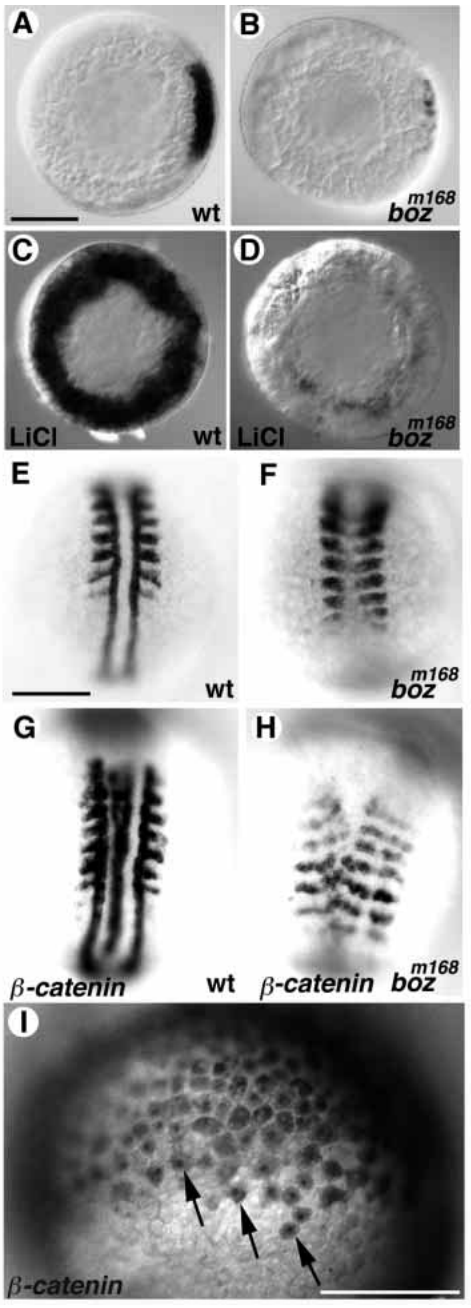


**Fig. 3.** *boz* acts downstream or in parallel to the maternal β-catenin pathway. (A,B) *gsc* expression in the dorsal margin of wild-type embryos (A) is reduced in *boz* mutants (B). (C,D) After treatment of embryos with LiCl, *gsc* is ectopically expressed at high levels around the blastoderm margin in wild-type (C), but only at very low levels in putative *boz* mutant siblings (D). (E,F) *myoD* expression in untreated wild-type embryos is detected in the somites and adaxial cells (E). In *boz*$^{m168}$ mutants, somitic *myoD* expression is fused and adaxial expression is absent (F). (G,H) After overexpression of β-catenin, wild-type embryos exhibit two complete arrays of *myoD* expression (G), while *boz* mutants have incomplete duplicated arrays of *myoD* expression with fused somites and absent adaxial cells (H). (I) Distribution of β-catenin at the blastula stage after injection of *β-catenin* mRNA. β-catenin (signal is brown) is detected in the cytoplasm and at higher levels in nuclei of numerous blastomeres (arrows). (A-D) Animal views; (A,B) Dorsal to the right; (E-I) Dorsal view, anterior to the top. Scale bar, 200 μm.

notochords, 12% displayed the *boz* phenotype, and 4% were unaffected wild type. Similarly, from 571 untreated control embryos, 11% were phenotypically *boz*. Since the frequency of *boz*$^{m168}$ mutants in the controls corresponds to those not responding to LiCl, we conclude that the *boz*$^{m168}$ mutation blocks the ability of LiCl to induce axial mesoderm.

The relationship between *boz* and β-catenin was investigated further by overexpression experiments. Injections of *β-catenin* mRNA (80-240 pg) dorsalized wild-type embryos and induced complete secondary axes, consistent with previous reports (Fig. 3E,G; Table 1; Kelly et al., 1995). Among the progeny of *boz*$^{m168/+}$ fish injected with *β-catenin* mRNA, *boz* mutants were still easily identifiable, with a penetrance comparable to uninjected controls (Table 1). Notably, a small fraction of embryos exhibited two *boz*-like axes, lacking notochord (Fig. 3F,H; Solnica-Krezel et al., 1996). In separate experiments, injection of *β-catenin* into wild-type embryos induced multiple thickenings of *ntl* expression, corresponding to ectopic shields. Conversely, 21% of the progeny of *boz*$^{m168/+}$ fish, injected with *β-catenin* exhibited uniform *ntl* expression in the margin (data not shown). Since *β-catenin* mRNA failed to induce an embryonic shield or notochord in *boz* mutant embryos, we concluded that β-catenin is unable to suppress the *boz* phenotype. Hence, *boz* acts downstream or in a parallel pathway to β-catenin.

To determine if the lack of suppression of the *boz* phenotype in β-catenin overexpressing embryos was due to interference of the *boz*$^{m168}$ mutation with nuclear accumulation of β-catenin, the subcellular localization of β-catenin was analyzed in such embryos at the late blastula stages (4-4.7 hpf) using a specific polyclonal antibody (Larabell et al., 1997; Schneider et al., 1996). Among wild-type embryos, 89% exhibited nuclear staining ($n$=88). Among the progeny produced by *boz*$^{m168/+}$ and/or *boz*$^{m168/m168}$ fish, 34% ($n$=167) were phenotypically *boz* mutant embryos and 93% ($n$=140) exhibited nuclear staining (Fig. 3J). These results indicated that *boz*$^{m168}$ does not interfere with the nuclear accumulation of β-catenin, further placing *boz* downstream or parallel to the maternal β-catenin pathway.

### *boz* acts upstream or in parallel to TGF-β signaling

The dorsalizing signals of the TGF-β superfamily, Nodal and zDVR1/Vg1, which are thought to act either downstream and/or in parallel to β-catenin, can induce incomplete secondary axes when overexpressed in zebrafish embryos (Dohrmann et al., 1996; Toyama et al., 1995). Similarly, overexpression of a constitutively active form of an activin/TGF-β-related type I receptor, *TARAM-A-D*, but not the wild-type receptor *TARAM-A*, can induce complete secondary axes (Renucci et al., 1996). Hence we tested the relationship between *boz* and dorsalizing TGF-β signals.

Overexpression of *TARAM-A* mRNAs did not produce any obvious morphological effects in wild-type embryos and failed to suppress the *boz* phenotype (Table 2). In contrast, injection of mRNA encoding the activated receptor TARAM-A-D was able to suppress the axial mesoderm deficiency in *boz* mutant embryos. *TARAM-A-D* mRNA-injected embryos obtained from *boz*$^{m168/+}$ parents could not be distinguished as *boz* or wild type; all embryos ectopically expressed the chordamesoderm marker *ntl* and a marker of the prechordal plate-derived hatching gland, *hgg1* (Fig. 4E; Table 2; Schulte-

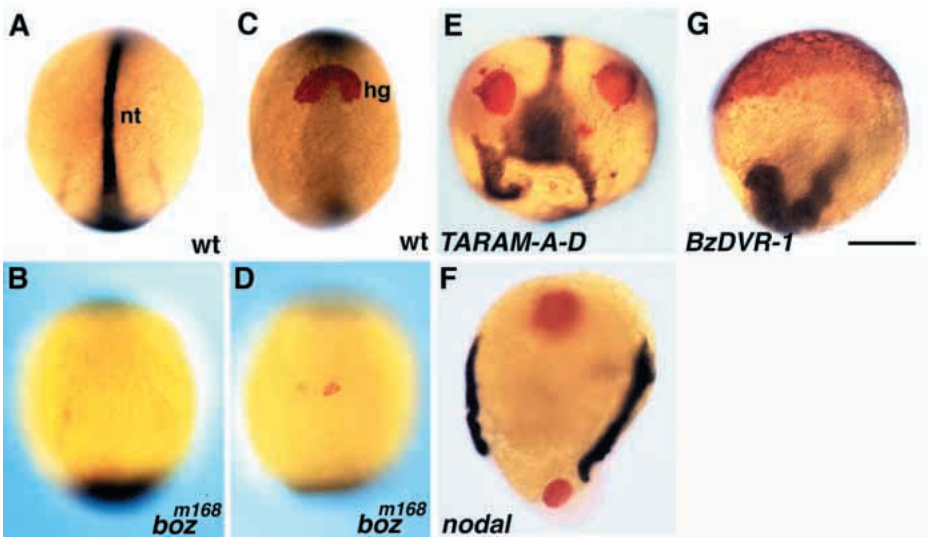


**Fig. 4.** TGF-β signaling suppresses the *boz* phenotype. (A,B) *ntl* expression (blue) normally marks notochord precursors (nt) and tail bud in wild-type embryos (A). In *boz* embryos, *ntl* expression in notochord precursors is absent (B) (5-7 somites). (C,D *hgg1* expression (red) in the prospective hatching gland (hg) of a wild-type embryo (C) is reduced in *boz*$^{m168}$ mutants (D). (E-G) Overexpression of TARAM-A-D (E), murine Nodal (F) and BMP-Vg1 (G) resulted in ectopic *ntl* and *hgg1* expression domains in the majority of embryos from wild-type and *boz*$^{m168/m168}$ parents. (A,B) Dorsal view, (C,D) Frontal view; in all images anterior is to the top. Scale bar, 200 μm.

Merker et al., 1992; Thisse et al., 1994), whereas *boz* embryos have reduced or absent *ntl* and *hgg1* expression (Fig. 4A-D).

To test the possibility of *boz* acting upstream of the receptor, we analyzed the response of *boz* embryos to two possible ligands, Nodal and the zebrafish homolog of Vg1, zDVR1. Overexpression of murine *nodal* mRNA in wild-type zebrafish dorsalized the embryos, inducing ectopic *ntl* and *hgg1* consistent with previous reports (Toyama et al., 1995). Similarly, when *nodal* mRNA was injected into progeny of *boz*$^{m168/+}$ fish the axial mesoderm deficiency in *boz* mutant embryos was suppressed (Fig. 4F; Table 2). A similar rescue of *hgg1* and *ntl* notochord expression in *boz* mutants was achieved by ectopic expression of BzDVR-1, a hybrid form containing the BMP pro region and Vg1 mature region (Table 2; Fig. 4G; Dohrmann et al., 1996). These studies indicate that both TGF-β ligands and a constitutively active type I receptor can suppress the deficiency of prechordal and chordamesoderm in *boz* mutants, suggesting that *boz* acts either upstream or in parallel to TGF-β signaling in the process of axis formation.

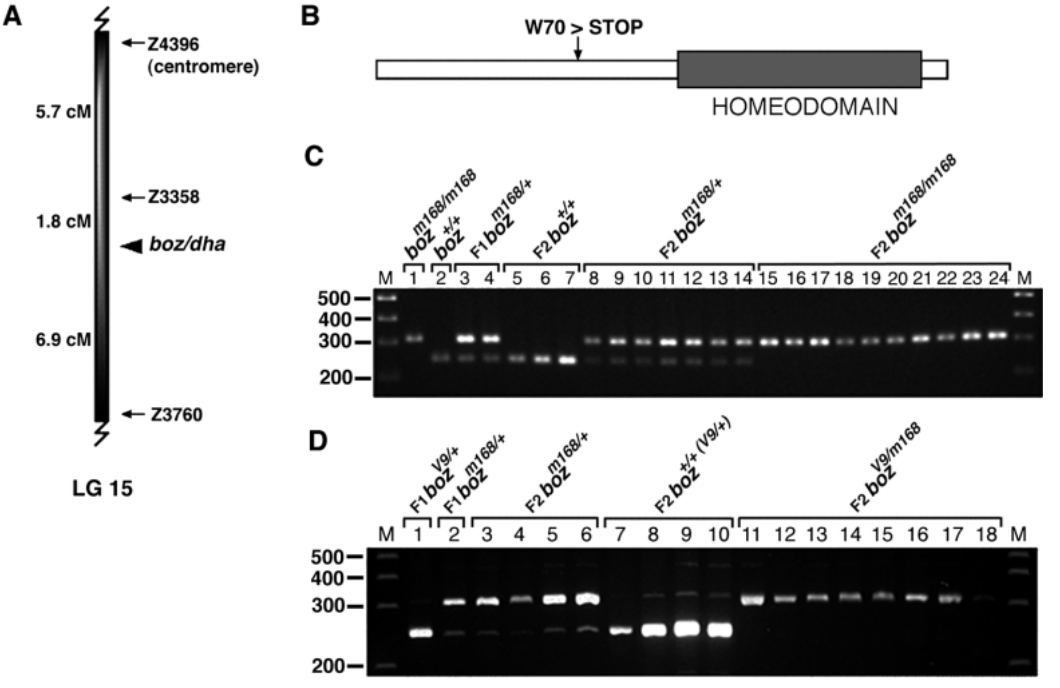


**Fig. 5.** *boz* maps to LG15 and is linked to *dharma*. (A) Schematic of LG15 showing *boz* localization to within 1.8 cM of marker Z3358 and 6.9 cM to marker Z3760. Mapping *dharma* with respect to Z3760 and Z470 revealed that *dharma* is on LG15 and linked to *boz*. (B) Schematic representation of Bozozok showing the position of the stop codon introduced by the *boz*$^{m168}$ mutation. (C) Identification of homozygous wild-type, *boz*$^{m168/+}$ wild-type and *boz*$^{m168/m168}$ mutant embryos by restriction fragment polymorphism analysis. A 312 bp PCR-amplified product from the *dharma* sequence is reduced in size to 247 bp by *Hae*III digestion in wild type. The *boz*$^{m168}$ mutant version was not cleaved. (Lanes 1,2) Homozygous *boz*$^{m168/m168}$ mutant and wild-type (+/+) embryos, respectively, identified by SSR markers Z3760 and Z3358, and phenotypic analysis. (Lanes 3,4) Heterozygous $F_1$, wild-type (*boz*$^{m168/+}$) embryos. (Lanes 5-7) Homozygous $F_2$ wild-type (+/+) embryos. (Lanes 8-14) Heterozygous $F_2$ wild-type *boz*$^{m168/+}$ embryos. (Lanes 15-24) Homozygous $F_2$ *boz*$^{m168/m168}$ mutant embryos. M, DNA size ladder. (D) Analysis of *boz*$^{v9}$ by restriction fragment polymorphism at the *Hae*III site as in (B). (Lane 1) $F_1$ heterozygous *boz*$^{v9/+}$. (Lane 2) $F_1$ heterozygous *boz*$^{m168/+}$. (Lane 3-6) $F_2$ wild-type heterozygous *boz*$^{m168/+}$. (Lane 7-10) $F_2$ wild-type homozygous +/+ (or heterozygous *boz*$^{v9/+}$). (Lane 11-18) $F_2$ *boz*$^{v9/m168}$. M, DNA size ladder as above.

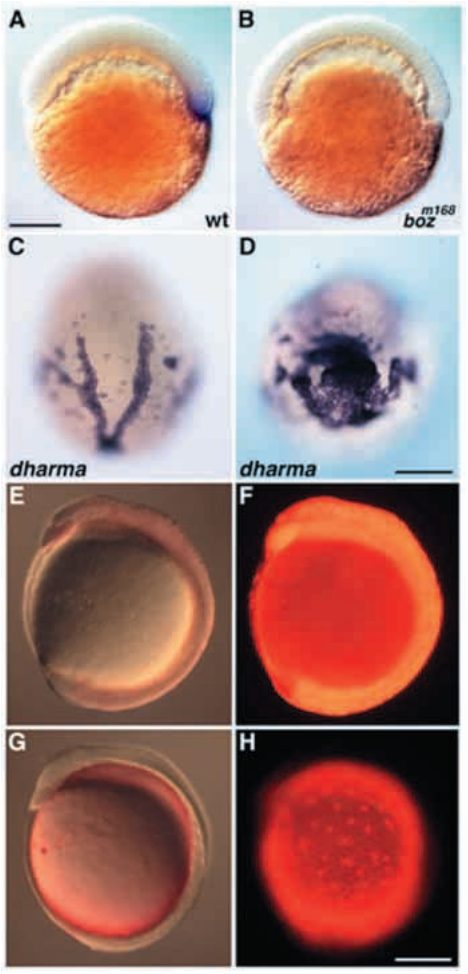


**Fig. 6.** Analysis of expression and rescuing potential of *dharma* RNA in *boz* mutants. (A,B) Whole-mount in situ hybridization reveals expression of *dharma* in the dorsal YSL of wild-type embryos at 50% epiboly (A). *dharma* was not expressed in sibling *boz* embryos (B). (C,D) Expanded *ntl* and *hgg1* expression in embryos from $boz^{m168/+}$ parents injected with *dharma* at the 1- to 4-cell stage. (E,F) Embryos coinjected with *dharma* and fluorescent dextran into one cell at the 16-cell stage. Nomarski (E) and fluorescent (F) images reveal that dextran is localized to the blastoderm. (G,H) After injections into the yolk at 1000-cell stage, fluorescent dextran remains confined to the YSL as seen in a Nomarski (G) and fluorescent (H) image. (A,B,E-H) Lateral views with animal pole to the top, (E-H) dorsal to the right; (C) animal view. Scale bar, 200 μm.

## The *bozozok* locus encodes Dharma, a paired type homeodomain protein

The early action of *boz* and the epistatic analyses presented here indicated that *boz* could be an early zygotic target of β-catenin/Tcf action. Recently, a homeobox gene called *dharma* with axis-inducing activity, was identified in zebrafish (Yamanaka et al., 1998). Expression of *dharma* is observed predominantly in the dorsal YSL from the early blastula stage and can be induced by LiCl treatment, suggesting that it is an early target of β-catenin (Yamanaka et al., 1998). The spatiotemporal expression pattern as well as the inductive potential of *dharma* made it a good candidate for corresponding to the *boz* locus.

The $boz^{m168}$ mutation was mapped to LG15 based on its linkage with the *chordino* locus (Schulte-Merker et al., 1997; E. Gonzales and L. S. K., unpublished observations) and subsequently by the use of simple sequence-length polymorphism (SSLP) markers (Knapik et al., 1998). This analysis positioned Z3358 approximately 1.8 cM (29 recombinants among 1620 meioses) and Z3760 about 6.9 cM (112/1620) to either side of the *boz* locus (Fig. 5A). Linkage analysis in wild-type and *boz* mutant mapping crosses placed *dharma* within the same interval of LG15 (Fig. 5A), prompting us to sequence the genomic DNA from $boz^{m168/m168}$ mutants to identify possible mutations in *dharma*. Sequence analysis revealed a guanine-to-adenine transition (TGG→TGA) that introduced a premature stop codon and truncated the *dharma* open reading frame (ORF) at codon 70, 45 amino acids upstream of the homeodomain (Fig. 5B).

To test linkage between the lesion in the *dharma* gene and the $boz^{m168}$ mutation, we analyzed a *Hae*III restriction site polymorphism introduced by the mutation in $boz^{m168}$ crosses. This *dharma* polymorphism segregated with *boz* in a DNA mapping panel of 810 diploid *boz* mutants. This indicated that *boz* and *dharma* are less than 0.06 cM apart (Fig. 5B), corresponding on average to a physical distance of 30 to 50 kb (Postlethwait et al., 1994). Furthermore, we found that the *dharma* sequences are deleted by $boz^{v9}$, a γ-ray-induced allele (Fig. 5D).

To test directly if Dharma could rescue the *boz* phenotype, *dharma* mRNA (20-500 pg) along with a rhodamine dextran lineage tracer was injected into the yolk of 1- to 4-cell-stage embryos generated by $boz^{m168/m168}$ and $boz^{m168/+}$ fish. These injections delivered mRNA to most cells of the developing embryos (Fig. 6E,F; Hammerschmidt et al., 1996). All doses dorsalized the embryos and induced ectopic axial mesoderm tissues (Fig. 6C,D). Furthermore, all doses suppressed the absence of notochord and prechordal plate in *boz* embryos (Table 3).

The close linkage of *dharma* and *boz*, presence of a nonsense mutation in the $boz^{m168}$ *dharma* ORF, deletion of *dharma* sequences by the $boz^{v9}$ allele and ability of *dharma* RNA to suppress the *boz* mutant phenotype provide conclusive evidence for Dharma being encoded by the *boz* locus. Therefore, we refer to this gene hereafter as *bozozok* (*boz*).

## Maintenance of *dharma* expression requires *boz* function

Among the progeny of $boz^{m168/+}$ and $boz^{m168/m168}$ fish at high stage (3.3 hpf), most embryos (97%) exhibited a normal *boz/dharma* expression pattern in the dorsal blastomeres (Yamanaka et al., 1998). However, already by sphere stage (4 hpf) and continuing during gastrulation, $boz^{m168/m168}$ mutants (retrospectively genotyped as described in Materials and Methods) did not express *boz/dharma* (Fig. 6A,B; Table 4). Therefore, the maintenance but not the establishment of *boz* expression requires its own function.

## Function of *boz* in the extraembryonic YSL is sufficient for normal development

Expression of *boz/dharma* in the dorsal YSL and in dorsal blastomeres at the blastula stages could indicate that *boz* function is required in both compartments (Yamanaka et al., 1998). To determine whether expression of *boz* in the YSL is

**Table 1. Analysis of the relationship between *boz* and the β-catenin signaling pathway**

| RNA injected | Genotype of parents | Quantity of mRNA injected (pg) | Total embryos | Affected* embryos (%) | *boz* embryos (%) | *boz* affected‡ (%) |
|---|---|---|---|---|---|---|
| β-*catenin* | *m168* (+/+) | – | 52 | 0 | | |
| | | 80 | 195 | 58 | | |
| | | 160 | 254 | 66 | | |
| | | 240 | 215 | 64 | | |
| | *m168* (+/–) | - | 143 | 0 | 15 | 0 |
| | | 80 | 193 | 23 | 13 | 0 |
| | | 160 | 406 | 35 | 14 | 19 |
| | | 240 | 226 | 46 | 13 | 45 |

*Affected embryos injected with β-catenin had expanded or ectopic *ntl* or *myoD* expression.
‡Percentage of *boz* embryos that had duplicated axes or anterior structures but lacked notochord and adaxial cells.

sufficient for its function, *boz/dharma* mRNA was coinjected with rhodamine-conjugated dextran into the YSL at 1000-cell stage, after the YSL became a separate compartment (Fig. 6G,H). Although injections of *boz/dharma* mRNA into the YSL were not as effective at dorsalizing wild-type embryos as injections into 1- to 8-cell-stage embryos (Yamanaka et al., 1998), they significantly reduced the penetrance of the $boz^{m168/m168}$ mutant phenotype (Table 3), as judged by analyzing injected embryos at day 1 of development for the presence of notochord, floor plate and anterior structures (data not shown). We conclude that overexpression of *boz* in the YSL of the *boz* mutant embryos can fully rescue the mutant phenotype. Hence, *boz* function in the extraembryonic YSL is sufficient for normal development of the overlying blastoderm.

## DISCUSSION

### The essential role of the homeodomain protein Bozozok in axis formation

Many genes have been identified in recent years that can induce axis formation when ectopically expressed in a vertebrate embryo, although only a few have been shown to be essential in this process (Moon and Kimelman, 1998). This work provides genetic evidence for the zebrafish *boz* locus being an early zygotic component required for development of a complete dorsal gastrula organizer, dorsal mesodermal tissues and anterior neural structures. We have found that the *boz* locus corresponds to the *dharma* gene encoding a paired-class homeodomain protein (Yamanaka et al., 1998). The presented molecular and genetic evidence argues that the $boz^{m168}$ mutation is a null allele.

The absence of the embryonic shield and the loss or reduction of organizer-specific gene expression in $boz^{m168/m168}$ mutants demonstrates that *boz* function is necessary for the formation of a complete gastrula organizer. Furthermore, *boz* affects specification of numerous organizer-derived dorsal midline structures, including notochord, prechordal mesendoderm, hypochord, floor plate and dorsal forerunner cells. This phenotype strikingly resembles defects obtained after surgical removal of the embryonic shield, consistent with the idea that the genetic and mechanical ablations of this important signaling center have similar developmental consequences (Shih and Fraser, 1996).

**Table 2. TGF-β signaling can suppress the *boz* phenotype**

| RNA injected | Genotype of parents | Quantity of RNA injected (pg) | Total embryos | Affected* embryos (%) | *boz* embryos (%) |
|---|---|---|---|---|---|
| *TARAM-A-D* | *m168* (+/–) | – | 189 | 0 | 14 |
| | | 2 | 158 | 92 | 0 |
| | | 5 | 96 | 94 | 0 |
| *nodal* | *m168* (+/+) | 16 | 25 | 44 | |
| | *m168* (+/–) | – | 87 | 0 | 21 |
| | | 16 | 44 | 70 | 5 |
| | | 32 | 45 | 93 | 2 |
| | | 48 | 31 | 94 | 6 |
| *BzDVR-1* | *m168* (+/+) | – | 23 | 0 | |
| | | 25 | 48 | 71 | |
| | | 50 | 22 | 91 | |
| | | 75 | 10 | 80 | |
| | (+/–) and/or (–/–) | – | 89 | 0 | 35 |
| | | 25 | 47 | 51 | 25 |
| | | 50 | 29 | 48 | 3.5 |
| | | 75 | 40 | 65 | 7.5 |

*Affected embryos had expanded or ectopic *ntl*, *myoD* and/or *hgg1* expression.

**Table 3: *dharma* can suppress the *boz* phenotype**

| Genotype of parents | Quantity of mRNA Injected (pg) | Stage of injection (cells) | Total embryos | Wild-type embryos | Dorsalized embryos | *boz* embryos (%) |
|---|---|---|---|---|---|---|
| *m168(–/–)* | – | – | 9 | 1 | 0 | 89 |
| | 170-500 | 1-4 | 12 | 0 | 11 | 8 |
| *m168 (+/–)* | – | – | 237 | 180 | 0 | 24 |
| | 20 | 1-4 | 120 | 1 | 119 | 0 |
| | 55 | 1-4 | 37 | 0 | 37 | 0 |
| | 170-500 | 1-4 | 108 | 5 | 102 | 1 |
| *m168 (+/–)* | – | – | 252 | 198 | 0 | 27 |
| | 100-300 | 1000 | 166 | 153 | 0 | 8 |

However, while removal of the embryonic shield does not lead to anterior-posterior patterning defects (Driever et al., 1997; Shih and Fraser, 1996), strongly affected *boz* mutants lack the most anterior neural structures. This indicates that *boz* mutations perturb development more extensively than extirpation of the embryonic shield. One possibility is that some organizer and *boz*-dependent patterning events occur before morphological manifestation of the embryonic shield. Accordingly, the dorsal expression of a number of genes like *gsc* and *lim1* precedes shield formation (Schulte-Merker et al., 1994; Stachel et al., 1993; Toyama et al., 1995) and is reduced or absent in *boz* mutants. Another intriguing possibility is that *boz* might specify anterior fates in an organizer/shield-independent fashion (Fig. 7).

## Bozozok acts in the extraembryonic YSL as a component of the Nieuwkoop center

Where and when in the embryo is *boz* function required for normal development? Since *boz/dharma* is expressed predominantly in the YSL and its ectopic expression in the YSL led to ectopic expression of the organizer-specific gene *gsc* in the overlying blastoderm, it was suggested that *boz* acts in the YSL to induce the gastrula organizer in a nonautonomous manner (Yamanaka et al., 1998). These studies could not exclude, however, a possible requirement for *boz* function in the blastoderm cells in this process. Here, we demonstrated that injections of wild-type *boz* mRNA exclusively in the YSL of *boz* mutant embryos fully rescued all aspects of the *boz* mutant phenotype, which strongly argues that *boz* function in the YSL is sufficient for normal development.

The nuclear localization of β-catenin in the dorsal YSL, together with the inductive properties of the yolk syncytium, led to the proposal that the dorsal YSL in fish embryos corresponds to the Nieuwkoop center of frog embryos (Mizuno et al., 1996; Schneider et al., 1996). We hypothesize that *boz* acts in the YSL as a component of the Nieuwkoop center and that it is essential for induction of the gastrula organizer in the overlying blastoderm. In *Xenopus*, the Nieuwkoop center is thought to induce the gastrula organizer in the overlying blastomeres in a non-autonomous fashion (Wylie et al., 1996). But, there is also evidence that the gastrula organizer is established directly by inheritance of dorsal determinants (Laurent et al., 1997). Our work provides evidence for a nonautonomous induction of the organizer by the dorsal YSL in zebrafish. However, Bozozok may also act in the blastoderm, e.g. in a dorsal group of non-involuting endocytic marginal (NEM) cells that remain in cytoplasmic confluence with the YSL even after formation of this extraembryonic layer and subsequently give rise to dorsal forerunner cells in the superficial layers of the shield (Cooper and D'Amico, 1996). Since forerunner cells are missing in strongly affected *boz* mutants, it is plausible that *boz* acts both in these cells and in the YSL during normal development as well as when ectopically expressed in the YSL. It remains to be determined if signals from NEM cells contribute and are necessary for organizer formation.

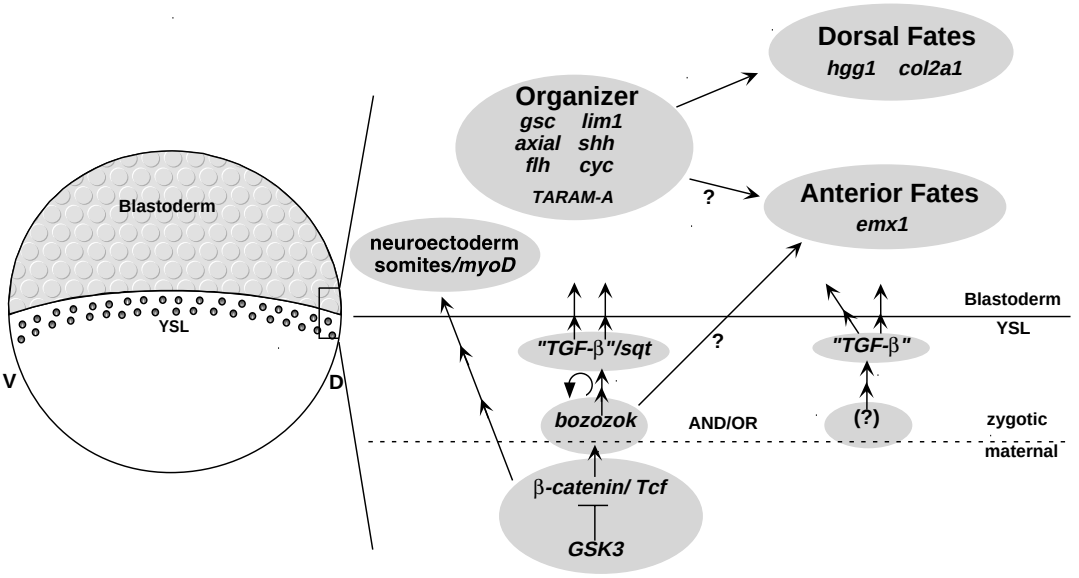


**Fig. 7.** Model for *boz* function in axis formation. (Left) A schematic of a zebrafish blastula (oblong stage) with blastoderm at the top and syncytial yolk cell at the bottom.

**Table 4. Expression of *dharma* mRNA in *boz* mutants**

| Genotype of parents | Stage | Total embryos | Fraction not expressing dharma* (%) |
|---|---|---|---|
| +/–male, +/–female | | | |
| | high | 91 | 3 |
| | sphere | 73 | 21 |
| | dome | 111 | 27 |
| | dome (Li treated) | 57 | 28 |
| –/–male, +/– female | sphere | 140 | 41 |
| | dome | 149 | 54 |
| | 50 % epiboly | 101 | 45 |
| –/–male, –/–female | high/oblong | 31 | 3 |

*Embryos were genotyped retrospectively as described in Materials and methods.

### *boz* acts downstream of the maternal β-catenin pathway

Several lines of evidence indicate that *boz* is a downstream target of dorsal maternal β-catenin and is essential for β-catenin-mediated organizer induction. Activation of the β-catenin pathway failed to suppress the *boz* phenotype, placing *boz* downstream or in parallel to β-catenin. The observation that ectopically injected β-catenin exhibits nuclear localization in *boz* mutants, that in LiCl-treated embryos expression of *boz* mRNA is increased (Yamanaka et al., 1998) and that there are several consensus Tcf/Lef binding sites in the *boz* promoter region (Y. Y., M. H. and T. H., unpublished data) strongly argue for *boz* being a downstream target of β-catenin.

Frog embryos in which maternal β-catenin mRNA was depleted fail to develop any dorsoventral asymmetry (Heasman et al., 1994). In contrast, *boz* mutant embryos exhibit an incomplete axis with somites and neural tube. Therefore, the loss of *boz* function most likely only partially blocks β-catenin signaling. We hypothesize that *boz* is required for specification of dorsoanterior fates but not for the ability of β-catenin to induce the remaining dorsal tissues, including somitic mesoderm and neuroectoderm (Fig. 7).

### Homeodomain proteins and organizer induction

The functional similarities between the frog homeobox genes *siamois* and *twin* and the zebrafish *boz* gene are striking. All three exhibit similar spatiotemporal expression patterns encompassing, but not limited to, the position of the Nieuwkoop center in frog and fish embryos, their expression is induced by β-catenin before organizer formation and they possess similar activities in gain-of-function experiments (Laurent et al., 1997; Lemaire et al., 1995; Yamanaka et al., 1998). Notably, inhibition of Siamois function via overexpression of a dominant repressor construct (SE) in which the Siamois homeodomain was fused to an active repression domain of *D. melanogaster* Engrailed inhibits Spemann organizer and axis formation in the frog embryo (Fan and Sokol, 1997; Kessler, 1997). This phenotype is similar to, but stronger than that described here for $boz^{m168}$. Considering that the homeodomains of Siamois and Twin are highly related, it is likely that the SE repressor interferes with the function of both proteins (Laurent et al., 1997). Similar to its failure to rescue the *boz* phenotype, β-catenin cannot suppress the loss of axial structures caused by the SE repressor. Therefore, like Bozozok, Siamois and Twin could be essential for gastrula organizer formation, acting downstream of β-catenin. However, definitive evidence for and identification of specific functions of Siamois and Twin in axis formation has to await the loss-of-function experiments for each of these two genes.

### *boz* may act upstream of TGF-β signaling

The key to understanding the mechanisms by which *boz* functions in the YSL in axis specification will be the identification of downstream targets encoding signaling molecules that can affect fates of overlying blastoderm cells. We provided evidence that ectopic expression of both ligands and an activated receptor of the TGF-β family, Vg1, mouse Nodal and TARAM-A-D can suppress the notochord and prechordal plate deficiency in *boz* mutants. One interpretation of these results is that Bozozok acts, directly or indirectly, upstream of a TGF-β-like signal that induces dorsal fates. Alternatively, *boz* could act in a parallel pathway, or downstream of TGF-β only partially inhibiting the signaling (Fig. 7). Consistent with *boz* acting upstream of TGF-β signaling, dorsal expression of two zebrafish *nodal*-related genes, *cyclops* and *squint*, is greatly reduced in *boz* mutants (Sampath et al., 1998; C. E. Erter, L. S. K. and C. V. E. Wright, unpublished observations). Notably, *cyclops* and *squint* fulfill partially redundant functions in establishment and function of the organizer (Feldman et al., 1998; Rebagliati et al., 1998a,b; Sampath et al., 1998). Furthermore, *squint*, like *boz*, is expressed in and can induce expression of organizer genes from within the YSL, making it an attractive candidate for a downstream *boz* target (Feldman et al., 1998; Erter et al., 1998).

While *boz* is the first gene shown to be required for development of both axial mesoderm and anterior-posterior neural patterning in the fish, mutations in several murine genes, such as *nodal*, *HNF-3β*, *lim1*, *otx2* and *smad2* lead to deficiencies in both dorsal and anterior fates (Acampora et al., 1995; Ang et al., 1996; Matsuo et al., 1995; Shawlot and Behringer, 1995; Waldrip et al., 1998). During murine development, formation of anterior structures requires function of the components of the Nodal signaling pathway, Nodal and Smad2, in the extraembryonic AVE (Varlet et al., 1997; Waldrip et al., 1998). While *boz* homologs have not been identified in mammalian embryos, it is worth noting that *boz* is required for expression of *axial* (*HNF3-β* homolog), *lim1* and *otx2*, and is also likely to act upstream of TGF-β signals like Nodal-related proteins. Therefore, it is tempting to speculate an existence of a mammalian *boz* homolog that would regulate a conserved signaling pathway inducing anterior fates by extraembryonic structures.

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